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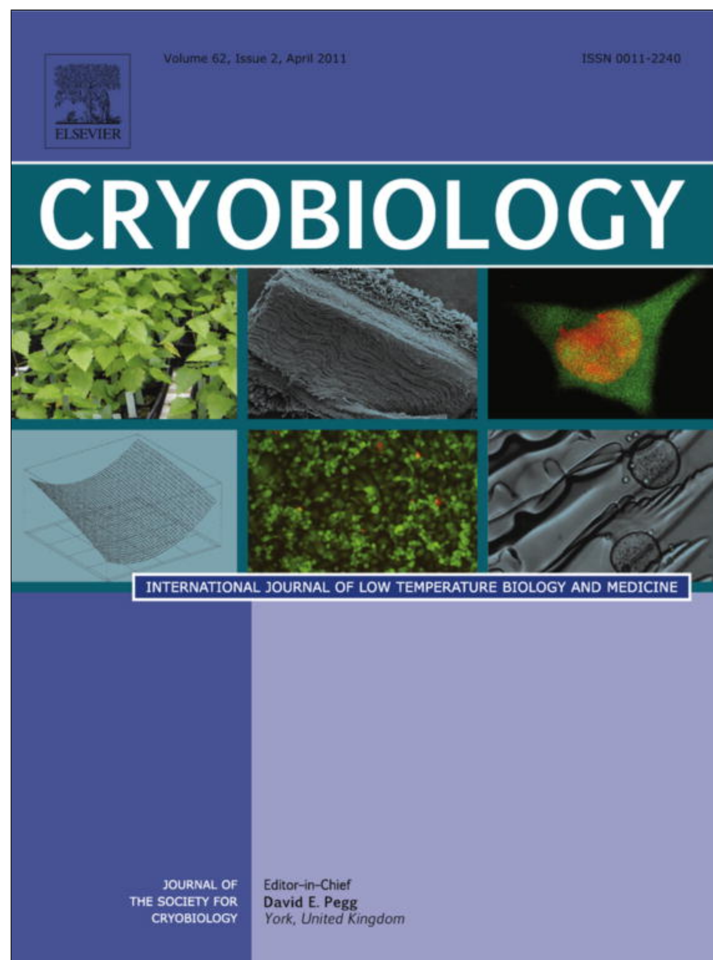

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Galacto-oligosaccharides as protective molecules in the preservation of *Lactobacillus delbrueckii* subsp. *bulgaricus* ☆

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ABSTRACT

In this work, the protective capacity of galacto-oligosaccharides in the preservation of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 was evaluated.

Lactobacillus bulgaricus was freeze-dried or dried over silica gel in the presence of three commercial products containing galacto-oligosaccharides. The freeze-dried samples were stored at 5 and 25 °C for different periods of time. After desiccation, freeze-drying or storage, samples were rehydrated and bacterial plate counts were determined.

According to the results obtained, all galacto-oligosaccharides assays demonstrated to be highly efficient in the preservation of *L. bulgaricus*. The higher content of galacto-oligosaccharides in the commercial products was correlated with their higher protective capacity.

Galacto-oligosaccharides are widely known by their prebiotic properties. However, their role as protective molecules have not been reported nor properly explored up to now. In this work the protective capacity of galacto-oligosaccharides in the preservation of *L. bulgaricus*, a strain particularly sensitive to any preservation process, was demonstrated.

The novel role of galacto-oligosaccharides as protective molecules opens up several perspectives in regard to their applications. The supplementation of probiotics with galacto-oligosaccharides allows the production of self-protected synbiotic products, galacto-oligosaccharides exerting both a prebiotic and protecting effect.

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Introduction

Lactic acid bacteria and bifidobacteria are the most extensively used probiotics in functional foods and pharmaceuticals. Their consumption produces several benefits to human health, namely, stimulation of the immune system, production of B vitamins, inhibition of pathogen growth, decrease of blood cholesterol levels, reduction of constipation and infantile diarrhea and increased resistance to infections [14,24,44].

Prebiotics are defined as selectively fermented ingredients that allow specific changes, both in composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health [13]. These compounds include oligosaccharides such as inulin, lactulose and fructo, gluco or galacto-oligosaccharides [7,30]. In particular, specific prebiotic mixtures composed of GOS and FOS included in certain infant formulas, have been described as responsible for the stimulation of bifidobacteria and lactobacilli in a similar way as oligosaccharides in human breast milk [2,3,45].

GOS are produced by transgalactosylation of lactose in a kinetically controlled reaction of synthesis with β-galactosidase (E.C.3.2.1.23) as catalyst, where the galactosyl–enzyme intermediate formed is attacked either by the water molecule, leading to the hydrolysis product, or by a nucleophile acceptor other than water (lactose in this case) to form new glycosidic bonds leading to GOS [27]. GOS are then composed of a variable number of galactose units linked to a glucose unit. The composition of GOS can vary quite markedly with respect to their degree of polymerization,

Abbreviations: GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; DP, degree of polymerization; CFU, cell forming units; MRS, De Man, Rogosa and Sharpe; UHT, ultra-high temperature; Tg, glass transition temperature.

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which can range from two to eight monomeric units. The main components of GOS are disaccharides other than lactose, trisaccharides and tetrasaccharides, with higher-molecular-weight oligosaccharides in much lesser proportions. The prebiotic effect is mainly associated with tri and tetrasaccharides [29].

It has been reported that the combination of probiotics and prebiotics in synbiotic products might improve the survival of the microorganisms crossing the upper part of the gastrointestinal tract, thus enhancing their effects in the large bowel. Moreover, these effects might be additive or even synergistic [33].

The important role of lactic acid bacteria as starters in the elaboration of dairy and pharmaceutical products highlights the requirement of appropriate processes for their preservation. Among them, freeze-drying has been the method of choice for the storage and delivery of microbial cultures from collections [25,26,35], and recently it has also been used for the preservation of functional foods, such as some type of yogurts [5,43]. However, during this process, the number of viable bacteria is dramatically reduced due to the decrease in water activity, loss of water being responsible for the cell damage. Membranes, nucleic acids and certain enzymes have been identified as the cellular targets for such damage [6,40].

To avoid these damages, the use of cryoprotectants is mandatory. Among them, polyhydroxylated compounds, such as sucrose and trehalose, have been used consistently as protecting agents during bacterial preservation [11,17,20–22,28]. Sugars can substitute water molecules upon dehydration by forming hydrogen bonds around the polar and charged groups present in phospholipid membranes and proteins, thereby stabilizing their native structure in the absence of water [8–10].

Taking into account the polyhydroxylated nature of prebiotics, it is reasonable to assume that they may prevent cells from dehydration, thus acting as protective molecules. In this regard, inulin and fructo-oligosacarides have demonstrated to be efficient cryoprotectants of lactic acid bacteria during freeze-drying [37,29]. In addition, Wieneke et al. reported that GOS from *Cyanobacterium nostoc commune*, are able to protect enzymes (i.e.: phosphoglucomutase and α-amylase) from heat and desiccation damage, and also *Escherichia coli* from desiccation [46].

Based on the above, GOS appear as important food ingredients, whose role as protective molecules should be further explored aiming to obtain "self-protected" synbiotic foods (synbiotics where prebiotics also act as protective molecules).

The goal of this work is to determine the efficiency of three commercial GOS preparations of different composition in the recovery of *Lactobacillus delbrueckii* subsp. *bulgaricus* after freeze-drying and desiccation over silica gel [46]. *Lactobacillus bulgaricus* has been chosen as the target organism because of its particular sensitivity toward any kind of stress [40]. Starters of *L. bulgaricus*, usually preserved by freezing, freeze-drying and spray drying, are widely used in the elaboration of dairy products. In order to perform a complete evaluation of the protectant properties of GOS, the acid production kinetics and the survival after storage at different temperatures were determined.

Material and methods

Gos

Three types of commercially available preparations consisting on a mixture of carbohydrates were studied: GOS A, GOS B and GOS C. The mixtures contained different percentages of GOS, with different DP, lactose, galactose and glucose, as determined by HPLC in a Perkin–Elmer Series 200 equipment with refractive index detector and autosampler, using BP-100 Ag^+ (300 × 4.6 mm) columns for carbohydrate analysis (Benson Polymerics, Reno, NV, USA) and Totalchrom software. 3α-4β-3α-galactotetraose and 4β-galactobiose were used as standards. The column and the detector were kept at 85 and 30 °C, respectively. Samples were eluted with 0.5 ml min^{-1} of Mili-Q water. The retention times were: galactose: 16.48 ± 0.2 min; glucose: 15.17 ± 0.3 min; lactose: 12.50 ± 0.2 min; DP-3 (GOS with DP = 3): 10.49 ± 0.2 min; DP-4: 9.23 ± 0.3 min; DP-5: 8.40 ± 0.2 min; DP-6: 7.90 ± 0.2 min. All standards were obtained from SIGMA (Sigma Chemical Co., St. Louis, MO). The composition of GOS A, GOS B and GOS C is shown in Table 1.

The commercial products GOS Qingdao (GOS A), kindly donated by Qingdao FTZ United International Inc (China), Vivinal® GOS (GOS B) (Friesland Foods, Zwolle, Holland), kindly donated by Friesland Foods Domo and Cup Oligo H-70® (GOS C) (Kowa Company, Tokyo, Japan), kindly donated by Kochi S.A. (Santiago, Chile) were also used as standards.

For the preservation experiments, three different concentrations of each GOS (GOS A, GOS B and GOS C) were used: 9, 19, and 38 g of commercial product (on dry basis) per 100 g of solution. They correspond to dilutions 1/2, 1/4 and 1/8 w/w of the original GOS sirups. 0.2 μm sterile filters were used to sterilize each GOS solution.

Bacterial strains and growth conditions

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 333 was isolated from a fermented product [15]. The strain was maintained frozen at −80 °C in 120 g l^{-1} non-fat milk solids. Cultures were grown in MRS broth [12] at 37 °C.

Drying over silica gel procedure

Cultures in the stationary phase (grown in MRS broth at 37 °C overnight, to attain approximately 10^9 CFU ml^{-1}) were harvested by centrifugation at 4000*g* for 10 min. One milliliter of each bacterial culture was washed twice in the presence of three different concentrations (9%, 19% and 38% w/w) of the three commercial preparations of GOS under study or in the presence of sodium chloride 0.85% w/v as control. The pellets were kept on the centrifuge tubes and dried over desiccators containing silica gel, until no changes in water desorption were detected as measured gravimetrically.

Freeze-drying procedure

Cultures in the stationary phase (grown in MRS broth at 37 °C overnight, to attain approximately 10^9 CFU ml^{-1}) were harvested by centrifugation at 4000*g* for 10 min. One milliliter of each bacterial culture was washed twice with sodium chloride 0.85% w/v. The pellets were resuspended in 0.1 ml of solutions containing the different GOS preparations at 19 or 38% w/w. The suspensions were frozen at −80 °C for 24 h. The freeze-drying process was carried out at −50 °C and lasted 24 h and a Heto FD4 (Heto Lab Equipment, Denmark) was used. The samples obtained in all conditions were stored at 5 or at 25 °C in desiccators containing silica gel. The recovery of cells after different times of storage was analyzed by plate counts.

Water activity and water content

Water activity was measured after drying the samples using an Aqualab water activity instrument (Aqualab, Model Series 3TE, USA). The residual water content of the dried powders was determined in a drying oven at 105 °C until a constant weight was attained.

Bacterial plate counts

Viable bacterial plate counts were determined before and after freeze-drying or desiccation and after storage. Dried microorganisms were resuspended in 1 ml of sodium chloride 0.85% w/v.

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Table 1
Composition of the commercial GOS.

	Qingdao (GOS A)	Vivinal (GOS B)[a]	Cup-Oligo H-70 (GOS C)[a]
Product	Powder	Sirup	Sirup
Water content (%)	–	25.0	25.0
Glucose (%)	9.4	20.4	2.2
Galactose (%)	1.2	2.1	2.6
Lactose + DP-2 (%)	17.9	37.6	22.9
DP-3 (%)	10.9	21.9	46.8
DP-4 (%)	6.1	10.4	21.3
DP-5 or higher (%)	3.9	7.6	4.2
Excipients (%)	50.6	–	–
Total GOS[b] (%)	⩾27	⩾44	⩾70

[a] The composition of GOS B and GOS C is expressed in dry basis.
[b] GOS minimum content as stated by the manufacturer.

Bacterial suspensions were serially diluted and plated on MRS agar plates. Bacterial counts were determined after 48 h of incubation at 37 °C.

Growth in milk after drying

After rehydration during 15 min, 0.5 ml of the rehydrated cells were used to inoculate 10 ml of UHT milk. Growth kinetics was followed by measuring the decrease of pH during incubation at 37 °C. pH were registered every each hour throughout a period of 24 h.

Reproducibility of the results

All experiments were done on duplicate samples using three independent cultures of bacteria. The relative differences were reproducible independently of the cultures used. Analysis of variance (ANOVA) of the viable counts and the *lag* times corresponding to the different treatments, was carried out using the statistical program Statistix 8 Software (Analytical Software, Florida USA). Differences were tested with paired sample *t* tests, and if $P < 0.05$ the difference was considered statistically significant.

Results

Lactobacillus bulgaricus CIDCA 333 was desiccated 48 h over silica gel desiccators until a constant weight was attained. The efficiency of GOS in the protection of *L. bulgaricus* against these dehydration conditions was then evaluated and results are presented in Fig. 1. When microorganisms were dehydrated in the absence of protectant, a decrease of 5 CFU logarithms with respect to the non-dehydrated cells (control) was observed.

The three commercial GOS under analysis induce a noticeable increase in the recovery of the strain after desiccation in all the concentrations used. However, the protective effect was different for the three GOS investigated. This observation can be attributed to differences in their composition regarding the chain length of the constitutive components of GOS (Table 1). The main differences among GOS A, B and C are related with the content of DP-3 and DP-4. It is interesting to note that the relative contents of DP-3 and DP-4 in GOS C are approximately the double of those in GOS B and the contents of these two oligosaccharides in GOS B are approximately the double of those in GOS A.

GOS B and GOS C clearly increased their protective effect when used at 19% and 38% w/w (Fig. 1). In fact, in these conditions, the plate counts of *L. bulgaricus* CIDCA 333 was only one logarithm lower than those corresponding to the non-dehydrated cells (control). No significant differences ($P > 0.05$) were observed in the bacterial recovery when GOS B and C were used in concentrations of 19% and 38% w/w.

GOS A also increased the recovery of *L. bulgaricus* after dehydration, but its protective effect was weaker than that of GOS B and GOS C. This different behavior can be attributed to the lower percentage of total oligosaccharides present in GOS A (27% *vs* 44% in GOS B and 70% in GOS C) (Table 1).

The protective effect of GOS has also been evaluated upon freeze-drying. Considering that GOS B and C were more efficient in the recovery of *L. bulgaricus* CIDCA 333, only these two commercial GOS were evaluated as cryoprotectants in freeze-drying (Fig. 2). After freeze-drying in the absence of cryoprotectants, a decrease of five logarithms in the cell forming units with respect to the control was observed. This indicates that *L. bulgaricus* is also very sensible to the hydric stress produced by freeze-drying. From Fig. 2, it can be concluded that after freeze-drying, both GOS B and GOS C at 19% and 38%, were very efficient cryoprotectants in the recovery of *L. bulgaricus*, a species particularly sensitive to both preservation process.

In order to evaluate differences in the protective role of GOS B and C, on the recovery of *L. bulgaricus* after freeze-drying, water activities and residual water contents after drying, growth kinetics and survival after storage at different temperatures were compared for both protectants.

The values of final water activities of the samples are displayed in Table 2. These water activities are lower than the water activity reported as critical for the recovery of *L. bulgaricus* after preservation [41]. In fact, in our previous work we have reported that this critical water activity is 0.55–0.7, depending on the preservation conditions. The water activity of microorganisms freeze-dried in the absence of GOS is slightly lower than that of bacteria freeze-

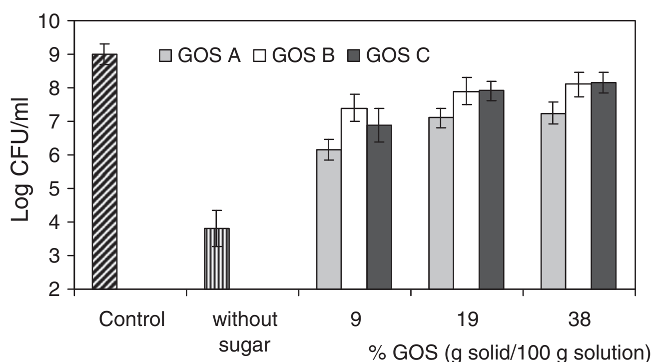

Fig. 1. Logarithm of CFU of microorganisms recovered after desiccation over silica gel. Bacteria grown in MRS were washed with different concentrations of GOS: GOS A (▒), GOS B (□) and GOS C (■). Counts of microorganisms before desiccation (control) (▨) and desiccated in the absence of GOS (▥) ($P < 0.05$).

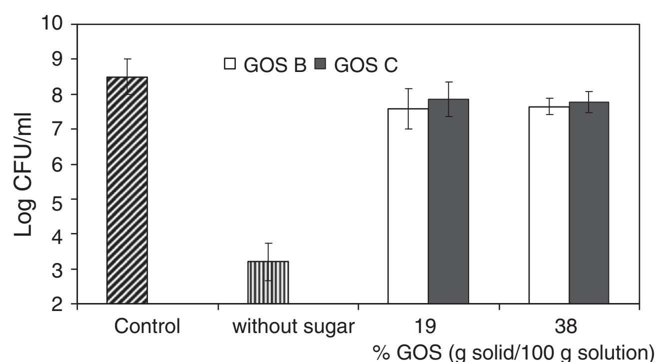

Fig. 2. Logarithm of CFU of microorganisms recovered after freeze-drying in the presence of different concentrations of GOS B (□) and GOS C (■). Counts of microorganisms before desiccation (control) (▨) and desiccated in the absence of GOS (▥) ($P < 0.05$).

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Table 2
Water activities after freeze-drying *L. bulgaricus* CIDCA 333 in the presence of different concentrations of GOS.

Freeze-drying conditions	Water activity (a_w)
Without GOS	0.37 ± 0.01
GOS B 19%	0.42 ± 0.01
GOS B 38%	0.42 ± 0.01
GOS C 19%	0.43 ± 0.02
GOS C 38%	0.43 ± 0.02

dried in the presence of GOS ($P < 0.05$). In the same way, the residual water content of samples dried in the absence of GOS was 0.051 ± 0.006 g/g dry mass and 0.068 ± 0.008 g/g dry mass in the presence of GOS in all the conditions assayed. This indicates that the presence oligo-saccharides may preserve the level of remnant water in freeze-dried samples, thus protecting the cells [42].

The growth kinetics in milk of the freeze-dried microorganisms in the presence of GOS B or GOS C were determined by registering the decrease of pH (Fig. 3A). As shown, freeze-drying in the absence of GOS produced a considerable increase in the *lag* time (*lag* time: 13 h) ($P < 0.05$), thus indicating serious bacterial damages. Freeze-drying in the presence of GOS reduced bacterial damage as revealed by the decrease in the *lag* times. In addition, the protective effect of GOS preparations against bacterial damage depended on the type and concentration of GOS, being GOS C at 38% the condition where the lowest level of damage was observed. In all other conditions, the protective effect of GOS was similar. Fig. 3B depicts the decrease in the *lag* time for *L. bulgaricus* CIDCA 333 grown in milk after freeze-drying. According to this figure, freeze-drying in the presence of 38% GOS C represents the condition where the lowest damage is observed (*lag* time: 5.5 h) ($P < 0.05$). When the strain was freeze-dried in the presence of 19% GOS C, the protective effect decreased considerably (*lag* time: 8.6 h). The cryoprotectant effect of GOS B was similar for both concentrations analyzed ($P < 0.05$).

In order to get an insight on the GOS components responsible for this protective effect, the increase in the *lag* times depicted in Fig. 3B were plotted against the relative concentrations of DP2–DP4 in the GOS mixtures (GOS B and GOS C 19 and 38%). Fig. 3C shows that the increase in the *lag* times is inversely related to the concentration of each component in the mixture, and that the protective effect of GOS components follows the pattern: DP2 < DP3 < DP4.

Fig. 4A and B show the decrease in the microbial survival after freeze-drying in the presence of GOS B and GOS C respectively, being further stored at 5 and 25 °C.

According to the storage experiments, the loss of viability can be described by simple exponential decay corresponding to first-order kinetics [18] (Eq. (1)).

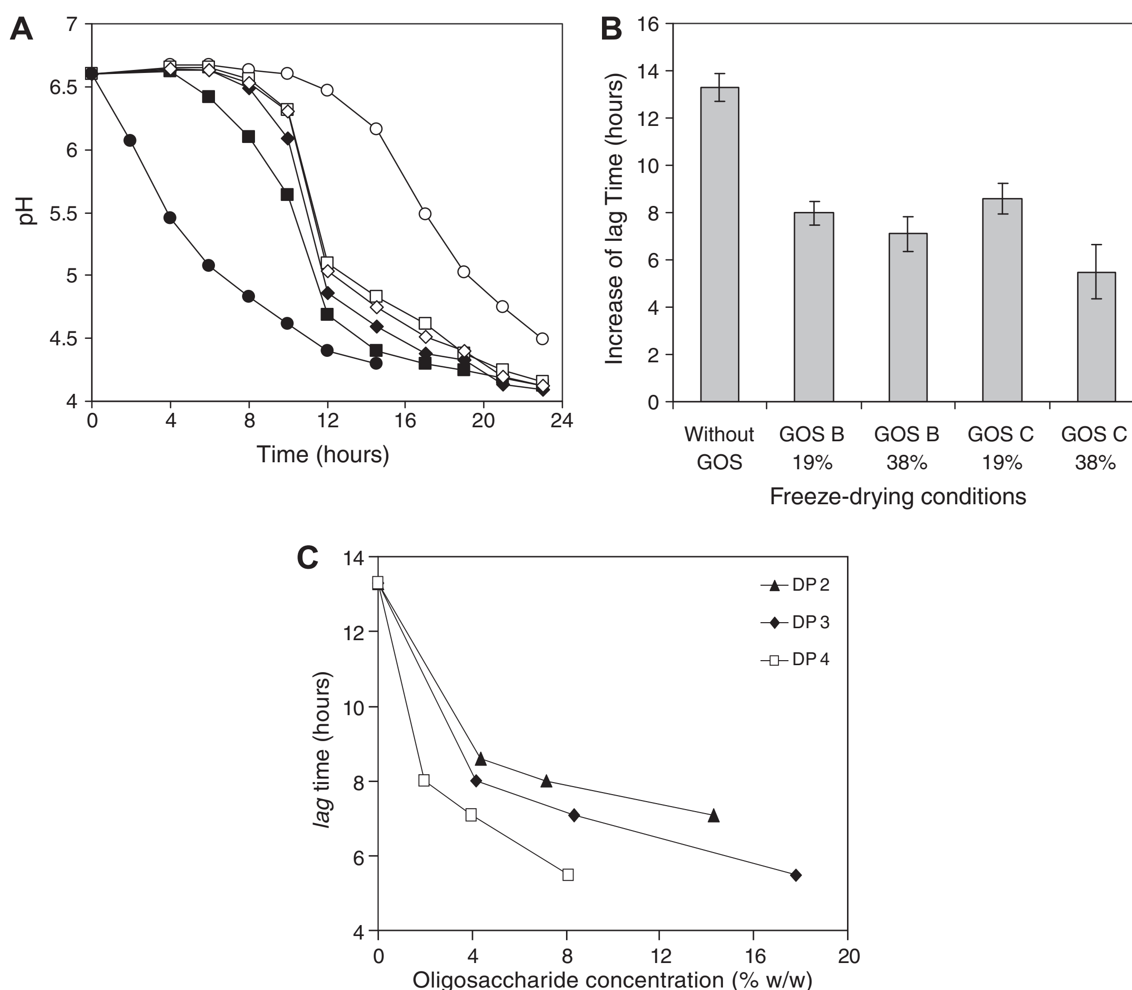

Fig. 3. (A) Growth kinetics of *L. delbrueckii* subsp. *bulgaricus* freeze-dried in different conditions: Control (●), in the absence of GOS (○), GOS B 19% (◇), GOS B 38% (♦), GOS C 19% (□), GOS C 38% (■). Kinetics were followed determining the decrease of pH as a function of time. Milk was used as culture medium. The concentration of GOS is expressed as g solid/100 g of solution; (B) Increase in the *lag* time with respect to the control (non- freeze-dried microorganisms) of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 grown in milk after being freeze-dried in different conditions ($P < 0.05$). (C) Decrease in the *lag* time as a function of the relative concentration of each galacto-oligosaccharide component in the each of the GOS mixtures (GOS B 19% and 38% and GOS C 19% and 38%). (▲), DP2; (♦), DP3; (□), DP4.

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$$\log N/N_0 = -kt \quad (1)$$

where N: CFU after storage; N_0: CFU after freeze-drying and before storage; k: rate constant of microbial inactivation; t: time of storage.

The rate constant "k" of microbial inactivation was obtained from the linear regression in each condition analyzed [GOS B and C 19% (broken line); GOS B and C 38% (continuous line), at a storage temperature of 5 or 25 °C].

Table 3 summarizes the values of "k" for each condition. According to these results, "k" is highly dependent on the storage temperature, low temperatures being the most suitable for conservation. The "k" values also indicate that cryoprotection mediated by GOS C 19% (storage temperature: 5 °C) is the best condition for storage. In addition, cryoprotectant effect of GOS B at both concentrations analyzed is similar and slightly higher than the one observed for GOS C at 38%.

Discussion

GOS are carbohydrate-based food ingredients whose prebiotic properties have been demonstrated [7]. They have been recognized as soluble dietary fiber and enclose interesting technological features [44]. Some authors have reported a protective role of prebiotic fibers (all of them being polyhydroxylated compounds) in the preservation of lactic bacteria during freeze-drying or vacuum drying [16,32,34,37,38].

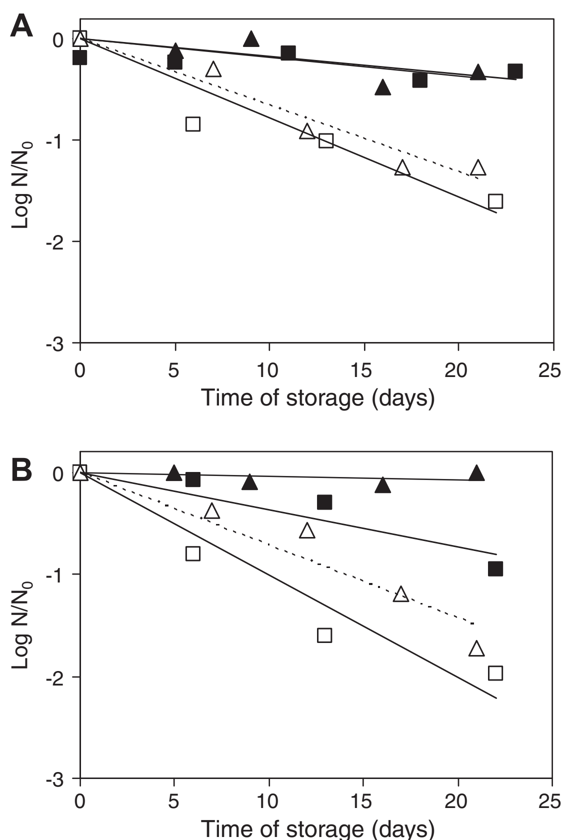

Fig. 4. Survival of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 after freeze-drying in the presence of GOS and stored in a desiccator over silica gel at 5 or 25 °C. The N/N_0 *vs* time after freeze-drying is plotted for all the conditions assayed. N: CFU after storage, N_0: CFU after freeze-drying and before storage. (A) Freeze-drying in the presence of GOS B: GOS B 19% at 5 °C (▲), GOS B 19% at 25 °C (△), GOS B 38% at 5 °C (■), GOS B 38% at 25 °C (□) ($P < 0.05$); (B) Freeze-drying in the presence of GOS C: GOS C 19% at 5 °C (▲), GOS C 19% at 25 °C (△), GOS C 38% at 5 °C (■), GOS C 38% at 25 °C (□) ($P < 0.05$).

Table 3
Constant of viability loss (k) of *L. bulgaricus* CIDCA 333 freeze-dried in the presence of GOS and stored at different temperatures.

Temperature (°C)	Freeze drying conditions			
	GOS k (day^{-1}) B 19%	GOS k (day^{-1}) B 38%	GOS k (day^{-1}) C 19%	GOS k (day^{-1}) C 38%
5	0.016 ± 0.004	0.018 ± 0.002	0.004 ± 0.002	0.037 ± 0.003
25	0.066 ± 0.005	0.078 ± 0.010	0.071 ± 0.010	0.101 ± 0.012

Three hypotheses have been raised to explain the protective role of polyhydroxylated compounds, such as sugars, in the preservation of biomolecules: (a) The direct interaction of the sugar moiety with the polar residues of macromolecules in the dried state, usually by hydrogen bonding, resulting in the maintenance of macromolecules in a physical state similar to that in the presence of a water excess. (b) The formation of glasses in the dried state (vitrification hypothesis). Sugar glass matrices can improve storage stability by raising the Tg (glass transition temperature) of the starter culture preparation, so that cells survive better during storage under a given condition [36]. (c) A third mechanism proposes that, in the presence of water, sugars are excluded from the surface, which may concentrate residual water molecules close to the biomolecular surface, thus preserving to a large extent its solvation and native properties [1,47]. It is possible that the three mechanisms operate simultaneously during the dehydration-rehydration processes.

Considering the latter mechanism and the values of water activities shown in Table 2, GOS may maintain the levels of residual water necessary to preserve the cellular structures from damage in the freeze-dried microorganisms, thus allowing for an appropriate preservation and avoiding unnecessary damage.

From a chemical point of view, GOS are polyhydroxylated compounds and their efficiency in bacterial preservation could be explained on the basis of the vitrification and water replacement hypotheses.

According to our results, the commercial GOS preparations containing the highest proportion of GOS (GOS B and C) were the most efficient in the protection of cells during desiccation, being DP3 and DP4, the GOS components with the highest cryoprotective capacity (Figs. 1 and 3C). On the contrary, GOS A was the one with the weakest protective capacity. This minor efficiency of GOS A is due to the lower concentration of galacto-oligosaccharides in this product (27%) (Table 1). The slight difference in the protection effect mediated by GOS B and C might be due to differences in the proportion of DP-3 and DP-4 with regard to the other GOS components (Table 1).

Cacela et al. [4] reported the effect of different families of oligosaccharides (fructans, malto-oligosaccharides and manno-oligosaccharides) on the preservation of liposomes upon freeze-drying. They found that structural characteristics of the different oligosaccharides and their chain-length may determine the extent to which they are able to interact with and protect membranes during drying. Though, the effect of GOS though, has not been studied up to now.

According to Fig. 2, the protective effect of the different GOS preparations during freeze-drying was quite similar. In regard to their capacity to grow in milk after rehydration, GOS C was more efficient than GOS B, thus indicating that the higher concentration of GOS has a strong influence in the recovery of microorganisms after damage. In spite of that, when considering long term preservation (Fig. 4), GOS B was more efficient, especially for low temperature storage. According to Figs. 3 and 4, the protective effect of GOS C is dependent on the concentration whereas that of GOS B does not. Noticeably, the protectant capacity of GOS C 19% is similar to that of GOS B at both concentrations, but is much lower than

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that of GOS C 38%. Considering that the difference in the composition of GOS B and GOS C is mainly due to balance between DP-3 and DP-4 (Table 1), it is reasonable to assign the greater protectant capacity of GOS C 38% to the higher concentration in DP-3. Structural differences between oligosaccharides play an important role not only in their ability to interact with biomolecules [4], but also in their ability to form glasses where biomolecules are embedded [18,36]. Further analyses are being carried out to correlate structural and thermo dynamical differences between the GOS preparations studied in this work with their effect in bacterial cryoprotection.

Conclusions

In this work, we have demonstrated that commercial GOS preparations are very efficient in the cryopreservation of *L. bulcaricus* CIDCA 333. This novel role of GOS as protective molecules opens up several valuable perspectives. Considering the physico-chemical and nutritional properties of GOS, their interaction with probiotics may be useful for the development of commercial synbiotic products, which could be incorporated into different foods (i.e.: infant formulas, powders containing probiotics in combination with prebiotics, which may be useful as functional food ingredients for the manufacture of probiotic foods).

In addition, the use of GOS has another important advantage. They are produced by enzymatic synthesis with β-galactosidases with transgalactosylation activity, using lactose as substrate [39], lactose being generally obtained from whey, which is a major by-product of cheese and casein industries and contains most of the lactose in milk [31]. The use of whey represents a very useful way to give an added value to effluents that because of their high biochemical oxygen demand (BOD) are costly to treat [23]. Therefore, the use of lactose from whey also represents an additional value from an environmental viewpoint and the production of functional food ingredients from it certainly represents a higher value than the conventional uses of lactose and whey [19].

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