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Effect of physical properties on the stability of *Lactobacillus bulgaricus* in a freeze-dried galacto-oligosaccharides matrix

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ABSTRACT

The ability of galacto-oligosaccharides (GOS) to protect *Lactobacillus delbrueckii* subsp. *bulgaricus* upon freeze drying was analyzed on the basis of their capacity to form glassy structures. Glass transition temperatures (T_g) of a GOS matrix at various relative humidities (RH) were determined by DSC. Survival of *L. bulgaricus* in a glassy GOS matrix was investigated after freezing, freeze drying, equilibration at different RHs and storage at different temperatures. At 32 °C, a drastic viability loss was observed. At 20 °C, the survival was affected by the water content, having the samples stored at lower RHs, the highest survival percentages. At 4 °C, no decay in the cells count was observed after 45 days of storage. The correlation between molecular mobility [as measured by Proton nuclear magnetic resonance (^{1}H NMR)] and loss of viability explained the efficiency of GOS as cryoprotectants. The preservation of microorganisms was improved at low molecular mobility and this condition was obtained at low water contents and low storage temperatures. These results are important in the developing of new functional foods containing pre and probiotics.

1. Introduction

Development of new functional foods containing probiotics and prebiotics, is of great interest because of their significant health benefits (Gibson et al., 2004; Wang, 2009). For this purpose, the preservation of lactic acid bacteria is essential to obtain concentrated viable starters, being freeze drying one of the most used methods (Meng et al., 2008; Morgan et al., 2006). During the preservation processes, the decrease of water activity produces damages on the cellular structures, decreasing the bacterial viability. To prevent these damages, sugars are usually incorporated as cryoprotectants (Carvalho et al., 2004). The protective effect of sugars has been ascribed to their ability to replace water during the dehydration, maintaining the biological structures in hydrated conditions (Crowe et al., 1992; Leslie et al., 1995; Santivarangkna et al., 2008). Another mechanism of protection is the ability of sugars to form glassy matrices, in which the high viscosity and low mobility restrict molecular interactions (Lodato et al., 1999). In this regard, the long term storage of dried products must be optimized taking into account the glass transition temperatures (T_g) at different water contents (Higl et al., 2007; Miao et al., 2008). The T_g value separates the supercooled from the glassy state (Roos, 1995) and is indicative of the degree of molecular mobility of the amorphous matrix. However, mobility of small molecules within the matrix is possible (*i.e.:* water, gases and small organic molecules) (Tromp et al., 1997; Schoonman et al., 2002). NMR relaxation has been proposed as a valuable method to understand the relationship between the molecular mobility of water and sugars, the moisture content and temperature (Hills et al., 2001).

GOS are carbohydrate-based well-known food ingredients with prebiotic properties, chemically synthesized by transgalactosylation of lactose (Playne and Crittenden, 2009; Neri et al., 2009; Vera et al., 2011). They are composed of a variable number of galactose units linked to a glucose unit, with a range from two to eight monomeric units (Neri et al., 2009). In addition to the prebiotic properties, the ability of GOS to act as cryoprotectants has been recently reported (Tymczyszyn et al., 2011). Tymczyszyn et al. (2011) demonstrated that commercial GOS preparations are very efficient in the cryopreservation of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Starters of *L. bulgaricus*, usually preserved by freezing, freeze drying and spray drying, are widely used in the elaboration of dairy products (Texeira et al., 1997). *L. bulgaricus* is known by its high sensitivity toward any kind of stress (Texeira et al., 1997) and according to our previous studies, strain CIDCA 333 is particularly sensitive (Tymczyszyn et al., 2007; 2011).

From a physical perspective, GOS are polyhydroxilated compounds and their efficiency as cryoprotectants could be explained on the basis of the vitrification and water replacement hypotheses.

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Nomenclature

ANOVA	Analysis of variance
CFU	Colony forming units
DP	Degree of Polymerization
DSC	Differential scanning calorimeter
FC	Flow cytometer
FID	Free induction decay analysis
GOS	Galacto-oligosaccharides
^{1}H NMR	Proton nuclear magnetic resonance
k	Inactivation constant
MRS	de Man, Rogosa, Sharpe
N	CFU of the sample under study
N_0	CFU of sample at time = 0
PI	Propidium iodide
RH	Relative humidity
t	time of storage
T	temperature of storage
T_2	Relaxation times
T_g	Glass transition temperature

However, the thermophysical properties of GOS have been little explored (Torres et al., 2011). Considering this, the aim of this paper was to get an insight on the thermophysical properties of GOS matrices containing *L. bulgaricus* CIDCA 333 as a support for future developments of new functional foods. For this reason, we determined the ability of commercial GOS to protect *L. bulgaricus* upon freeze drying and storage at different RHs, taking into account their effect on bacterial membranes and the formation of glassy structures. In order to perform a complete evaluation of the protectant properties of GOS, the recovery of cells after different times of storage at various RHs was correlated with the T_g and molecular mobility.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 333 was isolated from a fermented product (Gómez-Zavaglia et al., 1999). The strain was maintained frozen at −80 °C in 120 g L^{-1} non-fat milk solids. Microorganisms were grown in MRS broth at 37 °C (De Man et al., 1960).

2.2. GOS

A commercial syrup, Cup Oligo H-70® (Kowa Company, Tokyo, Japan) kindly donated by Kochi S.A. (Santiago, Chile) was used for the experiments. The syrup contained 75% of GOS of different DP: 4% of high-molecular-weight oligosaccharides (DP ≥ 5); 21% of tetrasaccharides DP 4; 47% of trisaccharides DP 3; 23% of disaccharides (DP2) and lactose, and 5% of monosacharides, including glucose and galactose (Tymczyszyn et al., 2011).

2.3. Preparation of samples for freeze drying

One-milliliter cultures in the stationary phase (grown in MRS broth at 37 °C overnight to obtain approximately 10^9 CFU/mL) were harvested by centrifugation at 4000 *g* for 10 min. The pellets were washed twice with sodium chloride 0.85% w/v, and resuspended in 1 mL of 20% (w/w) aqueous solutions of GOS, previously sterilized using 0.2 μm sterile filters. As reported before (Tymczyszyn et al., 2011), this GOS concentration allows the highest bacterial recovery.

2.4. Freeze drying procedure

Aliquots of 1 mL containing cell suspensions in the presence or absence of GOS and pure GOS solutions were transferred into 5 mL glass vials under aseptic conditions and frozen for 48 h at −20 °C. A freeze-drier Alpha 1–4 LD/2–4 LD-2 (Martin Christ, Gefriertrocknungsaniagen GmbH, Osterode, Germany) operated with the condenser at −84 °C at a chamber pressure of 0.04 mbar was used. The freeze drying process lasted for 48 h.

2.5. Humidification procedure

Freeze-dried matrices were equilibrated for 15 days in atmospheres of the following saturated salts: LiCl, KCH_3COO, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, NaCl and $(NH_4)_2SO_4$, giving relative humidities (RH) 11, 22, 33, 44, 52, 75 or 80%, respectively.

2.6. Glass transition temperatures

Glass transitions were determined by DSC (onset values, heating rate: 10 °C min^{-1}) using a DSC 822^e Mettler Toledo calorimeter (Schwerzenbach, Switzerland), calibrated with indium, lead and zinc. Hermetically sealed 40 μL medium pressure pans were used (an empty pan served as reference). Thermograms were evaluated using Mettler Star^e program. An average value of at least two replicates was reported. The standard deviation for the glass transition temperature measurement was ± 1 °C.

2.7. Bacterial plate counts

Viable bacterial plate counts were determined before and after freezing, after freeze drying and after equilibration at different RHs. Dried microorganisms were rehydrated in 1 mL of 0.85% (w/v) sodium chloride for 15 min. Bacterial suspensions were serially diluted and plated on MRS agar plates. Bacterial counts were determined after 48 h of incubation at 37 °C.

2.8. Storage

Equilibrated samples at 11, 22 and 33% RH were sealed and stored at 4, 20 or 32 °C for 45 days. The recovery of cells after different times of storage was analyzed by plate counts.

2.9. Water content determination

Karl Fischer (KF) titration was carried out at 25 ± 1 °C with a Karl Fischer titrator DL 31 from Mettler Toledo (Zurich, Switzerland), applying the one-component technique with Hydranal Titrant Composite 5 from Riedel-de Haën (Seelze, Germany). Methanol/formamide mixture 95 (1:1) was used as solvent, and they were purchased from Merck (Darmstadt, Germany). Sample sizes were approximately 100 mg. The water content for samples equilibrated at 11, 22 and 33% was 4.84 ± 0.03, 6.20 ± 0.01 and 7.53 ± 0.01% (dry basis), respectively.

2.10. Membrane damage

Cells were incubated with the DNA-binding probe propidium iodide (PI), which only penetrates bacterial cells when membranes are damaged. Stock solutions of PI (Molecular Probes, Leiden, The Netherlands) were prepared in distilled water to a final concentration of 10 mg/mL and stored in the dark at 4 °C. PI was added to a final concentration of 0.5 mg/mL. For flow cytometric analysis, the concentration of microorganisms in the samples was adjusted to approximately 10^6 CFU/mL. The cells were incubated with the probe for 5 min at room temperature and the PI uptake was performed by flow cytometry (FACSCalibur, CellQuest software; Becton Dickinson,

Mountain View, CA, USA), following the procedure reported by Wouters et al. (2001). Samples were processed so that 10,000 events were collected for each sample, and the event rate was less than 300 events/s. Non-treated stained cells were used as negative controls and cells heated for 3 min at 80 °C were used as positive controls.

2.11. Molecular mobility

A Bruker PC 120 Minispec pulsed proton nuclear magnetic resonance (^{1}H NMR) instrument, with a 0.47 T magnetic field operating at a resonance frequency of 20 MHz, was used for measurements. Equilibrated samples were removed from the desiccators, placed into 10 mm diameter glass tubes and returned to the desiccators for 24 h prior to analysis.

The spin–spin relaxation time (T_2) associated to the fast relaxing protons (related to the solid matrix and to water interacting tightly with solids) was measured using a free induction decay analysis (FID) after a single 90° pulse. The decay envelopes [protons signal intensity (I) versus experimental time (t)] were fitted to mono-exponential behavior with the following equation:

$$I = A \exp(-t/T_2) \quad (1)$$

where T_2 is the relaxation time of protons in the polymeric chains of the sample and of tightly bound water, and A is a constant. Since no 180° refocus pulse was used in the experiments, the spin–spin relaxation time constants are apparent relaxation time constants, *i.e.* T_2^*. However for solid samples (like ours), we can consider that the intrinsic T_2 is very close to the T_2^* as reported previously by Fullerton and Cameron (1988). Therefore, T_2 was used for convenience.

2.12. 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). Comparison of means by Tukey methods was tested, and if $P<0.05$ the difference was considered statistically significant.

3. Results and discussion

Fig. 1 shows the T_g values for the freeze-dried matrices after humidification at different RHs. The presence of microorganisms did not modify the T_g of the GOS matrix. The T_g values obtained were similar to those of other sugars used as cryoprotectants (*i.e.:* trehalose, lactose), and higher than T_g of sucrose (Miao et al., 2008). However, they were lower than the T_g reported by Torres et al. (2011) for another freeze-dried GOS system. The higher proportion of DP4-GOS (42%) and DP5-GOS (8%) in the samples reported by Torres et al. (2011) can explain the observed differences.

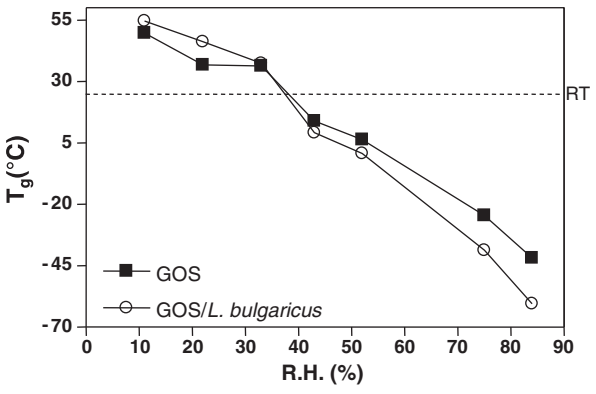

Fig. 1. Onset glass transition temperatures (T_g), of GOS (■) and GOS/*L. bulgaricus* (○) after equilibration at different RHs. Dashed line shows room temperature.

Since the glassy state at room temperature is maintained at RHs below 33%, (Fig. 1), the rest of the studies were performed at 11, 22 and 33% RH. Table 1 depicts the viable cell counts and membrane damage (measured as PI uptake) after different treatments. Non-treated microorganisms and freeze-dried samples in the absence of GOS were used as controls. The viability of cells after freezing and freeze drying with GOS did not show statistically significant differences when compared to the non-treated microorganisms ($P>0.05$). The bacterial counts for samples equilibrated at 11 and 22% were significantly lower than those of the non-treated microorganisms ($P<0.05$), but not than those of frozen and freeze-dried cells ($P>0.05$). The samples equilibrated at 33% RH showed a significantly lower bacterial count than those obtained after freezing, AND freeze drying ($P<0.05$), but not than those equilibrated at 11 and 22% RH ($P>0.05$).

Taking into account that the membrane is the main target of damage during dehydration (Lievense et al., 1994), and that sugars may interact with lipid membranes protecting them from damage (Crowe et al., 1992; Oldenhof et al., 2005; Schwab et al., 2007), the incorporation of PI after dehydration–rehydration was determined to evaluate the membrane integrity (Table 1). After freeze drying and even more after equilibration at different RHs, the high level of labeled cells (above 80%) denotes a severe damage in the bacterial membrane ($P<0.05$), thus indicating that GOS do not prevent membrane damage during dehydration. In spite of that, this damage is not lethal since microorganisms can recover and grow (Table 1). Analogous results were reported in dehydrated *L. bulgaricus* (Tymczyszyn et al., 2007), *Lactobacillus reuteri* (Schwab et al., 2007) and *Lactobacillus helveticus* (Santivarangkna et al., 2007) with different matrices. Texeira et al. (1997) have found that damages produced in membrane lipids are reversible, whereas damages produced in proteins are not. Protein–sugar and membrane–sugar interaction have been widely studied for many sugars to understand their protective effect (Cacela and Hincha, 2006; Crowe et al., 1992; Oldenhof et al., 2005; Santivarangkna et al., 2008; Schwab et al., 2007) but the interactions of GOS with these structures have not been studied yet.

To assess the stability of *L. bulgaricus* upon storage at different RHs, the viability loss was studied along time at 4, 20 and 32 °C (Fig. 2). The number of viable cells at 4 °C remained constant at least for 45 days (Fig. 2a). At high temperatures (32 °C), an important detrimental effect was observed, regardless the water content (Fig. 2c), and when cells were stored at room temperature the survival was clearly dependent on RH (Fig. 2b), being cells preserved at the highest water content, the most affected ones. Although the GOS matrix was in the glassy state at the three temperatures tested, vitrification was not sufficient to maintain bacterial viability. Similar results were

Table 1
Logarithm of CFU of microorganisms recovered and % of PI uptake before and after freezing, freeze drying and humification at different RHs in the presence of 20% (w/w) GOS. Different letters (a, b, c and d) denote statistically significant differences ($P<0.05$).

Treatment	Log CFU/mL	% PI uptake
Control (non-treated microorganisms)	9.00 ± 0.5 (a)	10.6 ± 4.2 (a)
Freeze dried in the absence of GOS	3.50 ± 0.3 (d)	99.25 ± 2.0 (d)
Freezing	8.41 ± 0.5 (a, b)	24.62 ± 0.9 (b)
Freeze drying with GOS	8.04 ± 0.7 (a, b)	83.12 ± 2.7 (c)
Freeze drying with GOS and humidification at 11% RH	7.70 ± 0.4 (b, c)	97.16 ± 1.6 (d)
Freeze drying with GOS and humidification at 22% RH	7.54 ± 0.3 (b, c)	98.33 ± 2.0 (d)
Freeze drying with GOS and humidification at 33% RH	6.60 ± 0.6 (c)	97.61 ± 2.0 (d)

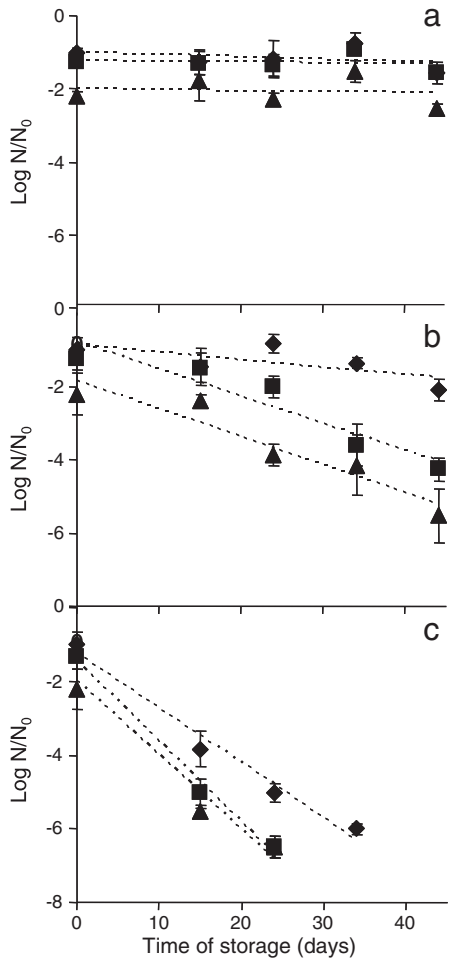

Fig. 2. Viability loss of *L. bulgaricus* freeze-dried in the presence of 20% (w/w) GOS and equilibrated at 11 (♦), 22 (■) and 33 (▲) % RH, as a function of storage time, at 4 °C (a), 20 °C (b) and 32 °C (c) . N = CFU of humificated samples after storage; N_0 = CFU after freeze drying. Dashed line shows linear regression for each condition.

obtained by Higl et al. (2007) during the storage of *L. paracasei* in a lactose matrix. Miao et al. (2008) observed the loss of viability of *L. rhamnosus* after 40 days of storage in trehalose and lactose glassy matrices. In a previous work carried out on dried commercial yeast, we proposed that the heterogeneity of amorphous matrices may also be responsible for the lack of the apparent relationship between T_g and stability (Schebor et al., 2000).

To understand the influence of molecular mobility and glass transition on the viability loss of *L. bulgaricus* upon storage, the survival rate at different temperatures was correlated with the molecular mobility and the T_g. Hence, we investigated the influence of RH and storage temperature on the molecular mobility of the GOS systems by measuring the transverse relaxation times (T_2) by ^{1}H NMR after the application of a single 90° pulse. Different authors reported that this fast decaying T_2 can be attributed to protons from solid polysaccharides and from water molecules strongly interacting with the solid matrix by hydrogen bonding (Kalichevsky and Blanshard, 1992).

The microbial survival from Fig. 2, was analyzed using Eq. (2):

$$\log N/N_0 = k\,t \qquad (2)$$

where N: CFU of humificated samples after storage at different temperatures; N_0: CFU after freeze drying; *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 assayed.

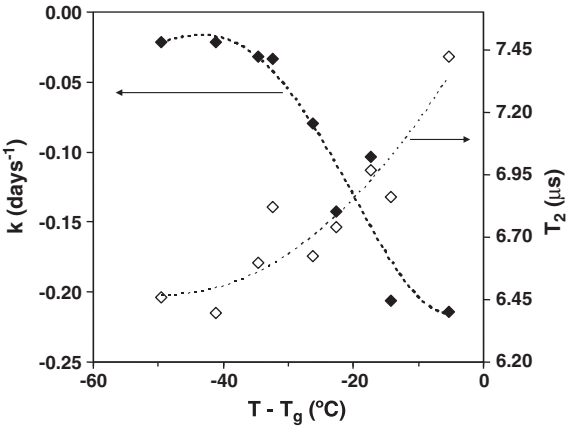

Fig. 3. Rate constant of microbial inactivation "*k*" (♦) and the T_2 relaxation times (◊) as a function of T–T_g.

In Fig. 3 the values of the rate constant of microbial inactivation (*k*) and the T_2 relaxation times were plotted versus the parameter T–T_g, where T is the storage temperature (4, 20 or 32 °C). This study was carried out at 11, 22 and 33% RH. There is a slight increase in the T_2 values with the increase of RH and temperature. The main increase in T_2 was observed at 33% RH (not shown). A clear trend in which the increase in molecular mobility coincides with the decrease in the viability constant was observed.

The molecular mobility as determined by ^{1}H NMR is a representative parameter of the matrix at a molecular level whereas the glass transition measurements are important as macroscopic parameters for the stabilization of the matrix. When biomaterials sensitive to degradation are considered, the molecular mobility can be directly correlated with the maintenance of biological properties in the dried systems. In the vitreous state, rotational and vibrational motions of molecules are present, and short moving with local relaxation may occur (Angell, 1988). These conditions would be enough to induce conformational changes in proteins and precipitation of salts, with deleterious effects on microorganisms. As is known, the water content and storage temperatures play an essential role when dehydrated products are to be developed.

The correlation between T_g and molecular mobility with the loss of *L. bulgaricus* viability found at different water contents and storage temperatures supports the cryoprotectant properties of GOS. There are few studies of thermophysical properties of cell–sugar matrix (Higl et al., 2007; Miao et al., 2008; Schebor et al., 2000), and there is only one work of thermophysical properties of GOS mixtures (Torres et al., 2011). In this work, we performed for the first time, an integrated research giving a scientific background for the development of new functional foods.

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