Influence of oligosaccharides on the viability and membrane properties of *Lactobacillus reuteri* TMW1.106 during freeze-drying

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Abstract

Freeze-drying is a process commonly used in starter culture preparation. To improve the survival rate of bacteria during the process, cryoprotectives are usually added before freezing. This study investigated the influence of the addition of sucrose, fructo-oligosaccharides (FOS), inulin and skim milk on the viability and membrane integrity of *Lactobacillus reuteri* TMW1.106 during freezing, freeze-drying and storage. The effect of drying adjuncts on survival was correlated to their interaction with bacterial membrane by determination of the parameters membrane fluidity and membrane lateral pressure. Sucrose, FOS and skim milk significantly enhanced survival of exponential-phase cells of *L. reuteri* during freeze-drying. Cellular viability during storage of exponential-phase cells remained highest for cells dried in the presence of skim milk and inulin. Membranes of these cells were completely permeabilized after freeze-drying. The application of FOS significantly improved survival of stationary phase cells of *L. reuteri* TMW1.106 after freeze-drying and storage. This increased viability of *L. reuteri* TMW1.106 in the presence of FOS correlated to improved membrane integrity. Fructo-oligosaccharides and fructans, but not gluco-oligosaccharides interacted with membrane vesicles prepared from *L. reuteri* TMW1.106 as indicated by increased membrane lateral pressure in the presence of FOS and fructans. Increased membrane integrity of stationary phase *L. reuteri* TMW1.106 was attributed to direct interactions between FOS and the membrane which leads to increased membrane fluidity and thus improved stability of the membrane during and rehydration.

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Freeze-drying is a common process for starter culture preparation in dairy and food industries. However, freeze-drying and subsequent storage is accompanied by a decline in cell viability. The extent of viability loss depends on strain properties as well as the growth conditions and growth state. Furthermore, technical parameters such as cooling rate and temperature, the presence of cryoprotectives, and the type of rehydration buffers affect cell survival.

Cryoprotectives are used to prevent or reduce cell death during freeze-drying and subsequent storage. Skim milk is widely applied as a cryoprotective in the starter culture industry. Moreover, the application of a variety of chemical compounds either alone or in addition to skim milk has been tested (for review, see [3,7]). For example, the monosaccharides glucose, sucrose, maltose, lactose and trehalose as well as maltodextrins increased viability of lactic acid bacteria during freezing and freeze-drying [11,22]. The protective effect of sugars during freeze-drying is attributed to direct interactions with proteins and membranes. Moreover, if the water content of the samples is low, sugars will form a glassy matrix that is characterized by high viscosity and low mobility [10]. The addition of the polysaccharides inulin, dextran and polydextrose to skim milk powder protected *Lactobacillus paracasei* NFBC338 during freeze-drying, the stabilizing effects became most apparent during storage [13]. The addition of the prebiotics FOS,
inulin and polydextrose also enhanced survival of lactobacilli during spray-drying [1,8].

Death of bacteria during freeze-drying is attributed to osmotic shock and membrane injury that are caused by intracellular ice formation and recrystallization [14]. In plants, recent evidence suggests that fructans play a role in stress tolerance during cold acclimatation and dessication [12,19].

Fructans of the inulin and the levan type as well as fructo-oligosaccharides, but not glucans, have been shown to directly interact with phospholipids of model mono- and bilayer membranes. These fructan–membrane interactions stabilize membranes during air-drying and freeze-drying [15,16,30,28]. Strains of L. reuteri harbour fructosyltransferases that form fructo-oligosaccharides, inulin and levan from sucrose [24,26,27]. L. reuteri TMW1.106 possesses at least one glucosyltransferase (GtfA) and one fructosyltransferase (Inu) and produces the fructo-oligosaccharides kestose and nystose from sucrose [24]. The expression of fructosyltransferases in L. reuteri is induced upon exposure to environmental stress, indicating a role of fructans in the bacterial stress response [24].

To evaluate the hypothesis that fructan synthesis plays a role in the protection against stress, this study investigated whether sucrose fructans differing in their degree of polymerisation (DP) act as cryoprotective substances during freeze-drying and dry storage of L. reuteri TMW1.106. The integrity of bacterial membranes was determined to ascertain that cryoprotective effects of sugars are related to membrane protection. Furthermore, the effects of sugars on two membrane parameters, lateral pressure and membrane fluidity, were established.

Materials and methods

Buffer

Inulin from chicory (SIGMA; Oakville, Canada), fructo-oligosaccharides (FOS) powder (Source natural; Scotts Valley, USA), sucrose (Difco; Oakville, Canada) and isomalto-oligosaccharides IMO (BioNeutra; Edmonton, Canada) were added to 50 mM phosphate buffer pH 6.2 to a level of 7.5% and sterilized by filtration. Skim milk was prepared rehydration of skim milk powder (Difco) to a level of 5% and sterilized. The light absorption of carbohydrates used as drying adjuncts was determined to verify that the carbohydrates do not interfere with the fluorescence measurements described below. Initially, levan purified from culture supernatant L. reuteri LTH5448 according to Schwab and Gänzle (24) was included in the experiments, however, coloured contaminants in the levan preparation interfered with the fluorescence-based assays of membrane properties (see below). Therefore, this high molecular weight fructan was not considered further.

**HPLC analysis of oligo- and polysaccharide preparations**

FOS, IMO and inulin were resuspended in ddH2O and analysed by HPLC. Carbohydrates were separated using a Supelecosil™ LC-NH2 (5 µm, Supelco; Oakville, Canada) column and detected with an evaporative light scattering detector (Alltech 500 ELSD, Alltech; Guelph, Canada). HPLC grade water (A) and acetonitril (B) were used as mobile phase (0 min 90% B, 1.5 ml min−1; 25 min 75%, 1.5 ml min−1; 26 min 50% B, 0.9 ml min−1; 32 min 50% B, 0.9 ml min−1; 33 min 30% B, 0.9 ml min−1; 40 min 30% B, 0.9 ml min−1; 45 min 90% B, 1.5 ml min−1). Peaks were assigned using glucose, maltose, isomaltose, maltotriose, isomaltotriose, fructose, sucrose (all from Sigma) and kestose (Fluka; Oakville, Canada) as standards.

Culture preparation

For preparation of exponentially growing cultures, overnight cultures of L. reuteri TMW1.106 were subcultured in modified de Man, Rogosa Sharpe medium (mMRS), [25] substituted with 10 g L−1 maltose, 5 g L−1 fructose, 5 g L−1 glucose (all Sigma) and grown at 37°C to an optical density (OD)595 nm of 0.40 corresponding to approximately 106 CFU ml−1. Cells were harvested, washed once in 60 mM phosphate buffer, pH 6.5 (PB), PB with additives or skim milk and resuspended in the corresponding buffer. stationary phase cells were harvested after 18 h incubation, washed in PB containing the additives and the optical density was adjusted to OD595 nm 0.4. Three milliliter of cell suspension were frozen at −80°C.

Freeze-drying

Frozen cells were freeze-dried using a Labconco FreeZone® 4.5 Liter Freeze Dry System (Labconco; Kansas City, USA) at a collector temperature of −50°C. Cell suspensions were freeze-dried in duplicate. Freeze-drying of stationary and exponential-phase L. reuteri TMW1.106 was repeated three and four times, respectively.

Dry storage

Duplicate samples of each treatment were wrapped in parafilm and stored at room temperature in the dark for 14 days.

Cell counts

Viable cell counts were determined before freezing, after freezing, after freeze-drying and after storage. Dried cells were resuspended in the appropriate volume of PB. Cell suspensions were serially diluted in phosphate buffered saline (SIGMA) and plated on mMRS agar plates. Cell counts were determined after 24 h of anaerobic incubation at 37°C.

Membrane integrity

The dye exclusion assay Live/Dead BacLight Bacterial Viability kit (Molecular Probes; Burlington, Canada) was applied to investigate membrane integrity. Heat-treated (10 min, 80°C) and untreated cells from exponential and
stationary growth phase (OD_{595nm} adjusted to 0.4) were mixed in increments of 20% to establish calibration curves. Frozen and rehydrated freeze-dried and stored cells were washed once and resuspended in PB. Propidium iodide (10 µM) and Syto9 (1.67 µM) were added and the suspensions were incubated at room temperature for 15 min in the dark. Fluorescence emission spectra (excitation 470 nm, emission 490–670 nm, BW 5 nm) were recorded using a Jasco FP6300 spectrophotometer (Jasco; Victoria, Canada) at room temperature. Membrane integrity was calculated as the ratio of Syto9 fluorescence (integrated intensity between 510 and 540 nm) to propidium iodide fluorescence (integrated intensity between 620 and 650 nm). Results are reported as percentage of intact membranes. Numerically negative membrane integrities are reported in those cases when the fluorescence ratio (membrane permeabilisation) of freeze dried samples was lower than that of the heat-treated controls. Membrane integrity could not be determined in samples containing skim milk because of the presence of nucleic acids in skim milk.

**Determination of membrane fluidity**

Membrane fluidity was determined using the fluorescence probe Laurdan (6-dodecanoyl-2-di-methyl-amino-naphtalene, Molecular Probes) according to [21] with modifications. Previous reports confirmed that the fluidity measurements in bacterial membranes with Laurdan fluorescence and Fourier-transformed infrared spectroscopy provide consistent results in the presence of lactose and sucrose [21]. Briefly, stationary cells were washed twice in PB and PB containing 7.5% of sucrose, FOS, inulin or IMO. The OD_{595nm} was adjusted to 1.5. Laurdan was added at a final concentration of 40 µM and cells were incubated at 30 °C for 30 min in the dark. Cells were washed twice in PB containing the additives and resuspended in the respective buffer. Emission spectra (emission 360 nm, excitation 380–520 nm, BW 5 nm) were recorded using a spectrophotometer. For each batch of labeled cells, spectra recorded at reference conditions (30 °C, no additives) were analysed to ascertain that Laurdan was quantitatively incorporated in the bacterial membranes. PB containing sucrose, FOS, inulin and IMO were added to obtain a final concentration of 7.5%, and emission spectra were recorded using a spectrophotometer (excitation 342 nm, emission 360–500 nm, BW 5 nm) over a temperature range from 10 to 50 °C. Changes in membrane lateral pressure were calculated as the ratio E/M (emission of excimer at 475 nm/emission of monomer at 435 nm). Five independent experiments were carried out. Because the numerical E/M values are subject to variation depending on the efficiency of dye incorporation into the membrane vesicles, results that are representative for all replicates are shown.

**Statistical analysis**

The difference between two means was calculated using the t-test software provided by the software package SigmaPlot 9.0. Means were considered significantly different when p < 0.05.

**Results**

**Purity of oligo- and polysaccharides**

The chicory inulin preparation contained no low-molecular weight compounds. The FOS powder was composed of sucrose (approx. 6%), kestose, nystose and higher fructo-oligosaccharides with a degree of polymerisation (DP) of up to 12. The IMO preparation contained isomaltose (approx. 23%), isomaltotriose and higher isomalto-oligosaccharides (data not shown).

**Survival and membrane integrity after freezing**

Stationary cells suspended in PB containing sucrose, FOS, inulin and skim milk showed a slight but not significant better viability after freezing than control samples (Fig. 1). All samples exhibited approximately 75–85% membrane integrity after freezing. Freezing of exponential-phase cells decreased the membrane integrity to less
than 15% and 25% independent of the presence of additives. In the absence of additives, the cell counts of dropped by two logs but about 10% of cells survived freezing in the presence of sucrose, inulin and FOS.

Survival and membrane integrity after freeze-drying

The addition of FOS provided the best protection during freeze-drying of stationary cells (Fig. 1) and the lowest viability and membrane integrity was observed in cells that were freeze-dried without additives. Stationary phase *L. reuteri* TMW1.106 freeze-dried in the presence of sucrose and inulin retained up to 10% membrane integrity, whereas cells freeze-dried in buffer containing FOS buffer had more than 20% membrane integrity. In contrast, numbers of exponentially phase cells dropped between 3.5 and 2 logs in the absence and presence of additives, respectively. Survival of *L. reuteri* TMW1.106 was significantly improved in samples containing sucrose, FOS and skim milk. The membrane integrity decreased to less than 5% in all treatments.

Survival and membrane integrity after storage

FOS significantly improved survival of stationary phase cells compared to control samples (Fig. 1). Even after storage, cell counts of stationary *L. reuteri* TMW1.106 decreased by 0.5 log only compared to the initial cell counts. Concurrently, membranes of these cells retained 20% membrane integrity, whereas membranes of cells freeze-dried and stored in the presence of sucrose and inulin where completely permeabilized. The viability of stationary *L. reuteri* TMW1.106 in PB, sucrose, inulin and skim milk decreased to a similar extend during storage. Cell counts of exponential-phase cells did not differ distinctly within treatments and their membranes were completely permeabilized after freeze-drying and storage.

Impact of fructans on membrane fluidity

The parameters membrane fluidity and membrane lateral pressure were determined in at least four independent experiments, and results shown represent the trends observed in all replicates. Temperature increase from 10 to 50°C resulted in decreased GP values in all samples, indicating increased membrane fluidity (Fig. 2). Inulin clearly reduced membrane fluidity compared to control samples. In contrast, the application of FOS resulted in increased membrane fluidity.

Impact of fructans on membrane lateral pressure

With increasing temperature, lateral pressure within the membrane increased (Fig. 3). Membrane pressure in vesicles in the presence of FOS and inulin were higher compared to controls at all temperatures. The presence of sucrose and IMO did not affect membrane lateral pressure.

![Fig. 1. Survival of stationary (a) and exponentially growing (b) *L. reuteri* TMW1.106 after freezing, freeze-drying and storage in PB or PB with 7.5% sucrose, FOS, inulin and skim milk powder. Membrane integrity of stationary (c) and exponentially (d) after freezing, freeze-drying and storage of *L. reuteri* TMW1.106 was determined using BactoLive/Dead Kit. *Means significantly (*p < 0.05) from control.](image-url)
Discussion

The impact of sucrose, FOS and inulin during, freezing freeze-drying and dry storage on the viability of L. reuteri TMW1.106 was investigated and compared with the protective effect of reconstituted skim milk. The effect of cryoprotectants on cellular viability was correlated to their effect on membrane integrity and to their interaction with bacterial membranes. In general, the addition of sugars protected L. reuteri TMW1.106 during freezing and freeze-drying compared to an isotonic solution. The protective effects of sucrose, FOS and inulin were similar or better than the effects of skim milk. The application of sucrose, trehalose and maltodextrins was previously shown to improve the viability of bacteria during drying [18,22].

Membranes are primary targets of freezing and desiccation injury and membrane damage is a catalyst of cell death during drying and dry storage [1,2,5,6]. In early stationary Escherichia coli and Bacillus thuringiensis, protective effects of sucrose and trehalose had been attributed to the stabilization of proteins and a decreased membrane phase transition temperature. The replacement of water between the lipid headgroups by trehalose prevented phase transition and leakage during rehydration [18]. The direct interaction of sucrose with membranes was suggested to contribute to the protective effects of sucrose on dried cells of Lactobacillus bulgaricus [22]. The decline in viability during storage of freeze-dried lactobacilli has been attributed to further membrane damage caused by oxidation and lipolysis [4]. In this study, a correlation between survival and the membrane integrity after freeze-drying of L. reuteri TMW1.106 was observed. Apparently, the protection of the bacterial membranes by cryoprotectants prevented sublethal membrane damage during freezing and minimised subsequent lethal cellular injuries during subsequent storage and rehydration.

The membrane integrity as well as the viability of stationary phase cells remained higher compared to exponentially phase cells. In many bacterial species, entry into the stationary growth phase is accompanied by profound structural and physiological alterations, including changes in expression levels of stress related proteins, membrane composition and cell wall structure that result in increased resistance to heat shock, oxidative, osmotic and acid stresses as well as high pressure [9,17,20,23]. The acquisition of a more resilient cell envelope upon entry into the stationary phase of growth likely contributes to the increased freezing and drying resistance in stationary phase L. reuteri TMW1.106. Moreover, these structural differences between exponential and stationary phase cells may account for variations in the protective effects of additives during storage. Starter cultures preparation in industrial fermentations generally employs cells grown to the stationary growth phase.

With stationary phase cells of L. reuteri TMW1.106, the highest values for membrane integrity and bacterial survival were obtained when the strain was freeze-dried in the presence of FOS. Fructans, but not glucans or gluco-oligosaccharides, directly interact with model monolayer and bilayer membranes and the interaction increases with increasing DP of the sugar molecule [15,22,28,30]. Using membrane vesicles prepared from L. reuteri TMW1.106, this study demonstrated that fructans also interact with complex bacterial membranes. The interaction increased with increasing chain length. In keeping with previous reports on the effect of fructans on the curvature stress in model membranes [30], fructans addition altered the curvature stress in L. reuteri membrane vesicles as indicated by increased membrane lateral pressure. The disaccharide sucrose or IMO did not affect membrane lateral pressure.
The beneficial effects of sucrose and trehalose during drying have been attributed to their ability to lower the liquid-crystalline/gel phase transition temperature [22]. Fructans maintain the liquid-crystalline state of anhydrous membranes and consequently prevent membrane damage during rehydration. This study provides evidence for an effect of FOS and inulin on the fluidity of bacterial membranes. The addition of inulin resulted in a shift to a more rigid membrane whereas addition of FOS increased the membrane fluidity. Sucrose addition did not alter the membrane state. Thus, the interaction between inulin-type fructans and the membrane appears to be optimal at specific degrees of polymerization. This observation is in contrast to earlier reports, stating that fructan does not influence the liquid-crystalline–gel phase shift under fully hydrated conditions [30]. However, these studies worked with high molecular weight levans rather than inulin and FOS and employed single component model membranes.

It was suggested that fructan synthesis contributes to the cold and drought tolerance of plants [12,19]. In L. reuteri, fructan and FOS synthesis is upregulated in response to environmental stressors, suggesting a comparable role of FOS and fructans in stress tolerance [24]. Accordingly, it was shown in this study that fructans protected L. reuteri TMW1.106 during freezing and drying. The protective effect of FOS, products of the fructosyltransferase reactions, was greater than the protective effect of its substrate, sucrose. Kestose production in liquid medium by L. reuteri did not exceed 4 g L⁻¹ [24], however, natural drought conditions are obviously connected with a concentration of bacterial metabolites. As fructosyltransferases are attached to bacterial cell membranes, which favours membrane–fructan interaction.

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References


