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Effect of lyoprotectants on β-glucosidase activity and viability of *Bifidobacterium infantis* after freeze-drying and storage in milk and low pH juices

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ABSTRACT

The benefit of disaccharide protectants for maintaining viability and β-glucosidase activity of *Bifidobacterium infantis* UV16PR during freeze-drying and storage in different food matrices was investigated. Protectants used were cellobiose, lactose, sucrose and trehalose.

At 5% concentration, cellobiose showed the best protective effect during freeze-drying. All protectants at 10% concentration significantly improved the viability and retention of β-glucosidase activity after freeze-drying and storage in food matrices. Regardless of the protectant used, no significant differences were observed after freeze-drying and storage for 2 weeks in milk, while in red-beet and grape juice, cellobiose and trehalose significantly enhanced β-glucosidase activity, viability and acid tolerance. Viability of cellobiose and trehalose protected cells in juices was comparable, whereas differences in retention of enzyme activity and acid tolerance after the storage in different juices were observed. In conclusion, various protectants increased the retention of β-glucosidase activity, viability and acid tolerance of freeze-dried *B. infantis* during storage in different food matrices. In order to choose protectants for probiotics for freeze-drying and food as probiotic carrier, not only their effect on survivability, but also the enzyme activity retention should be considered.

1. Introduction

Probiotics are defined as "live microorganisms, which when administered in sufficient numbers, confer a health benefit to the host" (FAO/WHO, 2001). Thus, probiotics are more and more frequently added to food. The most common food carriers for delivery of probiotics to the human body are dairy products, such as fermented milk, yoghurt, cheese and ice cream (Fritzen-Freire, Muller, Laurindo, & Prudencio, 2010; Ramchandran & Shah, 2010; Ranadheera, Baines, & Adams, 2010). Much less often, juices are used as carriers and much less has been reported regarding the stability of probiotics in juices at low pH values (Champagne & Gardner, 2008; Saarela, Virkajärvi, Alakomi, Sigvart-Mattila, & Mättö, 2006; Vinderola et al., 2012). Milk (non-fermented) and fruit juices are advantageous as food carriers for probiotics because they do not contain starter cultures which may compromise the survival of the probiotics (Dave & Shah, 1997; Ng, Yeung, & Tong, 2011). Fruit juices have the further advantage of being suitable for people with lactose intolerance.

Probiotics are added to food mainly as frozen or freeze-dried cultures. Freeze-dried cells are preferred because they are easier to handle than frozen cells. However, during drying the cells are exposed to stress which may lead to several injuries such as membrane injury, protein denaturation (including that of enzymes) and DNA damage (Lievense, Verbreek, Noomen, & van't Riet, 1994; Meng, Stanton, Fitzgerald, Daly, & Ross, 2008; Stummer et al., 2012). Incorporation of freeze-dried cells into food matrices is challenging because of the exposure to additional stress such as pH and oxygen (Saarela et al., 2006; Vinderola et al., 2012; Champagne et al., 2005). For preservation of probiotics during freeze-drying, sucrose, trehalose and lactose are widely used (Li et al., 2011; Stummer et al., 2012; Vinderola et al., 2012). Their protective effect during drying was shown to be strain-specific (Siaterlis,

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Deepika, & Charalampopoulos, 2009; Strasser et al., 2009). Sucrose and lactose also showed good protection of probiotics during storage in food matrices (Saarela et al., 2005; Vinderola et al., 2012).

Together with viability, the functionality of probiotics like enzyme activity and acid tolerance has to be considered. Some probiotic microorganisms including *Bifidobacterium* spp. exert β-glucosidase activity (Marotti et al., 2007; Otieno et al., 2006). Several groups reported that freeze-drying reduces enzyme activities in probiotic bacteria (Heljo, Jouppila, Hatanpää, & Juppo, 2011; Li et al., 2011; Vasiljevic & Jelen, 2003). Addition of various protectants may preserve enzyme activities during freeze-drying of probiotics to different extents (Li et al., 2011).

The β-glucosidase activity of different probiotics declines during storage in fermented soymilk at different temperatures; the extent of decline varies with the strain used (Otieno et al., 2005). To the best of our knowledge, no studies have been reported on the stability of β-glucosidase and the effect of different protectants on activity retention after storage of freeze-dried *Bifidobacterium* in different food matrices.

Storage of probiotic bacteria in food matrices, especially in low pH juices affects their survival in the gastric environment (Champagne & Gardner, 2008; Saarela et al., 2006; Vinderola et al., 2012). Lankaputhra, Shah, and Britz (1996) reported that *Bifidobacterium infantis* lose more than 83% of their viability during storage in fermented milk at 4 °C. Therefore, reducing the loss of viability of *B. infantis* is an important challenge when this species is to be incorporated into functional food.

The aim of the present study was to investigate the role of disaccharide protectants (cellobiose, trehalose, sucrose and lactose) on viability and β-glucosidase activity of the probiotic *B. infantis* during freeze-drying and during storage in different food matrices. In addition, the influence of protectants on the acid tolerance of *B. infantis* after storage in different food matrices was investigated. Furthermore, the role of cellobiose as new potential probiotic lyoprotectant, in comparison to other common protectants on the stability and functionality of *B. infantis* was compared. To the best of our knowledge, this is the first study to test cellobiose for protecting viability and functionality of a probiotic strain.

2. Materials and methods

2.1. Growth of microorganisms

B. infantis (UV16PR) was kindly provided by Medipharm (Kågeröd, Sweden). The microorganisms were grown in Reinforced Clostridial Medium (RCM, Oxoid Ltd., Hampshire, UK) by incubation at 37 °C in an anaerobic jar using Anaerogen kits (Oxoid Ltd., Hampshire, UK).

2.2. Freeze-drying

Fresh cells were harvested by centrifugation (10,000 × *g*, 4 °C, 15 min) and washed twice with 50 mM phosphate buffer, pH 6.5. The pellets were resuspended in phosphate buffer solution containing 5 or 10% (w/v) sucrose (Sigma–Aldrich, St. Louis, MO, USA), trehalose (Merck, Darmstadt, Germany), lactose (Sigma–Aldrich) or cellobiose (Glycon Biochemicals, Luckenwalde, Germany). The suspensions were divided into 500 μl aliquots and frozen at −80 °C prior to lyophilization. Freeze-drying was undertaken under vacuum (0.3 mbar) at a condenser temperature of −54 °C using a HetoPowerDry LL3000 lyophilizer (Thermo Scientific, Waltham, MA, USA) according to previous studies (Li et al., 2011; Stummer et al., 2012). Freeze-dried cells without protectants were prepared as control.

2.3. Storage of freeze-dried bifidobacteria in juices and milk

B. infantis cells freeze-dried in the presence of different protectants were incorporated into 100 ml of pasteurized low fat milk containing ≤1.5% fat prepared from skim milk powder (pH 6.7; Merck, Darmstadt, Germany) and commercial juices including grape juices (pH 3.4) and red-beet juice (pH 4.2) purchased from local stores in Austria. The juices were stored at 4 °C for 4 weeks and the milk for 2 weeks.

2.4. Cell enumeration

Viable cells were determined using the plate count method prior to freezing and after freeze-drying and storage in food matrices.

Dried cells were resuspended at initial volume with phosphate buffer and left for rehydration at room temperature. 1 ml of rehydrated cells or 1 ml of milk or juices were serially diluted with phosphate buffered saline (PBS) and plated onto Reinforced Clostridial Agar plates in triplicate. The plates were incubated under anaerobic conditions at 37 °C for 48–72 h. The viability was expressed as colony forming units (CFU) per millilitre. The survival of cells after freeze-drying and storage was calculated in percent.

The pH of food matrix was measured before and after storage using pH meter (Hanna Instruments, Woonsocket, RI, USA).

2.5. Cell free extract preparation

β-Glucosidase activity was determined in cell free extracts before and after freeze-drying, and before and after storage in different food matrices.

After freeze-drying cells were rehydrated and harvested by centrifugation, washed twice with 50 mM phosphate buffer (pH 6.5) and then resuspended in the same buffer. From food matrices cells were harvested by centrifugation (10,000 × *g*, 4 °C, 10 min) and washed with 50 mM phosphate buffer pH 6.5. For preparing the cell free extract, resuspended cells were mechanically disrupted by mixing (1:1.5 w/v) with sterile glass beads (200–300 μm) using a vortex for 5 cycles of 2 min with 2 min break on ice. Following cell disruption, the suspensions were centrifuged (17,000 × g, 30 min, 4 °C) using a Sorvall® RC5C centrifuge (Thermo Scientific, Waltham, MA, USA) to remove the cell debris and glass beads. The supernatant was then filtered using a 0.45 μm filter (Merck Millipore, Darmstadt, Germany).

2.6. Determination of β-glucosidase activity retention after freeze-drying and after storage in different food matrices

The cell free β-glucosidase activity was determined before and after freeze-dying, and before and after storage for 4 weeks in juices and 2 weeks in milk. p-Nitrophenyl-β-D-glucopyranoside (p-NPG, Sigma–Aldrich) was used as substrate. p-NPG solution (5 mM, 150 μl) in 50 mM phosphate buffer pH 6.5 was mixed with 600 μl of cell free enzyme and the mixture was incubated for 15 min at 37 °C, according to Dianawati and Shah (2011) with minor modification. The reaction was stopped on ice by adding 375 μl of cold NaOH (0.1 M). The amount of p-nitrophenol released was measured at 410 nm using a Hitachi U-1100 spectrophotometer (Hitachi Ltd. Tokyo, Japan). The enzyme activity at baseline (before storage) was defined as 100% activity. The enzyme activity after storage was compared to the initial enzyme activity at the baseline. The remaining enzyme activity after storage in different food matrices was expressed as relative retention in percent.

The β-glucosidase activity was expressed in units (1 unit is the amount of enzyme that releases 1 μM p-nitrophenol per ml per