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Stability and functionality of freeze-dried probiotic *Bifidobacterium* cells during storage in juice and milk

Maria Saarela[a,*], Ilkka Virkajärvi[a], Hanna-Leena Alakomi[a], Pia Sigvart-Mattila[b], Jaana Mättö[a]

[a] *VTT Biotechnology, P.O. Box 1500, 02044 VTT Espoo, Finland*
[b] *Valio Ltd. R&D, P.O. Box 30, 00039 Helsinki, Finland*

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Abstract

We investigated the stability of differently produced (variables being fermentation time, pH during drying, and cryoprotectant) freeze-dried *Bifidobacterium animalis* subsp. *lactis* E-2010 (Bb-12) cells in fruit juice and low-fat milk. In addition, the effect of the food matrix on the acid and bile tolerance of the cells was studied. Cells produced in different ways had comparable stability in milk, whereas in juice, sucrose-protected cells survived better than reconstituted skim milk-protected cells. The acid and bile tolerance was better in cells added to milk compared with those in phosphate buffered saline or juice. Despite good culturable stability in milk the acid and bile tolerance of cells decreased during the storage. Apparently, culturable stability data alone do not give an accurate enough prediction of the probiotic functionality in adverse conditions (e.g. survival in acid and bile stress). When choosing a cryoprotectant for a probiotic also the stability in target food applications should be considered.

Keywords: *Bifidobacterium*; Freeze-drying; Stability; Acid tolerance; Bile tolerance

1. Introduction

Probiotics are normally added to foods either as dried or frozen concentrated cultures. Freeze-drying has been the classical way of producing dry bacterial powders (Heckly, 1985). Freeze-dried powders are easier to handle than frozen products, but drying exposes the cells to an additional stressful processing step. The important contributors to viability loss during freeze-drying are osmotic shock and membrane injury resulting from intracellular ice formation and recrystallisation (Heckly, 1985). Skim milk and sucrose have been commonly used as cryoprotectants (Hubalek, 2003); skim milk is considered to be capable of preventing cellular injury by stabilizing the cell membrane and providing a protective coating for the cells (Carvalho et al., 2004), whereas the protective activity of sucrose is suggested to be due to its ability to prevent injurious eutectic freezing of cell fluids by trapping salts in a highly viscous or glass-like phase (Hubalek, 2003).

The most typical food matrices for probiotic bacteria are different kinds of fermented milk products, especially yoghurts (Fasoli et al., 2003; Temmerman, Pot, Huys, & Swings, 2003; Gueimonde et al., 2004). During the past few years the diversity of probiotic foods on the market has increased: probiotics can nowadays be found in non-fermented milk, in fruit and berry juices and in cereal-based products. However, there is relative little published information on the survival of probiotics in non-fermented food matrices, whereas the stability of probiotics in yoghurts has been widely studied (Kailasapathy & Rybka, 1997). Stability data for various probiotic strains are available for cereal puddings, frozen soy dessert, ice-cream and milk (Hughes & Hoover 1995; Sanders, Walker, Walker, Aoyama, & Klaenhammer, 1996; Abu-Taraboush, Al-Dagal, & Al-Royli, 1998; Shin, Lee, Petska, & Ustunol,

*Corresponding author. Tel.: +358 20 722 4466; fax: +358 20 722 7071.
E-mail address: maria.saarela@vtt.fi (M. Saarela).

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2000; Alamprese, Foschino, Rossi, Pompei, & Savani, 2002; Heenan, Adams, Hoskenm, & Fleet, 2004; Helland, Wicklund, & Narvhus, 2004).

The aim of the present study was to assess whether different methods of production affect the culturable stability of freeze-dried *Bifidobacterium animalis* subsp. *lactis* VTT E-012010 (Bb12) preparations during storage in milk and fruit juice. In addition, the effect of the food matrix storage on the acid and bile tolerance of the cells was investigated to reveal possible changes in the cell functionality.

2. Materials and methods

2.1. Bacterial strains

B. animalis subsp. *lactis* VTT E-012010 (E2010; originally isolated from Bb-12 cell concentrate purchased from Chr. Hansen, Denmark) was obtained from the VTT culture collection. The culture was stored at –70 °C and revived on De Man Rogosa Sharpe agar (MRSA) (Oxoid, Bakingstoke, UK) supplemented with 0.5 g L^{-1} L-cysteine-HCl (Merck, Darmstadt, Germany) (MRSA + cys) at 37 °C under anaerobic conditions.

2.2. Fermentation and freeze-drying

B. animalis subsp. *lactis* E2010 cells were grown in 30 L fermenters (New Brunswick IF 40, New Brunswick Scientific, Edison NJ, USA) at 37 °C for 15 or 22 h. A non-milk-based general edible medium (GEM) consisting of glucose (40 g L^{-1}), soy peptone (30 g L^{-1}), yeast extract (7 g L^{-1}) and $MgSO_4 \times 7\ H_2O$ (1 g L^{-1}) was used in the fermentations.

The cultivations were initiated from stock culture plated on MRSA + cys and incubated at 37 °C for 2 or 3 d under anaerobic conditions. A single colony was transferred to MRS broth + cys (MRSB + cys) and incubated at 37 °C for 24 h followed by transfer of 2% inoculum of the cell suspension to GEM. Finally, the fermenter was inoculated by using a 2% inoculum of 17 to 18 h incubated GEM suspension. The fermenter was stirred (50 rpm) and pH controlled at 5.8 with 12.5% NH_4OH. No aeration was used and N_2 was fed to the head space of the fermenter at a low rate.

Cells were concentrated at 15 h (late logarithmic growth phase) and 22 h (early stationary growth phase) (harvested from the same fermentation batches) by centrifugation at $4000 \times g$, 10 min, 4 °C (Sorvall RC 12, Wilmington, DE, USA) and re-suspended in equal volume of non-neutralised (pH 5.8) or NH_4OH-neutralised (pH 7) spent medium (the medium where the cells were grown). The re-suspended cell mass was mixed with reconstituted skim milk (RSM; 5% w/w; Difco, Detroit, MI, USA) or with sucrose solution (5% w/w; BDH, Dorset, UK) prior to freeze-drying. For the freeze-drying the cell mass was frozen at −20 °C. Freeze-drying was performed in an Epsilon 2-25 freeze-dryer (Martin Christ, Duingen, Germany) with a standard program by increasing the temperature gradually from −45 to 20 °C at 0.12 mbar pressure (49 h) followed by 15 h at 0.01 mbar. Two separate fermentation and freeze-drying experiments (with RSM and sucrose cryoprotectants) were performed.

Bacterial numbers were determined during fermentation, concentration and freeze-drying on reinforced clostridial medium (RCMA; Difco, Detroit, MI, USA) by plate count analysis. RCMA was used instead of MRS since our studies have shown that RCMA is better able to support the growth of stressed *B. animalis* subsp. *lactis* cells (e.g. freeze-dried cells) than MRS. Fermentation and freeze-drying experiments as well as stability studies and bile and acid tolerance tests (see below) were performed in duplicate.

2.3. Storage stability

Freeze-dried cultures (11.5–11.6 log cfu g^{-1}) were incorporated as such into 125 or 200 mL portions of commercial fruit juice (three fruits' juice: orange, grape, passion fruit; pH 3.7; Valio, Helsinki, Finland), commercial pasteurised milk containing 1% fat (pH 6.6–6.7; Valio, Helsinki, Finland) and phosphate-buffered saline (PBS), pH 7.2 (only RSM-protected cells) in polystyrene beakers with aluminium foil lids. The initial cell numbers were 7.5–8.4 log cfu mL^{-1}. Juices were stored at 4 and 20 °C up to 6 weeks and milk and PBS at 4 °C up to 2 weeks followed by plate count on RCMA as described above.

2.4. Acid and bile tolerance

Acid and bile tolerance of the cells produced with RSM cryoprotectant was assessed at baseline i.e. before storage (juice, milk and PBS) and after 2 weeks storage (milk and PBS only). For the acid and bile tolerance assay cells were harvested from 10 mL of juice/milk/PBS by centrifugation ($2400 \times g$ for 10 min) and resuspended in 2 mL of 0.01 mol L^{-1} PBS, pH 7.2. Acid and bile tolerance was determined as previously described (Saarela, Hallamaa, Mattila-Sandholm, & Mättö, 2003). Briefly, 0.3 mL of resuspended cells (approximately 10^7 cells mL^{-1}) was transferred to 3 mL of pH 3.0 and pH 2.5 PBS (pH adjusted with HCl) with and without 3 g L^{-1} pepsin (Sigma P7000, St Louis, MO, USA) or to 1% bile acids (Sigma B8381) in pH 7.2 PBS or in PBS pH 7.2 (control). After 0 h (control), 2 h (control and acid tolerance tests) and 3 h (control and bile tolerance tests) of incubation at 37 °C cell numbers were determined by plate count with a spot technique (10 μL drops were pipetted on agar surface and microcolonies enumerated after the incubation) on RCMA, and the pH was measured after the incubation period. Acid and bile tolerance tests were performed in duplicate from two fermentation batches.

Since during the acid tests, we noted that adding cells to the low pH buffer increased the pH, we repeated some of