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Acid tolerant mutants of *Bifidobacterium animalis* subsp. *lactis* with improved stability in fruit juice

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ABSTRACT

The potential of UV mutagenesis combined with a specific selection step to generate more acid-resistant *Bifidobacterium animalis* subsp. *lactis* Bb-12 strains with improved viability in low pH food matrices was investigated in this study. A total of 144 Bb-12 UV-mutants were initially characterised. After prolonged storage in apple juice (pH 3.5) and various pheno- and genotypic tests (acid and bile tolerance, substrate utilisation, antibiotic susceptibility, aerotolerance, RAPD) two mutants (2.20 and 2.56) were chosen for further studies including cell surface morphology, stability of various traits after repeated inoculations, and performance in a fermenter and during down-stream processing. 2.20 and 2.56 showed over two Log-values better viability in pH 3.5 juice compared to Bb-12. Alterations in cell surface structures of 2.20 and 2.56 were detected with AFM, whereas other studied traits remained unchanged.

In conclusion, UV mutagenesis and subsequent incubation in acidic medium enabled improving the stability of *B. animalis* subsp. *lactis* in low pH juice. Acid tolerance testing (HCl, pH 2.5, 2 h) results did not predict long-term stability of the strains in acidic food matrix (apple juice pH 3.5).

1. Introduction

In addition to the more traditional dairy products probiotics are nowadays available for the consumers in an increasing variety of foods. Novel probiotic foods include e.g. fruit and berry juices and drinks, recovery drinks, cereal-based drinks and snacks. Although various *Bifidobacterium* species have been studied for probiotic properties, *Bifidobacterium animalis* subsp. *lactis* has typically been the only *Bifidobacterium* (sub)species detected in probiotic foods (Jayamanne & Adams, 2006; Masco, Huys, De Brandt, Temmerman, & Swings, 2005). The popularity of *B. animalis* subsp. *lactis* is due to its superior technological properties compared to other *Bifidobacterium* species (Matsumoto, Ohishi, & Benno, 2004; Mättö et al., 2004).

Good viability is a prerequisite for optimal probiotic functionality and therefore probiotic products should contain high enough levels of the specific probiotic strain(s) throughout the storage and during consumption. The health benefits of probiotics have been shown in human intervention studies using fairly high doses of viable probiotic cells (typically 10^9–10^{11} cfu in a daily dose) (Saarela, 2007). Thus the initial quality (regarding the levels of viable bacteria) and the storage stability of the probiotic product need be high to ensure that consumers can get the health benefits associated with the product.

In beverages one of the most important factors affecting probiotic viability and stability is the pH of the product. Shelf-stable beverages typically have pH-values below 4.4 to ensure their microbial stability (Eckert & Riker, 2007); e.g. fruit juices usually have a pH below 4 or even 3 (Saarela, Alakomi, Puhakka, & Mättö, 2009; Saarela, Virkajärvi, Nohynek, Vaari, & Mättö. 2006). pH-values below 4 are typically detrimental to most probiotic strains (Champagne, Raymond, & Gagnon, 2008; Saarela et al., 2009; Sheehan, Ross, & d Fitzgerald, 2007). Probiotic bacteria vary in their tolerance to organic acids and low pH. Bifidobacteria are reported to be sensitive to pH-values below 4.6 (Boylston, Vinderola, Ghoddusi, & Reinheimer, 2004), and thus their viability and stability in fruit juices is typically poor. However, the acid-resistance of bifidobacteria varies and *B. animalis* strains are clearly more acid-resistant than the strains of other *Bifidobacterium* species (Mättö et al., 2004). It is also well-known that the properties of *Bifidobacterium* species and strains can vary widely and therefore all processing steps with probiotic bacteria should be performed in conditions that suit best the strain in question (Saarela et al., 2004).

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The aim of the present study was to investigate whether UV mutagenesis technique combined with a specific selection step in low pH enables the isolation of more acid-resistant *B. animalis* subsp. *lactis* Bb-12 strains with improved viability in low pH food matrices.

2. Materials and methods

2.1. Strain and growth conditions

B. animalis subsp. *lactis* Bb-12, originating from a commercial Bb-12-culture, was obtained from Valio Ltd's culture collection. The strain was stored at −70 °C and revived on pre-reduced reinforced clostridial medium agar (RCMA; Difco, Detroit, MI, USA) anaerobically ($H_2/CO_2/N_2$; 10/10/80%, Anoxomat, MART, Lichtenvoorde, the Netherlands). Pre-reduced RCMA was also used in various viability determinations throughout the study (2–3 d at 37 °C). Bb-12 was always grown in anaerobic conditions whereas fruit juice stability studies were performed under aerobic conditions.

2.2. Random mutagenesis B. animalis subsp. lactis Bb-12

B. animalis subsp. *lactis* Bb-12 was grown on pre-reduced RCMA, inoculated into 50 ml of General Edible Medium, GEM (Saarela et al., 2004) and incubated for 18 h at 37 °C. Cells were harvested and resuspended into 5 ml of GEM. For UV mutagenesis cell suspensions were mixed with 10 ml of peptone saline (with cysteine–HCl 0.3 g l^{-1}; PS + cys) on a Petri dish and treated in a UV crosslinker (Stratalinker 2400, Stratagene, CA, USA) for 0–700 μJ $cm^{2\ -1}$. After the UV-treatment cells were harvested, resuspended into PS + cys, added into GEM broth (vol. 200 μl, pH 3.5, 3.7, 3.9, 4.1, 4.3, 4.5 or 6.3) on a microtiter plate, and incubated anaerobically at 37 °C for 3 d and subsequently at room temperature for up to 3 weeks. Selected isolates (those growing the best in the low pH broths) were plated on RCMA and re-inoculated into GEM broth as above. All UV treatments were performed in duplicate and the untreated Bb-12 was used as a control in the experiments.

The best performing isolates (altogether 144) were purified and stored at −70 °C for further analysis. Of these 36 and eventually 22 were included in further studies. Isolates were fingerprinted using randomly amplified polymorphic DNA (RAPD) using primers OPA-02, OPA-03, OPA-13 (and OPV-7 for selected isolates/strains) as previously described (Mättö et al., 2004; Saarela et al., 2007) for preliminary confirmation of the similarity of the isolate fingerprint with that of the wild-type (untreated) *B. animalis* subsp. *lactis* Bb-12.

2.3. Screening of the mutants – stability studies in fruit juice

The selected 144 isolates were taken from the frozen stock and grown in GEM (37 °C for 18 h). Cells were harvested, resuspended in PS + cys, added into apple juice (pH 3.5; Pirkka, Finland) (500 μl cell suspension per 10 ml of juice in sterile plastic tubes) in duplicate and stored protected from light at 4 °C for 6 weeks. The numbers of viable *B. animalis* cells were determined in parallel by culture on RCMA on a weekly basis.

2.4. Further characterisation of mutants

Altogether 17 mutant strains (including strains 2.20 and 2.56) as well as the wild-type strain Bb-12 were studied for pheno- and genotypic properties by performing the following tests: acid and bile tolerance (according to Saarela, Virkajärvi, Alakomi, Sigvart-Mattila, & Mättö, 2006), substrate utilisation (using API 50 CHL panels according to manufacturer's instructions; BioMerieux, France), susceptibility testing of 13 antibiotics (using VetMIC™ –E-cocci (version 3) kit, National Veterinary Institute, Uppsala, Sweden), according to van Hoek, Mayerhofer, Domig, and Aarts (2008), aerotolerance (optical density in shaken GEM cultures, 150 rpm, after 24 and 48 h), stability in pH 3.5 apple juice (as above), and genetic fingerprinting by RAPD (as above).

Pheno- and genotypic stability of 2.20, 2.56 and Bb-12 was additionally studied by inoculating and growing the strains repeatedly in GEM (10 rounds in GEM, freezing at −70 °C, another 10 rounds in GEM starting from the frozen stocks) and performing the above tests for the repeatedly inoculated strains. In addition, growth in GEM broth supplemented with Oxyrase® (Oxyrase, Inc. Mansfield, Ohio, USA) on microtiter plate during 48 h incubation was determined in an automated turbidometer (Bioscreen, Labsystems, Finland) (Saarela, Hallamaa, Mattila-Sandholm, & Mättö, 2003). Also genetic fingerprinting by pulsed field gel electrophoresis, PFGE, with restriction enzymes *Xba*I and *Spe*I (according to Mättö et al., 2004) was performed.

2.5. Fermenter growth and down-stream processing

Mutant strains 2.20 and 2.56 as well as the wild-type strain Bb-12 were grown in fermenters in Fermentation Medium (FM; glucose 40 g l^{-1}; casein hydrolysate 10 g l^{-1} (obtained from Valio Ltd.); yeast extract 10 g l^{-1} (Lab M); $MgSO_4 \times 7\ H_20$ 1 g l^{-1}; cysteine–HCl 0.3 g l^{-1}) under pH control (pH 5.5). Fermentations were performed in 7 l fermenters (BioFlo IV, New Brunswick Scientific, Edison, N.J., USA) under nitrogen flow. In 7 l fermenters alkaline feeding (NH_4OH) was stopped after 20 h incubation and the pH was allowed to drop freely for until it reached pH 5.2 (about 1 h). Cells were cooled to 10 °C, harvested by centrifugation (Sorvall RC12, Wilmington, DE, USA, 4000 g, 20 min), resuspended in supernatant and mixed with sucrose (10% w vol^{-1}). After 1 h incubation at room temperature, cell-carrier concentrates were either frozen at −40 °C for stability studies or freeze-dried with a standard programme with an Epsilon 2–25 freeze-dryer (Martin Christ, Duingen, Germany). Freeze-dried powders were packed in portion-size (approximately 1 g) aluminium foil sachets for storage stability tests (storage at 10, 25 and 37 °C up to 24 weeks). Residual moisture content of the powders was measured by Karl–Fisher titration.

2.6. Atomic force microscopy

Concentrate samples of fermenter-grown cells were thawed in 37 °C water bath and suspended in 10 mmol l^{-1} PBS buffer (pH 7.2). After homogenization cells were diluted 1:10 in 10 mmol l^{-1} PBS buffer (pH 7.2), harvested by centrifugation (10,000 g) in an Eppendorf microcentrifuge for 1 min at room temperature and washed twice with PBS buffer. For Atomic Force Microscopy (AFM) analysis cells were diluted in sterile ultrapure water and applied on a freshly cleaved mica surface and allowed to dry before imaging (Alakomi, Paananen, Suihko, Helander, & Saarela, 2006). Images were acquired in air under ambient conditions using a NanoScope IIIa Multimode AFM (Digital Instruments, Santa Barbara, CA) equipped with a "J"-scanner. The tapping mode was used with scan rates of 0.5–1.2 Hz and as little force as possible, and the ratio of set point amplitude and free amplitude was usually 0.8 to 0.9 with a target amplitude 1 V. Noncontact silicon cantilevers (NSC15/AlBS; μMasch) with the nominal resonance frequency of 350 kHz and a tip radius better than 10 nm were used. The topography and phase-contrast images were captured simultaneously. On average 5 different images on different areas for each sample were imaged. The phase-contrast image shows the phase difference between the oscillations of the cantilever-driving piezo and the detected