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Growth, organic acids profile and sugar metabolism of *Bifidobacterium lactis* in co-culture with *Streptococcus thermophilus*: The inulin effect

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ABSTRACT

The organic acids profile, sugar metabolism and biomass growth of *Streptococcus thermophilus* (St) and *Bifidobacterium lactis* (Bl) have been studied in pure cultures or binary co-culture (St–Bl) in skim milk either containing 40 mg/g of inulin or not. With inulin, the time required by St, Bl and St–Bl to complete fermentation (i.e., when the pH reached 4.5) was about 14, 8 and 49% shorter than without inulin, respectively. This prebiotic also enhanced the levels of lactic and acetic acids and volatile compounds, showing a positive synbiotic effect between pre- and probiotics. In particular, the St–Bl co-culture showed final concentrations of both microorganisms about 15 and 38% higher than in their respective pure cultures, thus highlighting a clear synergistic effect between these microorganisms due to mutual interactions. In addition, the well-known bifidogenic effect of inulin was confirmed.

1. Introduction

In recent years, the number of functional dairy products enriched with live probiotic strains belonging mainly to the *Bifidobacterium* and *Lactobacillus* genera has remarkably increased. According to FAO/WHO (2002), probiotics are live microorganisms which, when administered in adequate amounts, confer health benefits on the host. As a significant part of the world population suffers gastrointestinal diseases caused by pathogenic bacteria, numerous studies are currently in progress (Donkor, Nilmini, Stolic, Vasiljevic, & Shah, 2007).

Available evidence indicates that ingestion of probiotic bacteria may reduce the severity and frequency of diarrheal diseases and improve lactose digestibility among lactose-intolerant individuals (Mattila-Sandholm et al., 2002). These therapeutic effects are the reason for which most probiotics are used in yogurts, fermented milks, ice creams and nutraceutical products (Cruz et al., 2010; Oliveira, Florence, et al., 2009).

Nowadays, to enhance their therapeutic effects, it has recently been studied the ability of probiotics to partially ferment oligosaccharides and inulin (prebiotics) in fermented milk (Oliveira, Perego, Oliveira, & Converti, 2011; Rodrigues et al., 2011; van den Broek & Voragen, 2008). In fact, these compounds may be also added to foods in attempts to increase fiber ingestion and obtain low-calorie dairy products (Oliveira, Florence, et al., 2009; Roberfroid, 2005).

Donkor et al. (2007) reported that, in set-type yogurt, prebiotics exert a protective effect and potentiate probiotics activity. The capability of these microorganisms to ferment prebiotic ingredients may in fact be an especially important characteristic, because the availability of carbohydrates that escape metabolism and adsorption in the small intestine has a major influence on the colon microbiota (Mattila-Sandholm et al., 2002).

On the other hand, although the homofermentative and heterofermentative pathways involved in the metabolism of lactic acid bacteria (LABs) and bifidobacteria are well documented (Axelsson, 1998; Cronin, Ventura, Fitzgerald, & van Sinderen, 2011; Mayo et al., 2010), there is limited published information on the microbial metabolic interactions in probiotic co-cultures used to ferment non-fat milk.

From a technological standpoint, these interactions are very important for the correct development of flavor and texture of functional fermented dairy product (Cruz et al., 2010; Martin et al., 2011; Østlie, Helland, & Narvhus, 2003; Salazar et al., 2009), being responsible for variations in the amounts of organic acids, volatile compounds and exopolysaccharides released during manufacture. Metabolically, they are relevant so as to allow microorganisms to better obtain energy and maintain their NAD^+/NADH ratio (Axelsson, 1998; Mayo et al., 2010).

Species and strains of *Bifidobacterium* genus are classified as Gram-positive, non-spore forming, non-motile and catalase-negative anaerobes, which may assume various shapes. Bifidobacteria differ from other colonic genera in the mechanism employed to ferment carbohydrates. Lacking the enzymes aldolase and glucose-6-phosphate $NADP^+$ oxidoreductase (Mayo et al., 2010), they are in fact unable to carry out either the usual glycolytic pathway or the hexose monophosphate shunt. In particular, the bifidus pathway, which is characterized by the presence of fructose-6-phosphate phosphoketolase (Fandi, Ghazali,

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Yazid, & Raha, 2001), allows bifidobacteria to produce more ATP from carbohydrates (2.5 mol ATP/mol glucose) than conventional hetero- and homofermentative pathways, leading to a theoretical yield of 1.5 mol acetic acid and 1 mol lactic acid (Cronin et al., 2011).

These bacteria bring about several benefits to their human hosts, such as vitamin production, anticarcinogenic activity, immunostimulating effects, hypocholesterolemic power and pathogen inhibition (Ejtahed et al., 2011). In particular, *Bifidobacterium animalis* subsp. *lactis* has been found in children's intestine, where it promotes production of IgA that is important in their immune system; it also possesses a number of desirable technological features, i.e. tolerance to oxygen, acid and bile resistance and ability to grow on milk-based media (Chen, Ruan, Zhu, & He, 2010; Janer, Arigoni, Lee, Pelaez, & Requena, 2005).

In the present study, the associative behaviors of *Streptococcus thermophilus* TA040 and *B. animalis* subsp. *lactis* BL 04 have been investigated in skim milk through a metabolic study based on the a) lactic and acetic acids formation from glucose and partly from galactose, b) release of unmetabolized galactose, c) diacetyl and acetoin formations, and d) biomass growth. Finally, we examined the effect of inulin, as one of the most attracting prebiotics in functional food preparation, on the fermentation patterns either of pure cultures of *S. thermophilus* and *B. lactis* or of their co-culture.

2. Materials and methods

2.1. Microorganisms

Two commercial starter freeze-dried strains (Danisco, Sassenage, France) were used in this study, specifically *S. thermophilus* TA040 (St) and *B. animalis* subsp. *lactis* BL 04 (Bl).

2.2. Milk preparation

Milk was prepared adding 13 g of skim powder milk (Castroni, Reggio Emilia, Italy) in 100 g of distilled water. Skim milk base was either used as such (control) or supplemented with 40 mg of inulin/g (trade name: Beneo TM) (Orafti Active Food Ingredients, Oreye, Belgium). The above solid content of milk corresponds to the average value for integral cow milk (Restle, Pacheco, & Moletta, 2003), while the concentration of inulin is in the range admitted by the Brazilian legislation for yogurts (ANVISA, 2002). Both milks were thermally treated at 90 °C for 5 min in water bath, model Y14 (Grant, Cambridge, United Kingdom), and transferred to 1.0 L-sterile flasks, cooled in ice bath, distributed into 250 mL-sterile Schott flasks inside a laminar flow chamber, and stored at 4 °C for 24 h before use.

2.3. Inoculum preparation

B. lactis pre-culture was prepared by dissolving 45 mg of freeze-dried culture in 50 mL of milk (10% of total solids; autoclaved at 121 °C for 20 min). After blending and activation at 42 °C for 30 min, 1.0 mL of the pre-culture was inoculated in 500 mL-Erlenmeyer flasks containing 250 mL of skim milk. *S. thermophilus* pre-culture was prepared in the same way by adding 90 mg of its freeze-dried culture to 50 mL of milk.

Cell counts of these pre-cultures were made in triplicate, as previously described by Oliveira, Perego, Converti, and Oliveira (2009), and ranged from 6.0 to 6.3 LogCFU/mL. In particular, samples (1.0 mL) were added to 9.0 mL of 0.1% sterile peptonated water; then, appropriate dilutions were made. After verifying the inability of the *S. thermophilus* strain employed in this study to grow on MRS medium, it was plated into M17 Agar (Oxoid, Basingstoke, UK) and then submitted to aerobic incubation at 37 °C for 48 h (Liu & Tsao, 2009). *B. lactis* was counted in MRS agar medium supplemented with 50 g/L cysteine (Sigma Chemical Co., St. Louis, MO, USA) and incubated anaerobically in anaerobic jar at 37 °C for 72 h (Oliveira, Perego, et al., 2009). Anaerobic conditions were ensured by the use of AnaeroGen (Oxoid).

2.4. Fermentations

After inoculation, the flask content was transferred to a 3.0 L-fermenter, model Z61103CT04 (Applikon, Schiedam, The Netherlands), with 2.0 L-working volume and provided with an electronic device, model ADI1030 (Applikon). Dissolved oxygen (DO) concentration was measured by a sterilized galvanic electrode, InPro6000 Series (Mettler-Toledo, Novate Milanese, Italy). Batch fermentations were carried out with single and mixed cultures, in triplicate, without any agitation, at 42 °C, and stopped when the pH reached 4.5, according to the common practice of yogurt preparation.

2.5. Analytical methods

Determination of organic acids was carried out using the method described by Donkor et al. (2007). Five 3.0 mL-fermented milk samples were taken in different values of pH (6.5, 6.0, 5.5, 5.0 and 4.5) and then mixed with 80 μL of 15.5 M nitric acid. Subsequently, the samples were diluted with 1.0 mL of the mobile phase (0.01 M sulfuric acid). The resulting mixture was centrifuged at 15,000 × g for 20 min using an Eppendorf 5415R centrifuge (Eppendorf, Milan, Italy) for removal of proteins. The supernatant was filtered through membrane filters with 0.20 μm-pore diameter (Millipore™, Milan, Italy) in a HPLC vial. A high-performance liquid chromatograph, model 1100 (Hewlett Packard, Palo Alto, CA), was used to analyze lactose, glucose, galactose, acetic acid, formic acid, ethanol, diacetyl, acetoin, and lactic acid. The system consisted of a HP-1050 Intelligent Auto Sampler, a HP-1047A Refractive Index Detector, a HP-1050 UV Detector and a HP-1050 pump. Separation was achieved using a Supelcogel H59304-U column (Sigma Aldrich, Bellefonte, PA) at 50 °C with 0.01 M sulfuric acid as eluent at 0.4 mL/min flow rate. The column was stabilized for at least 3 h before use utilizing the same solution under the same conditions as the separation. Quantification of organic acids was performed from standard curves obtained using solutions of pre-determined concentrations (Donkor et al., 2007). Each analysis was done in triplicate.

After dilution of samples and casein precipitation by acidification to pH 4.5 with HCl, except the sample which was taken at pH 4.5 (Hipp, Groves, Custer, & McMeekin, 1950), biomass concentration was determined in pure cultures by optical density (OD) measurements at 640 nm using a UV–vis spectrophotometer (Model Lambda 25, Perkin Elmer, Wellesley, MA) and an OD versus dry weight calibration curve. For dry weight determinations, cells were harvested by centrifugation cycles (16,000 × g for 10 min) in Eppendorfs, then separated from casein by filtration on membranes with 0.20-μm-pore diameter, washed with 2 volumes of distilled water and dried to constant weight at 101 °C. In co-cultures, biomass was determined by the same selective cell counts procedure earlier described for pre-cultures, and the results were expressed in g/L dry weight using LogCFU/mL versus g/L dry weight calibration curves previously-prepared for each microorganism.

2.6. Statistical analysis

The experimental data of biomass concentration collected in pure or binary co-cultures of *S. thermophilus* (St) and *B. lactis* (Bl) at the end of fermentations were presented as mean values. Variations with respect to the mean values were presented as standard deviations. Mean values of concentrations were submitted to analysis of variance (ANOVA) by the Statistica Software 6.0 (Padova, Italy). They were compared using the Tukey's test at significance level (P)<0.05, and different letters were used to label values with statistically significant differences among them.