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Prebiotic Effect of Inulin on the Growth and Organic Acid Profile of *Bifidobacterium lactis* in Co-culture with *Streptococcus thermophilus*

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The associative behaviors of *Bifidobacterium lactis* (Bl) and *Streptococcus thermophilus* (St) have been investigated in skim milk through a study based on their growth and organic acid profile. Finally, the effect of inulin was examined, as one of the most attracting prebiotics in functional food preparation, on the fermentation patterns either of pure cultures of Bl and St or of their co-culture. In the presence of inulin, the time that St, Bl and St-Bl lasted to complete fermentation (i.e., to reach pH 4.5) was shorter than in its absence. Biomass growth and levels of lactic and acetic acids and volatile compounds also enhanced in the presence of inulin, showing a positive synbiotic effect between pre- and probiotics.

1. Introduction

The benefits on health of probiotic bacteria mainly belonging to the *Lactobacillus* and *Bifidobacterium* genus have led to their increased incorporation in functional dairy products (Lourens-Hattingh and Viljoen, 2001). Today, most probiotics (FAO/WHO, 2002) are used in yoghurts, fermented milks, ice creams and pharmaceutical products, because of their therapeutic effects (Mattila-Sandholm et al., 2002). Nowadays, to improve therapeutic effects, dairy foods usually contain probiotics in association with prebiotics (Di Criscio et al., 2010), i.e. non-digestible oligosaccharides that resist hydrolysis and absorption in the upper gastrointestinal tract and are metabolized selectively by at least one type of probiotic in the colon (Mattila-Sandholm et al., 2002). Among these, inulin was shown to exert a protective effect on the lactic acid bacteria by stimulating their survival and activity during storage of the final product (Donkor et al., 2007). Inulin is a soluble and fermentable fructan that cannot be digested by α-amylase or other hydrolytic enzymes in the upper section of the intestinal tract (Villegas and Costell, 2007) and is mainly applied to get non-fat fermented milk (Oliveira et al., 2011).
From a technological point of view, the association between probiotics and prebiotics are very important for the correct development of flavor and texture of functional fermented dairy product (Martin et al., 2011), being responsible for variations in the amounts of organic acids, volatile compounds and exopolysaccharides released during manufacture (Ong and Shah, 2009).
Bifidobacterium strains 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). These bacteria have 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 resistant and ability to grow on milk-based media (Chen et al., 2010).
The objective of this work was investigate the associative behaviors of *Bifidobacterium lactis* (Bl) and *Streptococcus thermophilus* (St) in skim milk through a study based on their growth and organic acid profile.

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2. Materials and methods

2.1 Microorganisms

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

2.2 Milk and inoculum preparations

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). Both milks were thermally treated at 90 °C for 5 min in water bath, model Y14 (Grant, Cambridge, United Kingdom). *S. thermophilus* and *B. lactis* pre-culture were prepared by dissolving 90 and 45 mg of freeze-dried culture, respectively, in 50 mL of sterilized skim milk (121 °C for 15 min) and activating at 42 °C for 15 min before use.

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 co-cultures, in triplicate, without any agitation, at 42 °C, and stopped when the pH reached 4.5, which were selected as the conditions to stop the fermentation. In particular, batch fermentations were monitored by means of a pH meter, model pH 210 (Hanna Instruments, Padua, Italy).

2.5 Analytical methods

Biomass concentration was determined by optical density (OD) measurements at 640 nm using a UV-Vis spectrophotometer (Model Lambda 25, Perkin Elmer, Wellesley, MA) and a OD versus dry weight calibration curve. For dry weight determinations, cells were harvested by centrifugation cycles (16,000×g for 10 min) in Eppendorfs, washed twice with distilled water and dried to constant weight at 101 °C.

Determination of organic acids was carried out using the method described by Donkor et al. (2007). First, the samples, containing single and binary co-culture, were mixed with 80 μL of 15.5M nitric acid and after that diluted with 1.0mL of the mobile phase of 0.01M sulphuric 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 a 0.20 μm membrane filter (MilliporeTM, Milan, Italy) into a HPLC vial. A high-performance liquid chromatograph, model 1100 (Hewlett Packard, Palo Alto, CA), was used to analyze lactose, glucose, galactose, acetic acid and lactic acid. The HPLC equipment 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 flowrate.

2.6 Statistical analysis

Biomass and organic acid 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 $(P) < 0.05$ (Sokal and Rohlf, 1979).

3. Results and discussion

3.1 Organic acids content

Table 1 illustrates the metabolic end-products determination of St and Bl, either in pure cultures or binary co-culture, which was monitored in fermented skim. Metabolic products concentrations of these cultures increased during the fermentations mainly due to metabolization of the glucose moiety of lactose, while a relevant portion of the galactose one was excreted in the medium. In general, Bifidobacteria are saccharolytic organisms that have been shown to possess the ability to ferment this

sugar (Cronin et al., 2011) through the activity of the enzymes lacto-N-biose phosphorylase and gal 1-P uridylyl transferase (Urashima et al., 2009).

According with the homofermentative pathway of St and the heterofermentative one of Bl, the concentration of lactic acid in St mono-culture was 26 and 8 % higher than in Bl and St-Bl, respectively. On the other hand, the concentration of acetic acid in St-Bl was less than 20 % of that in Bl, likely due to a slight inhibition of the heterofermentative features of *B. lactis*. like that proposed by Rodrigues et al. (2011). According to the same authors, this behavior contributes to a better stability and organoleptic quality of the functional dairy products.

With presence of inulin, the concentrations of lactic acid were approximately 10 % (St), 15 % (Bl) and 7 % (St-Bl) higher than those obtained in the absence of this prebiotic. So, in general, the presence of inulin lactic and acetic acid levels. According to the definition proposed by Holzapfel and Schillinger (2002), these results demonstrate that the fermented skim milk produced in the present study can be considered as a potential synbiotic one.

Table 1: Concentration versus pH profiles of skim milk fermentations by pure cultures of S. thermophilus (St) and B. lactis (Bl) or a binary co-culture of S. thermophilus with B. lactis (St-Bl) in the absence or in the presence of inulin.

Without inulin				**With inulin**			
ST							
pH	**Lactose (gL^{-1})**	**pH**	**Lactose (gL^{-1})**	**pH**	**Lactose (gL^{-1})**	**pH**	**Lactose (gL^{-1})**
6.5	62,3 ± 0,2	6.5	62,3 ± 0,2	6.5	62,3 ± 0,2	6.5	62,3 ± 0,2
6.0	51,5 ± 0,2	6.0	51,5 ± 0,2	6.0	51,5 ± 0,2	6.0	51,5 ± 0,2
5.5	40,8 ± 0,3	5.5	40,8 ± 0,3	5.5	40,8 ± 0,3	5.5	40,8 ± 0,3
5.0	35,4 ± 0,2	5.0	35,4 ± 0,2	5.0	35,4 ± 0,2	5.0	35,4 ± 0,2
4.5	30,1 ± 0,2	4.5	30,1 ± 0,2	4.5	30,1 ± 0,2	4.5	30,1 ± 0,2
BL							
pH	**Lactose (gL^{-1})**	**pH**	**Lactose (gL^{-1})**	**pH**	**Lactose (gL^{-1})**	**pH**	**Lactose (gL^{-1})**
6.5	62,3 ± 0,3	6.5	62,3 ± 0,3	6.5	62,3 ± 0,3	6.5	62,3 ± 0,3
6.0	53,9 ± 0,2	6.0	53,9 ± 0,2	6.0	53,9 ± 0,2	6.0	53,9 ± 0,2
5.5	42,4 ± 0,2	5.5	42,4 ± 0,2	5.5	42,4 ± 0,2	5.5	42,4 ± 0,2
5.0	38,0 ± 0,2	5.0	38,0 ± 0,2	5.0	38,0 ± 0,2	5.0	38,0 ± 0,2
4.5	32,3 ± 0,1	4.5	32,3 ± 0,1	4.5	32,3 ± 0,1	4.5	32,3 ± 0,1
ST-BL							
pH	**Lactose (gL^{-1})**	**pH**	**Lactose (gL^{-1})**	**pH**	**Lactose (gL^{-1})**	**pH**	**Lactose (gL^{-1})**
6.5	62,4 ± 0,2	6.5	62,4 ± 0,2	6.5	62,4 ± 0,2	6.5	62,4 ± 0,2
6.0	53,9 ± 0,1	6.0	53,9 ± 0,1	6.0	53,9 ± 0,1	6.0	53,9 ± 0,1
5.5	42,4 ± 0,2	5.5	42,4 ± 0,2	5.5	42,4 ± 0,2	5.5	42,4 ± 0,2
5.0	38,0 ± 0,2	5.0	38,0 ± 0,2	5.0	38,0 ± 0,2	5.0	38,0 ± 0,2
4.5	29,5 ± 0,2	4.5	29,5 ± 0,2	4.5	29,5 ± 0,2	4.5	29,5 ± 0,2

3.2 Biomass growth

The St-Bl co-culture showed final concentrations of both microorganisms approximately 15 and 39 % higher than in their respective pure cultures (Figure 1), showing a synergistic effect between these strains due to mutual interactions (Oliveira et al., 2008). Maximum growth of microorganisms may be obtained more quickly when dairy mixed cultures are used together (Wang et al., 2003). The possible causes of such a synergism are concerned because free amino acids (FAA) are formed by some LABs' proteolytic activities which can stimulate the growth of both lactobacilli and bifidobacteria (Donkor et al., 2007). Altieri et al. (2008) demonstrated that bifidobacteria are able to reduce oxygen content in the medium, thus reducing a stressing element for streptococci; but, at the same time, they seemed to get a benefit from the consociation with *S. thermophilus*, as proved by higher acidification of the medium and lower viability loss. When inulin was added, the concentrations of St and Bl in the St-Bl co-culture were approximately 35 and 53 % higher than their respective pure cultures. So, these results confirm the synbiotic and bifidogenic effects already mentioned by Šimunek and Evačić (2009).

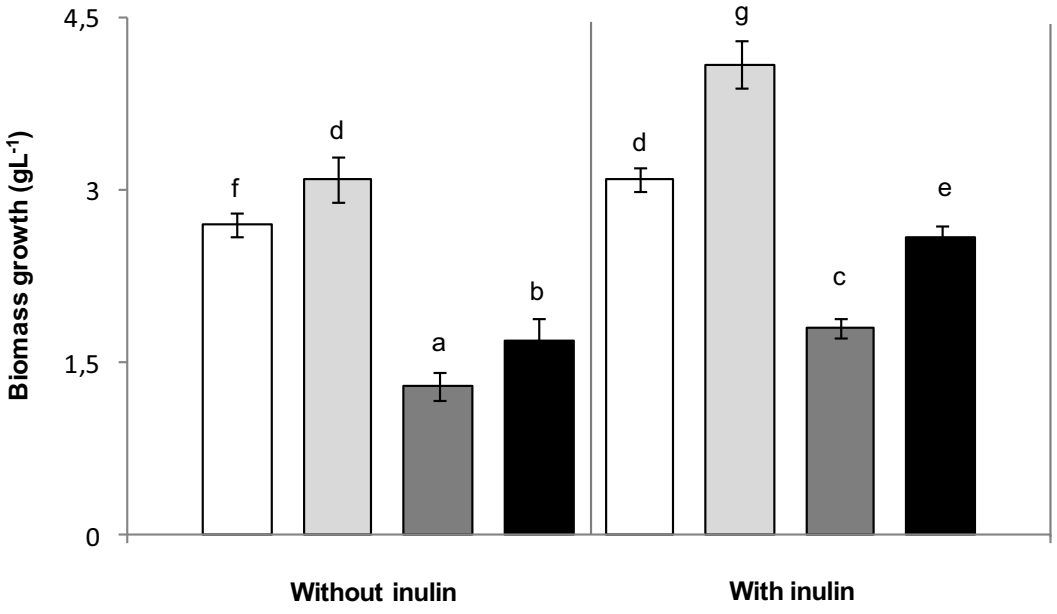

Figure 1: Biomass growth of Streptococcus thermophilus (white color - single culture; light gray color - binary co-culture) *and Bifidobacterium lactis* (dark gray color - single culture; black color - binary co-culture) *in the absence or in the presence of inulin at the end of fermentation (pH= 4.5). Different letters mean statistically significant difference according to the test of Tukey (α < 0.05).*

4. Conclusions

The associative behaviors of *Bifidobacterium lactis* (Bl) and *Streptococcus thermophilus* (St) in skim milk through a study based on their growth and organic acid profile was investigated. In addition, the effect of inulin as a prebiótico was studied. In fact, with inulin, biomass growth, lactic and acetic acid levels were improved. In particular, the concentrations of lactic acid were approximately 10 % (St), 15 % (Bl) and 7 % (St-Bl) higher than those obtained in the absence of this prebiotic, and the ones of acetic acid 19 % (St-Bl) and 22 % (Bl) higher.

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