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Effect of inulin as a prebiotic to improve growth and counts of a probiotic cocktail in fermented skim milk

Ricardo Pinheiro De Souza Oliveira [a,b], Patrizia Perego [b], Maricê Nogueira De Oliveira [a], Attilio Converti [b,*]

[a] Biochemical and Pharmaceutical Technology Department, Faculty of Pharmaceutical Sciences, São Paulo University, Av Prof Lineu Prestes, 580, Bl 16, 05508-900 São Paulo, Brazil
[b] Department of Chemical and Process Engineering, Genoa University, Via Opera Pia 15, I-16145 Genova, Italy

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ABSTRACT

Inulin was used as a prebiotic to improve quality of skim milk fermented by pure cultures of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus* and *Bifidobacterium lactis*, binary co-cultures with *Streptococcus thermophilus*, or a cocktail containing all them. Inulin supplementation to pure cultures lowered the generation time, with particular concern to *S. thermophilus* and *L. acidophilus*. The generation time of all micro-organisms decreased in the following order: mono-cultures, co-cultures, cocktail. It was demonstrated a synergism between *S. thermophilus* and the other strains and a bifidogenic effect of inulin. Enumerations of *L. rhamnosus* in cocktail markedly decreased compared to co-cultures likely because of greater competition for the same substrates. The results of this work highlight the industrial potential of the cocktail, mainly in terms of fermentation acceleration.

1. Introduction

Probiotic foods, including dairy products, have been classically defined as "foods containing live micro-organisms believed to actively enhance health by improving the balance of micro-flora in the gut" (Tamime, Saarela, Søndergaard, Mistry, & Shah, 2005). Lactic-acid bacteria are well-known probiotics that, when used in large amounts in the preparation of foods, are able to survive the passage through the upper digestive tract and adhere to intestinal cells, helping in the intestinal balance. Probiotics used in functional dairy products belong to the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Saccharomyces*. To produce the desired benefits, they should be present in the product in viable counts during their whole shelf-life (7–9 LogCFU/mL); however, their viability in commercial preparations is affected by several factors, among which the presence of other micro-organisms (Kailasapathy & Rybka, 1997).

Prebiotics are non-digestible carbohydrates that resist hydrolysis and absorption in the upper parts of the gastrointestinal tract and are metabolized selectively by at least one type of probiotic in the colon (Mattila-Sandholm et al., 2002). Among these, inulin proved to exert a protective effect towards *Lactobacillus acidophilus* and *Lactobacillus casei* improving their survival and activity during storage (Donkor, Nilmini, Stolic, Vasiljevic, & Shah, 2007). Nevertheless, other lactobacilli would deserve attention due to their health-promoting effects.

Important information about physiology of industrial strains can be obtained by the study of the influence of culture conditions upon growth kinetics. The generation time was proposed as a tool to investigate the microbial dynamics either in pure or mixed cultures, and in this way the stimulating effect of inulin on the growth of bifidobacteria was confirmed (Bruno, Lankaputhra, & Shah, 2002). Reference to lactobacilli is very poor also to this respect, and only a few attempts were made in pure cultures with *L. acidophilus* (Brizuela, Serrano, & Pérez, 2001), *Lactobacillus rhamnosus* (Jyoti, Suresh, & Venkatesh, 2004), and *Lactobacillus bulgaricus* (Kimmel & Roberts, 1998).

On the basis of this background, inulin appears an important food ingredient that would merit to be additionally explored for the production of functional foods. However, to advance in the field, a number of questions should be solved. Comparing the cell counts and the generation times of *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus* and *Bifidobacterium lactis* in pure culture or in binary co-cultures with *Streptococcus thermophilus* or in a cocktail culture containing all of them, the present study aims at shedding light on the: a) synergistic effects among the selected micro-organisms; b) capability of *L. rhamnosus* to be used as further probiotic in fermented milk production; c) prebiotic effect of inulin on probiotics, with particular concern to the poorly investigated *L. rhamnosus* and *L. acidophilus*; d) preservation of cell viability during short-term cold storage (Table 1).

* Corresponding author. Tel.: +39 010 3532593; fax: +39 010 3532586.
E-mail address: converti@unige.it (A. Converti).

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2. Materials and methods

2.1. Microbial cultures

Five pure commercial starter freeze-dried cultures (Danisco, Sassenage, France) were used, specifically the yoghurt micro-organisms *S. thermophilus* TA040 (St) and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340, from here onwards called *L. bulgaricus* (Lb); and the probiotics *L. acidophilus* LAC4 (La), *L. rhamnosus* LBA (Lr) and *Bifidobacterium animalis* subsp. *lactis* BL 04, from here onwards called *B. lactis* (Bl).

2.2. Milk preparation

Milk prepared adding 13 g of skim powder milk (Molico, Nestlé, Araçatuba, Brazil) in 100 g of distilled water was either used as such (M) or supplemented (SM) with 4 g of inulin/100 g (trade name: Beneo TM) (Orafti Active Food Ingredients, Oreye, Belgium), as previously suggested (Oliveira, Perego, Converti, & Oliveira, 2009). Both milks were thermally treated at 90 °C for 5 min in water bath, model 550 THE (Fisatom, São Paulo, Brazil). Heated milks were transferred to 1.0-L sterile flasks, cooled in ice bath, distributed into 250-mL sterile Schott flasks inside laminar flow chamber, and stored at 4 °C for 24 h before use.

2.3. Inoculum preparation

The La pre-culture was prepared by dissolving 100 mg of freeze-dried culture in 50-mL of skim milk (10 g/100 g 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 into 250-mL of skim milk. Lb, Lr, Bl, and St were prepared in the same way adding to 50 mL of milk 400, 130, 45 and 90 mg of their respective freeze-dried cultures. Enumerations of these pre-cultures ranged from 6.1 to 6.5 LogCFU/mL.

2.4. Fermentations

After inoculation, flasks were transferred to a water bath assembled to a CINAC (Cynetique d'acidification, Ysebaert, Frépillon, France) system that allowed continuously measuring and recording the pH and evaluating the acidification rate. Batch fermentations were carried out in quadruplicate at 42 °C and stopped when the pH reached 4.5.

Table 1
Counts of *L. bulgaricus, L. acidophilus, L. rhamnosus* and *B. lactis* in pure cultures, in binary co-cultures with *S. thermophilus*, and in cocktail containing all of them[a].

	M[b]		SM[c]	
	D1[d] (Log CFU/mL)	D7[e] (LogCFU/mL)	D1[d] (LogCFU/mL)	D7[e] (LogCFU/mL)
Pure culture				
La	7.85t	7.84t	7.87t	7.87t
Lb	7.51jk	7.51jk	7.53jk	7.52jk
Lr	7.12d	7.14d	7.12d	7.13d
Bl	7.25e	7.23e	8.03u	8.02u
Co-culture				
La	7.70op	7.53k	7.81rs	7.79r
Lb	7.80rs	7.68no	7.81rs	7.63m
Lr	7.30f	7.13d	7.50jk	7.51jk
Bl	7.60m	7.48j	9.06z	9.06z
Cocktail culture				
La	7.66o	7.38gh	7.73p	7.32f
Lb	7.62m	7.40hi	7.77q	7.56l
Lr	6.88b	6.70a	7.12d	7.02c
Bl	7.43i	7.36g	8.28x	8.21v

[a] Different letters mean statistically significant difference among the values, according to the test of Tukey ($p < 0.05$).
[b] Skim milk without inulin.
[c] Skim milk supplemented with inulin.
[d] Storage of fermented milk at 4 °C for 1 day.
[e] Storage of fermented milk at 4 °C for 7 days.

2.5. Counts of micro-organisms

Bacteria were enumerated after storage of the fermented skim milk at 4 °C either for 1 day (D1) or for 7 days (D7). Aliquots (1.0 mL) of each sample were diluted with 9 mL of 1 g/L sterile peptonated water. After serial dilutions, bacteria were counted by the pour plate technique. St colonies in co-cultures were counted in M17 agar after aerobic incubation at 37 °C for 48 h. Lb, La and Lr were enumerated in MRS agar, after pH adjustment at 5.4 with acetic acid and aerobic incubation at 37 °C for 48, 72 and 72 h, respectively. Bl was enumerated in MRS agar containing 50 g/L cysteine without any pH adjustment (IDF, 2003).

St and Lb in cocktail were enumerated in M17 and MRS at pH 5.4, respectively, after aerobic incubation at 37 °C for 48 h. After incubation at 37 °C for 72 h in anaerobic jar, La, Bl and Lr were enumerated in MRS plus 10 μL/mL clindamycin (pH 6.2), RCA plus 1 μL/mL dicloxacillin (pH 7.1) and MRS plus 0.5 μL/mL vancomycin (pH 6.2), respectively. M17 and MRS (pH 5.4) were prepared according to Dave and Shah (1996) and Jordano, Serrano, Torres, and Salmeron (1992). All media were obtained from Merck (Darmstadt, Germany).

Anaerobic conditions were ensured by the use of AnaeroGen (Oxoid, Basingstoke, UK). Colony forming units (CFU) were enumerated in plates containing 30 to 300 colonies, and cell concentration was expressed as LogCFU/mL.

2.6. Growth kinetics

Growth kinetics of each microorganism was investigated throughout the milk fermentation, either in the absence or in the presence of 4 g inulin/100 g, by a) pure cultures of Lb, La, Lr, Bl and St; b) binary co-cultures of them with St, or c) a cocktail of all these micro-organisms. In particular, the maximum specific growth rate (μ_{max}) was calculated during the exponential growth phase as $\mu_{max} = \ln(X_2/X_1)/(t_2-t_1)$, being X_2 and X_1 the counts (CFU/mL) at time t_2 and t_1, respectively. The generation time ($t_g = \ln 2/\mu_{max}$) was calculated for each culture from the corresponding value of μ_{max}.

2.7. Statistical analyses

The experimental data of t_g and bacterial counts, either after D1 or D7, were expressed as mean values. Variations with respect to the mean values were presented as standard deviations. Mean values of these parameters were submitted to analysis of variance (ANOVA) by the Statistica Software 6.0. They were compared using the Tukey test at a significant level ($p < 0.05$), and different letters were used to label values with statistically significant difference among them.

3. Results and discussion

3.1. Generation time

As shown in Fig. 1A, inulin supplementation lowered the generation time (t_g) significantly ($p < 0.05$), with particular concern to St and La (by about 30%), which means it exerted a prebiotic effect. For bifidobacteria (Bruno et al., 2002) and lactobacilli (Desai, Powell, & Shah, 2004) this effect was ascribed to the release in the milk of additional nutrients like aminoacids or to the reduction of a negative environmental impact.