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Growth and Survival of Mixed Probiotics in Nonfat Fermented Milk: the Effect of Inulin

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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 microorganisms decreased in the following order: mono-cultures, co-cultures, mixed 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 et al., 2005). 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 microorganisms (Kailasapathy and Rybka, 1997). In the other hand, 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).

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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. 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 microorganisms; 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.

2. Materials and Methods

2.1 Experimental Procedure

Five pure commercial starter freeze-dried cultures (Danisco, Sassenage, France) were used, specifically the yoghurt microorganisms *Streptococcus thermophilus* TA040 (St), *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340, *Lactobacillus acidophilus* LAC4 (La), *Lactobacillus rhamnosus* LBA (Lr) and *Bifidobacterium animalis* subsp. *lactis* BL 04 (Bl). Enumerations of these pre-cultures ranged from 6.1 to 6.5 LogCFU/mL.

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 (Orafti Active Food Ingredients, Oreye, Belgium), as previously suggested (Oliveira et al., 2009).

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^{o}C and stopped when the pH reached 4.5.

2.2. Counts of microorganisms

Bacteria were enumerated after storage of the fermented skim milk at 4°C either for 1 day (D1) or for 7 days (D7). St colonies in co-cultures were counted in M17 agar after aerobic incubation at 37ºC for 48 h. Lb, La and Lr were enumerated in MRS agar, after pH adjustment at 5.4 with acetic acid and anaerobic incubation at 37ºC 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.

2.3. Growth kinetics

Growth kinetics of each microorganism was investigated throughout the milk fermentation, 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 microorganisms. 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}.

2.4. Statistical analyses

Results were submitted to analyses of variance (ANOVA) using the Statistica Software 6.0. Mean values were compared using the Tukey test at $P < 0.05$.

3. Results and Discussion

3.1. Generation time

Table 1 shows the generation time (t_g) of *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus* and *B. lactis* in pure cultures, in binary co-cultures with *S. thermophilus*, and in mixed cultures. In pure culture, the inulin addition 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.

The t_g values of all the microorganisms were remarkably lower in co-cultures, either in the presence of inulin (by 27-60%) or not (by 22-70%), confirming the occurrence of a synergism between St and the other strains.

The simultaneous presence of all the microorganisms in the mixed cultures (cocktail) lowered furthermore all the t_g values even compared to co-cultures, either in the presence of inulin (by 11-55%) or not (by 23-64%). It is possible that the presence of several microorganisms could have led to larger synergistic or suppressive effects among them, associated to the release of metabolic products, such as organic acids, inferring with the metabolism of the others, increased proteolytic activity and so on.

*Table 1: Generation time (t_g) 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**

	M	SM
Pure culture		
St	0.45^{c}	0.33^{b}
La	0.98^{g}	0.87^{f}
Lb	1.49^{j}	0.94^{f}
Lr	1.01^{e}	0.93^{f}
Bl	0.85^{f}	0.79^{e}
Co-culture		
St	0.27^{a}	0.24^{a}
La	0.53^{c}	0.42^{c}
Lb	0.45^{c}	0.37^{b}
Lr	0.76^{e}	0.55^{d}
Bl	0.57^{d}	0.37^{b}
Cocktail culture		
St	0.31^{b}	0.23^{a}
La	0.24^{a}	0.15^{a}
Lb	0.39^{b}	0.20^{a}
Lr	0.51^{c}	0.42^{c}
Bl	0.30^{b}	0.20^{a}

M: Skim milk without inulin.
SM: Skim milk supplemented with inulin.
*Different letters mean statistically significant difference among the values, according to the test of Tukey ($P < 0.05$).

3.2. Microbiological analysis

Enumeration of St in all these cultures varied within a very narrow range (8.91-9.11 LogCFU/mL) and was not significantly influenced by the microorganism, the presence of inulin and the storage duration (data not shown).With regards to pure cultures, all the strains, but St, exhibited comparable counts in the absence of inulin, irrespective of the storage time (7.12-7.85 LogCFU/mL) (Fig. 1A). These results are almost coincident with that reported by Oliveira et al. (2008).

In binary co-cultures without inulin, although statistically significant ($P < 0.05$), the variations in the counts were not so large either after D1 or D7 (7.13-7.80 LogCFU/mL), the highest and lowest values having been detected for Lb (D1) and Lr (D7), respectively (Fig. 1B). On the other hand, apart from Lb, inulin stimulated the growth of the other lactic bacteria, with particular concern to Bl, whose counts dramatically increased, from 7.60 LogCFU/mL (D1) or 7.48 LogCFU/mL (D7) to 9.06 LogCFU/mL (D1 and D7) ($P < 0.05$).

In the cocktail (Fig. 1C), the average counts of Lr (6.79 LogCFU/mL) were about 6% lower than in its respective co-culture, while those of La (7.52 LogCFU/mL), Lb (7.51 LogCFU/mL) and Bl (7.39 LogCFU/mL) were only 1-3% lower, as the likely result of

greater competition of these microorganisms for the same substrates. Although only Lr in M showed counts in the cocktail below the recommended limit (7.0 LogCFU/mL), the addition of inulin to skim milk appears to be a fundamental requisite to get fermented milk with all the characteristics of functional products.

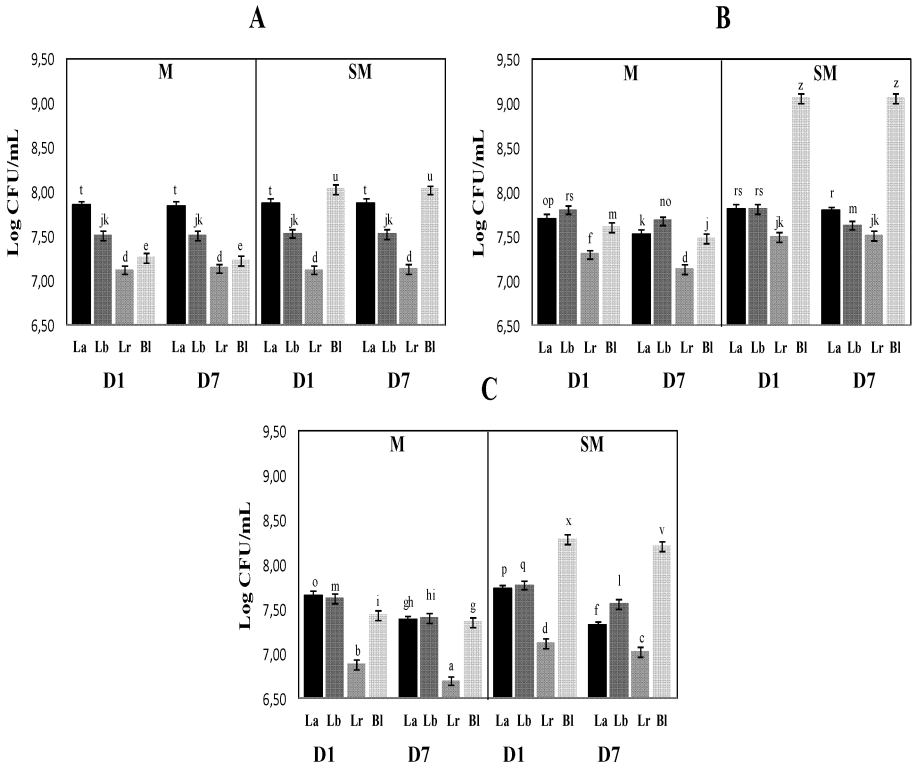

Figure 1: Counts of A) L. acidophilus (La), L. bulgaricus (Lb), L. rhamnosus (Lr) and B. lactis (Bl) in pure cultures; of B) La, Lb, Lr and Bl in binary co-cultures with St; of C) cocktail containing all the strains together. M = skim milk without inulin; SM = skim milk supplemented with 4 g of inulin/100 g. D1 = Storage of fermented milk at 4°C for 1 day; D7 = Storage of fermented milk at 4°C for 7 days.

4. Conclusions

The present work dealt with the effect of inulin as a prebiotic to improve the quality of skim milk fermented by pure cultures, by binary co-cultures of *L. acidophilus, L. rhamnosus, L. bulgaricus* and *B. lactis* with *S. thermophilus* or by a cocktail containing all of them.
The supplementation of inulin in binary co-cultures and in the cocktail led to generation times significantly shorter than in pure cultures, while it was not observed any valuable effect on the counts of pure cultures.
After storage of fermented milk at 4°C for 1 day, the prebiotic effect of inulin was remarkable only for the *S. thermophilus-B. lactis* co-culture, while, after 7 days, it was so for all the binary co-cultures. Either after 1 or 7 days, the enumerations of *L. rhamnosus* and *B. lactis* in the cocktail markedly decreased compared to their respective co-cultures, as the consequence of greater competition for the same substrates.

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