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Interactions Among Lactic Acid Starter and Probiotic Bacteria Used for Fermented Dairy Products

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ABSTRACT

Interactions among lactic acid starter and probiotic bacteria were investigated to establish adequate combinations of strains to manufacture probiotic dairy products. For this aim, a total of 48 strains of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium* spp. (eight of each) were used. The detection of bacterial interactions was carried out using the well-diffusion agar assay, and the interactions found were further characterized by growth kinetics. A variety of interactions was demonstrated. *Lb. delbrueckii* subsp. *bulgaricus* was found to be able to inhibit *S. thermophilus* strains. Among probiotic cultures, *Lb. acidophilus* was the sole species that was inhibited by the others (*Lb. casei* and *Bifidobacterium*). In general, probiotic bacteria proved to be more inhibitory towards lactic acid bacteria than vice versa since the latter did not exert any effect on the growth of the former, with some exceptions. The study of interactions by growth kinetics allowed the setting of four different kinds of behaviors between species of lactic acid starter and probiotic bacteria (stimulation, delay, complete inhibition of growth, and no effects among them). The possible interactions among the strains selected to manufacture a probiotic fermented dairy product should be taken into account when choosing the best combination/s to optimize their performance in the process and their survival in the products during cold storage.
(**Key words:** bacterial interactions, lactic acid bacteria, probiotic bacteria)

Abbreviation key: CFS = cell-free supernatant, **CCFS** = concentrated cell-free supernatant.

INTRODUCTION

An increasing commercial interest in the addition of probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus casei*, and bifidobacteria) to fermented dairy products has been observed since recent discoveries in several aspects of bioscience support the hypothesis that, beyond nutrition, diet may modulate various functions in the body (Sanders and in't Veld, 1999). Properly formulated probiotic foods offer consumers a low cost dietary component that has the potential to promote health in a variety of ways (Goldin, 1998). In the fermentative dairy industry, the current trend is to add cultures composed of defined single strains to fermented milks (Gilliland, 1998) and cheeses (Stanton et al., 1998; Vinderola et al., 2000); to do so, a wide variety of lactic acid bacteria and probiotic strains are commercially available. Different combinations of starter lactic and probiotic cultures allow the production of fermented dairy products with target technological characteristics, and potential nutritional and health benefits (Juillard et al., 1987). However, microbial interactions, either beneficial (protocooperation) or unfavorable (antagonism) among these cultures may generate undesirable changes in the composition of the bacterial flora during the manufacture and cold storage of fermented dairy products (Bellengier et al., 1997).

For lactic acid bacteria, it has been found that some rod/coccus culture combinations were inhibitory, stimulatory, or neutral with regard to the rate of lactic acid production compared with single-strain cultures. Although a symbiotic relationship between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* is generally assumed, not all strains are actually compatible, and growth imbalance in fermentations with mixed cultures may occur (Radke-Mitchell and Sandine, 1984). There is little information regarding possible interactions among lactic acid starter and probiotic bacteria. It was established that interaction among species is a factor affecting the viability of *Lb. acidophilus* and bifidobacteria in yoghurt (Kailasapathy and Rybka, 1997; Vinderola et al., 1999). Regarding interactions among probiotic bacteria strains added to

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fermented dairy products, there is no information available, with the exception of some cases such as the synergistic growth-promoting effects observed between *Lb. acidophilus* and *B. bifidum* strains (Kneifel et al., 1993) and the growth inhibition among probiotic species due to bacteriocin production (Yang, 1998; Yildirim and Johnson, 1998). Besides, in Argentina, the current practice is to add *Lactococcus* cultures to probiotic (Vinderola et al., 2000) or traditional cheeses manufactured by thermophilic technologies, without knowledge of the compatibility with the other genera used (*Streptococcus*/*Lactobacillus*) for this product kind. Further studies on interactions among strains would be appreciated (Juillard et al., 1987; Rajagopal and Sandine, 1990) because these bacteria must arrive alive to the intestinal tract to perform their probiotic role (Kailasapathy and Rybka, 1997).

The aim of this work was to screen and characterize the interactions among lactic acid starter and probiotic bacteria to establish adequate combinations of strains to manufacture probiotic dairy products.

MATERIALS AND METHODS

Lactic Acid Starters and Probiotic Strains

Five commercial strains (identified as A4, A5, A10, DC1, and CC1) and three wild strains (identified as strains 43, 175, and 176, isolated from Argentinian natural milk cultures) of *Streptococcus thermophilus*, eight commercial strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (identified as Ab1, Bb1, Cb1, Db1, Eb3, Eb4, Gb1, and Hb2) and eight commercial strains of *Lactococcus lactis* (identified as 13-3, 15-1, 15-4, C12, SL3, SD5, Mo12, and A6) were used for this study. The following probiotic cultures were also assayed: *Lactobacillus acidophilus* (commercial strains A3, A9, 08, 53, La5, CSL, and strains CNRZ 1881 and CNRZ 1923, obtained from the CNRZ collection-INRA, Jouy-en-Josas, France), *Lactobacillus casei* (commercial strains A13, A14, A15, A16, strains BRA, LS, and LB isolated from fermented dairy products and strain CNRZ 1874), *B. bifidum* (commercial strains A12, BBI and Bb12 and ATCC 35914), *Bifidobacterium longum* (commercial strains A1 and A7 and strain BL isolated from a fermented dairy product) and *Bifidobacterium* sp. (commercial strain A2). All commercial strains were provided by local industries. All strains were kept frozen (–80°C) in the PROLAIN collection.

Preparation of Cell-Free Supernatants

Lactic acid starter bacteria were grown in 10% reconstituted skim milk (Merck, Darmstadt, Germany) at 37°C (except lactococci that were incubated at 25°C) for 24 h. Probiotic bacteria were grown in both MRS broth and 10% reconstituted skim milk under aerobic conditions (except bifidobacteria that were incubated under anaerobiosis, Gaspak System) at 37°C for 24 h. Bifidobacteria required the addition of 0.25% yeasts extract (Britania, Buenos Aires, Argentina) to coagulate milk. *Lactobacillus casei* LS was the only strain that did not coagulate skim milk, even incubated anaerobically in the presence of 0.25% yeasts extract. Cell-free supernatants (**CFS**) were obtained by centrifugation (3300 × *g*, 20 min, 5°C) of overnight cultures and sterilization by filtration through a 0.45-μm pore filter (Millipore, Biopore S.R.L., Buenos Aires, Argentina). Cell-free supernatants were kept frozen (–80°C).

Detection of Bacterial Interactions (Well-Diffusion Agar Assay)

Interactions among lactic acid strains, among probiotic strains and between both type of strains were investigated. Twenty milliliters of MRS or Elliker agar (Biokar, Beauvais, France) melted and tempered at 45°C were vigorously mixed with 200 μl of an overnight culture of lactobacilli/bifidobacteria and cocci, and poured into Petri dishes. Wells of 10 mm in diameter were made in the agar layer, and 180 μl of the cell-free supernatant of each strain was placed into each well. The plates were incubated aerobically overnight (except for bifidobacteria that were incubated under anaerobiosis) at 37°C (lactococci plates were incubated at 25°C). Experiments were replicated three times.

Detection of Bacterial Interactions (Growth Kinetics)

According to the results obtained by the screening method described above, growth kinetics were performed. The strains were grown at 37°C in broth in the presence (test curve) or not (control curve) of a concentrated cell-free supernatant (**CCFS**) of another species. The CCFS were obtained as described above, but before passage through the filter, they were fivefold-concentrated (4 h, 43°C, under vacuum) so its addition (5% vol/vol) to the broth would not significantly dilute it. Growth kinetics were performed by optical density (560 nm) measurements. Viable cell counts were carried out in MRS agar (3 d at 37°C, aerobiosis for lactobacilli and anaerobiosis for bifidobacteria) or Elliker agar (3 d at 37°C, aerobiosis for streptococci) after 24 h of growth to estimate the differences in cell counts between test and control kinetics. Experiments were carried out in triplicate.

Inhibition of *Lb. delbrueckii* subsp. *bulgaricus* Strains by *Lb. acidophilus* Supernatants

To elucidate the nature of the complete growth inhibition of *Lb. delbrueckii* subsp. *bulgaricus* strains by *Lb. acidophilus* supernatants observed in both the well-diffusion assay and the growth curve of *Lb. delbrueckii* subsp. *bulgaricus* Ab1 in the presence of CCFS of *Lb. acidophilus* CNRZ 1881, a CCF was submitted to the following treatments: heating at 121°C for 15 min, neutralization (NaOH) and incubation (4 h, 37°C) in the presence of proteolytic enzymes (Pepsin, Merck, Darmstadt, Germany and Proteinase K, Sigma, St. Louis, MO). Then, they were concentrated, sterilized as previously described, and assayed for remaining activity by the growth kinetics method (measuring OD_{560nm} after 24 h of incubation). CCFS without any treatment was used as a control. Experiments were carried out in triplicate.

Stimulation of *Lb. acidophilus* Strains by *B. bifidum* Supernatants

To determine whether the growth stimulation of *Lb. acidophilus* CNRZ 1881 and A3 by *B. bifidum* BBI and A12 supernatants observed in growth kinetics was due to the acetate (Marshall, 1991) produced by bifidobacteria, *Lb. acidophilus* strains were grown at acetate concentrations (0.05, 0.3, and 1.2%) normally produced by bifidobacteria in liquid media (Ventling and Mistry, 1993; Samona et al., 1996; Dubey and Mistry, 1996a, 1996b; Gomes et al., 1998; Mlobeilli et al., 1998). The effect was investigated by the growth kinetics method (measuring optical density at 560 nm after 24 h of incubation). Experiments were carried out in triplicate.

Statistical Analysis

All data were analyzed using the one-way ANOVA procedure of SPSS. The differences among means were detected by the Duncan's multiple range test (Lizasoain and Joaristi, 1995).

RESULTS

Well-Diffusion Agar Assay

Interactions among lactic acid starter bacteria strains. A total of 24 strains of lactic acid starter bacteria belonging to the species *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* (eight strains of each) were tested for interactions among them. Three behaviors were detected with this methodology: complete (a clear absence of growth of the test strain around the well) and weak (the presence of a partial inhibition halo around the well) inhibitions, and absence of interaction. Cell-free supernatants of *Lc. lactis* did not present evident effects on the growth of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains. The same behavior was observed when *Lb. delbrueckii* subsp. *bulgaricus* and *Lc. lactis* strains developed in the presence of CFS of *S. thermophilus*, except for *Lc. lactis* 15-4 and SL3 that were weakly inhibited by streptococci supernatants. *Lactobacillus delbrueckii* subsp. *bulgaricus* supernatants weakly inhibited the growth of *Lc. lactis* strains, except for *Lb. delbrueckii* subsp. *bulgaricus* Ab1 and Gb1 on *Lc. lactis* SD5, 15-1, 15-4, 13-3 and SL3 and *Lb. delbrueckii* subsp. *bulgaricus* Cb1 on *Lc. lactis* 15-1. In these cases, no effect was observed (data not shown). The most variable results were observed when *S. thermophilus* strains were grown in the presence of *Lb. delbrueckii* subsp. *bulgaricus* supernatants (Table 1). In this case, the absence of interaction and the results of complete and weak inhibitions were recorded. The most inhibitory strains were *Lb. delbrueckii* subsp. *bulgaricus* Db1, Eb3, Eb4, and Hb2, while *Lb. delbrueckii* subsp. *bulgaricus* Ab1 and Gb1 showed a reduced inhibition spectrum. On the other hand, the most sensitive *S. thermophilus* strains were 175 and DC1.

Table 1. Effect of cell-free supernatants (CFS) of *Lactobacillus delbrueckii* subsp. *bulgaricus* strains on the growth of *Streptococcus thermophilus* strains (well-diffusion agar assay).

S. thermophilus (strain)	*L. delbrueckii* subsp. *bulgaricus* (strain)							
	Ab1	Bb1	Cb1	Db1	Eb3	Eb4	Gb1	Hb2
A4	–	–	–	+	+	+	–	+
A5	–	+	+	+	+	+	–	+
A10	–	+	+	+	+	+	–	+
43	–	+	+	+	+	+	–	+
175	+	x	x	x	x	x	+	x
176	–	+	+	+	+	+	–	+
CC1	+	+	+	+	+	+	+	+
DC1	+	x	x	x	x	x	+	x

x = Complete inhibition.
+ = Weak inhibition.
– = No effect.

Interactions among probiotic bacteria strains. A total of 24 strains of probiotic bacteria belonging to *Lb. casei*, *Lb. acidophilus*, and *Bifidobacterium* (eight of each) were tested for their antimicrobial activity with the well-diffusion assay. Cell-free supernatants of *Lb. acidophilus* obtained in MRS broth or skim milk did not show any effect on the growth of *Lb. casei* or *Bifidobacterium* strains nor did CFS of *Lb. casei* obtained in MRS broth or skim milk on the growth of *Bifidobacterium* species or vice versa (data not shown). *Lactobacillus acidophilus* proved to be the most sensitive species among probiotic bacteria because seven of the strains

Table 2. Effect of cell-free supernatants (CFS) of *Bifidobacterium* and *Lactobacillus casei* strains obtained from skim milk (left sign in each box) and MRS broth (right sign in each box) cultures on the growth of *Lactobacillus acidophilus* strains (well-diffusion agar assay).

	Bifidobacterium (strain)								*Lb. casei* (strain)							
Lb. acidophilus (strain)	A1	A2	A7	35914	A12	BL	BBI	Bb12	A13	A14	A15	A16	BRA	LB	LS[1]	1874
A3	+ −	− −	+ −	+ −	+ −	+ −	+ −	+ −	− +	+ +	− +	+ +	− +	+ +	+	− +
A9	+ +	− +	+ +	+ +	+ +	+ +	+ +	+ +	− +	+ +	− +	+ +	+ +	+ +	+	− +
08	+ −	− −	+ −	+ −	+ −	− −	+ −	+ −	− +	+ +	− +	− +	− −	− −	+	− +
53	+ +	− +	− +	+ +	− −	+ −	+ +	+ −	− +	− +	− +	− +	− −	− −	+	− +
CNRZ 1881	+ +	− +	+ −	+ −	+ +	+ +	+ +	+ +	− +	+ +	− +	+ +	+ +	+ +	+	− +
CNRZ 1923	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	−	− −
CSL	+ −	− +	+ −	+ −	+ +	+ +	+ +	+ +	− +	+ +	− +	+ +	− +	+ +	+	− +
La5	+ −	− −	+ −	+ −	+ −	− −	− −	+ −	− +	− +	− +	+ +	− +	+ +	+	− +

x = Complete inhibition.

+ = Weak inhibition.

− = No effect.

[1]CFS obtained only from a MRS broth culture.

assayed were inhibited to a certain extent by all strains of *Bifidobacterium* and *Lb. casei* strains (Table 2). Cell-free supernatants of *Lb. casei* strains proved to be more inhibitory on *Lb. acidophilus* strains when obtained from MRS broth than from skim milk, while the opposite was observed for CFS of *Bifidobacterium* strains. *Lb. acidophilus* CNRZ 1923 showed to be insensitive to CFS of *Lb. casei* and *Bifidobacterium*. The less inhibitory strains were *Bifidobacterium* A2, A12, and BL and *Lb. casei* BRA and LB.

Interactions among lactic acid starter and probiotic bacteria strains. A total of 24 strains of lactic acid starter bacteria belonging to the species *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lc. lactis* (eight of each) and 24 strains of probiotic bacteria belonging to *Lb. casei*, *Lb. acidophilus*, and *Bifidobacterium* (eight of each) were tested for interactions among them, using CFS obtained from skim milk cultures (Table 3). As a general statement, probiotic bacteria proved to be more inhibitory toward lactic acid bacteria than vice versa since the latter did not exert any effect on the growth of the former, except for CFS of some strains of *Lb. delbrueckii* subsp. *bulgaricus* that inhibited weakly the growth of some *Lb. acidophilus* strains. *Bifidobacterium* and *Lb. casei* strains did not show effects on the growth of *Lb. delbrueckii* subsp. *bulgaricus* strains. On *S. thermophilus* and *Lc. lactis*, probiotic strains showed variable results, depending on the strain considered. When probiotic strains CFS obtained from MRS broth cultures were used, a wider and stronger inhibition was observed on lactic acid starter strain growth than when CFS obtained from skim milk cultures were used, except for *Bifidobacterium* and *Lb. delbrueckii* subsp. *bulgaricus*, since no interactions were detected between them (data not shown). In this case, all *Bifidobacterium* and *Lb. casei* strains inhibited the growth of *S. thermophilus* and *Lc. lactis* strains weakly, except CFS of *B. bifidum* ATCC 35914 that showed no effect in the development of *Lc. lactis* strains, and also *Lc. lactis* SD5 and Mo12 proved to be resistant to CFS of *Bifidobacterium* strains Bb12, A1, BBI, and BL. It was also observed that *Lb. delbrueckii* subsp. *bulgaricus* Bb1 and Hb2 were weakly inhibited by CFS from all strains of *Lb. casei*, while the other *Lb. delbrueckii* subsp. *bulgaricus* strains were inhibited in variable ways by the latter. All *Lb. delbrueckii* subsp. *bulgaricus* strains were completely inhibited by all *Lb. acidophilus* strains and by some strains (A5, A10, DC1, 175, and 176) of *S. thermophilus.*

Growth Kinetics

Taking into account the results obtained by the well-diffusion agar assays, some strain combinations were chosen to perform growth kinetics. The results obtained revealed different effects when a strain was grown in the presence (5%, vol/vol) of a CCFS of another one. The behaviors observed were: 1) no effect, 2) growth stimulation, 3) growth delay and 4) complete growth inhibition (Table 4).

Figure 1 shows a typical case for which no effect on the test strain growth was detected. The test organism was *Lb. acidophilus* CNRZ 1881 and the CCFS used was obtained from a culture of *S. thermophilus* A10. At different times, no significant differences were detected ($P > 0.05$) between OD_{560nm} for both kinetics.

The stimulation of growth of *Lb. acidophilus* A3 caused by the addition of a CCFS of *B. bifidum* BBI is shown in Figure 2 as a typical case of this kind of behavior. The viable cell count values were significantly different ($P < 0.05$; 7.73 ± 0.09 and 8.38 ± 0.23 $\log_{10}$ orders for the control and test kinetics, respectively) after 24 h. The final OD_{560nm} values were 1.95 (control kinetics) and 2.44 (test kinetics).

Table 3. Effect of cell-free supernatants (CFS) obtained from skim milk cultures of lactic acid starter on probiotic bacteria (right sign in each box) and of probiotic on lactic acid starter bacteria (left sign in each box) (well-diffusion agar assay).[1]

	Streptococcus thermophilus								*Lactobacillus delbrueckii* subsp. *bulgaricus*								*Lactococcus lactis*							
Probiotic strains	CC1	43	A4	A5	A10	DC1	175	176	Ab1	Bb1	Cb1	Db1	Eb3	Eb4	Gb1	Hb2	13-3	15-1	15-4	SL3	SD5	A6	C12	Mo12
Bifidobacterium																								
Bb12	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
A1	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	− −	+ −	+ −	− −
BBI	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	− −	+ −	+ −	+ −
BL	+ −	+ −	− −	+ −	+ −	− −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	− −	− −	− −	+ −	− −
A2	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	− −	− −	− −	− −	− −	− −
A12	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
A7	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	− −	− −	+ −	− −
35914	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
Lactobacillus acidophilus																								
A3	+ −	+ −	+ +	+ +	+ −	+ −	+ −	+ −	− +	− +	− −	− −	− −	− −	− +	− +	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
A9	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
08	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− +	− −	− +	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
53	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− +	− +	− +	− +	− +	− +	− +	− +	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
1881	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	x +	x +	x +	x −	x −	x −	x +	x +	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
1923	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	x −	x −	x −	x −	x −	x −	x −	x −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
CSL	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
La5	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
Lactobacillus casei																								
BRA	+ −	+ −	− −	+ −	+ −	− −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	− −	− −	+ −	− −
LB	+ −	+ −	+ −	+ −	+ −	− −	+ −	+ −	− −	− −	− −	− −	− −	− −	+ −	− −	+ −	+ −	+ −	+ −	− −	− −	+ −	− −
LS[2]	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	+ −	− −	+ −	− −	− −	− −	+ −	−	−	−	−	−	−	−	−
1874	− −	+ −	+ −	+ −	+ −	+ −	− −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	− −	− −	+ −	− −	− −	− −	− −
A13	− −	+ −	− −	+ −	− −	− −	− −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	− −	− −	− −	+ −	− −
A14	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
A15	+ −	+ −	− −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	− −	− −	− −	+ −	− −
A16	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	− −	− −	− −	− −	− −	− −	− −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −

[1]x = Complete inhibition; + = Weak inhibition; − = No effect.

[2]No growth in skim milk.

Table 4. Interactions among lactic acid starter and probiotic bacteria (growth kinetics).[1]

Growing organism	CCFS added (from a culture of[1])	Interaction
Lb. acidophilus CNRZ 1881	*B.b.* A12 (MRS broth)	Stimulation
	L.b. Ab1 (MRS broth)	Delay
	L.c. A14 (MRS broth)	No effect
	B.b. BBI (MRS broth)	Stimulation
	S.t. A10 (Elliker broth)	No effect
	S.t. A10 (RSM)	No effect
Lb. acidophilus A3	*B.b.* BBI (MRS broth)	Stimulation
Lb. casei A14	B.b. A12 (MRS broth)	No effect
	L.b. Ab1 (MRS broth)	Delay
	L.a. CNRZ 1881 (MRS broth)	No effect
Lb. delbrueckii subsp. *bulgaricus* Ab1	*L.a.* CNRZ 1881 (MRS broth)	Complete inhibition
	L.c. A14 (MRS broth)	Delay
	S.t. A10 (RSM)	Delay
	S.t. A10 (Elliker broth)	Delay
S. thermophilus A10	*L.b.* Ab1 (MRS broth)	Complete inhibition
	L.b. Ab1 (RSM)	Complete inhibition

[1]Parentheses indicate the culture medium from which CCFS were obtained. CCFS = Concentrated cell-free supernatant. RSM = 10% reconstituted skim milk. *B.b.* = *B. bifidum. L.b.* = *Lb. acidophilus. L.b.* = *Lb. delbrueckii* subsp. *bulgaricus*. *L.c.* = *Lb. casei. S.t.* = *S. thermophilus.*

Figure 3 shows the delay in growth caused by the addition of a CCFS of *Lb. delbrueckii* subsp. *bulgaricus* Ab1 on a culture of *Lb. casei* A14. During the first 10 h a bacteriostatic effect was observed. Then, the growth was exponential and no significant differences ($P > 0.05$) were detected after 24 h between the viable cell counts and OD_{560nm} values for the control and test kinetics.

Finally, a complete growth inhibition of *Lb. delbrueckii* subsp. *bulgaricus* Ab1 was observed when it was cultured in the presence of a CCFS of *Lb. acidophilus* CNRZ 1881 (Figure 4). There were no significant differences ($P > 0.05$) between colony counts of *Lb. delbrueckii* subsp. *bulgaricus* Ab1 at time zero and after 24 h of incubation in the presence of the CCFS of *Lb. acidophilus* CNRZ 1881, while the control grew from 6.21 ± 0.10 up to 8.15 ± 0.12 $\log_{10}$ orders (cfu/ml) after 24 h at 37°C. The results of culturing *Lb. delbrueckii* subsp. *bulgaricus* Ab1 in the presence of CCFS of *Lb. acidophilus* CNRZ 1881 submitted to different treatments, measured as OD_{560nm} values at 24 h (growth kinetics) are shown in Figure 5. There was no reduction in the antibacterial activity after the neutralization or heating (121°C for 15 min) of the CCFS, but this activity produced by *Lb. acidophilus* CNRZ 1881 completely disappeared after the treatments with proteinase K and pepsin. These results indicate that the growth inhibition of *Lb. delbrueckii* subsp. *bulgaricus* Ab1 by CCFS of *Lb. acidophilus* CNRZ 1881 was due to the presence

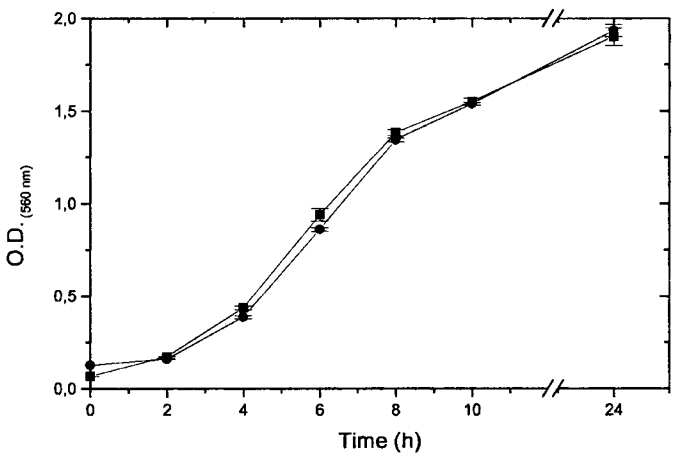

Figure 1. Growth (37°C) kinetics of *Lactobacillus acidophilus* CNRZ 1881 with (●) and without (■) the addition of concentrated cell-free supernatant of *Streptococcus thermophilus* A10 (mean values ± SD of three experiments).

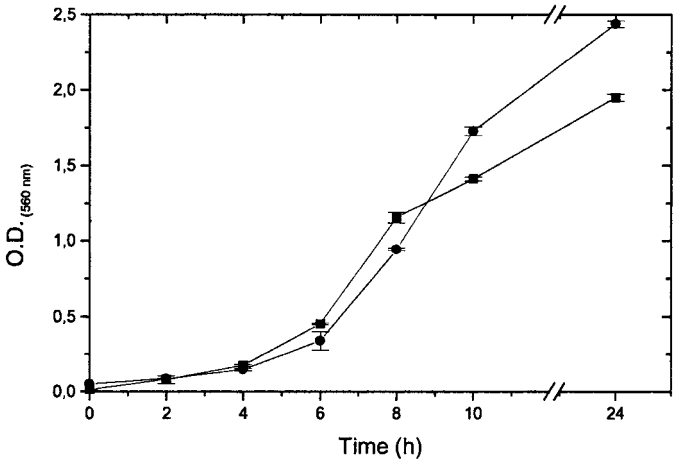

Figure 2. Growth (37°C) kinetics of *Lactobacillus acidophilus* A3 with (●) and without (■) the addition of concentrated cell-free supernatant of *Bifidobacterium bifidum* BBI (mean values ± SD of three experiments).

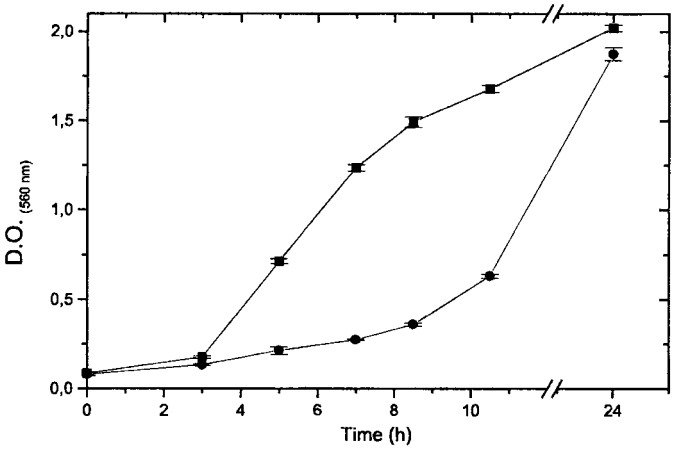

Figure 3. Growth (37°C) kinetics of *Lactobacillus casei* A14 with (●) and without (■) the addition of concentrated cell-free supernatant of *Lactobacillus delbrueckii* subsp. *bulgaricus* Ab1 (mean values ± SD of three experiments).

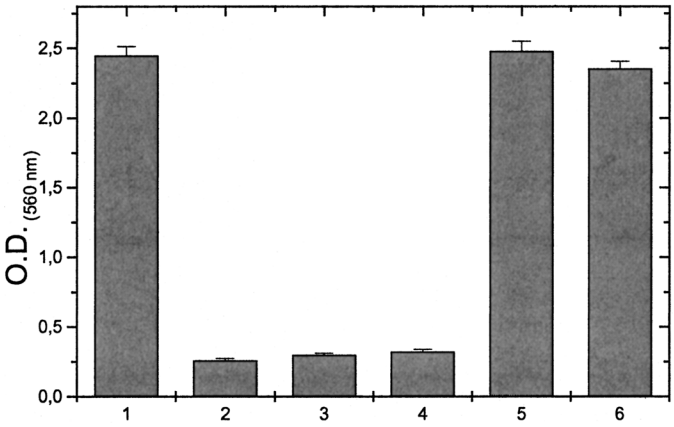

Figure 5. Influence of a concentrated cell-free supernatant (MRS broth, 24 h at 37°C) of *Lactobacillus acidophilus* CNRZ 1881 (2), neutralized (3), heated (121°C, 15 min) (4) and treated with proteinase K (5) and pepsin (6), on the growth (after 24 h at 37°C in MRS broth) of *Lactobacillus delbrueckii* subsp. *bulgaricus* Ab1 (1, control) (mean values ± SD of three experiments).

of a bacteriocin-like substance produced by the latter. On the other hand, it was found that the growth stimulation of *Lb. acidophilus* CNRZ 1881 and A3 was not due to the presence of acetate, as it had been previously reported (Marshall, 1991) because the growth stimulation of pure cultures of *Lb. acidophilus* CNRZ 1881 (Figure 6) and A3 was not achieved by the addition of sodium acetate as observed in the presence of a CCFS of *B. bifidum* BBI and A12 (Table 4).

DISCUSSION

Mixed-strain cultures of lactic acid starter and probiotic bacteria are commonly used in the manufacture of probiotic fermented milks and cheeses. In these bacterial combinations, interactions among different strains can result in stimulation, inhibition, or the absence of effects on microbial growth rate and metabolic activity.

Even though a protocooperative interaction between *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* has always been recognized, some rod/coccus culture combinations were found to inhibit the rate of lactic acid production compared with single strain cultures (Radke-Mitchell and Sandine, 1984; Zárate et al., 2000). In our work, *Lb. delbrueckii* subsp. *bulgaricus* was found to be able to marginally inhibit the growth of

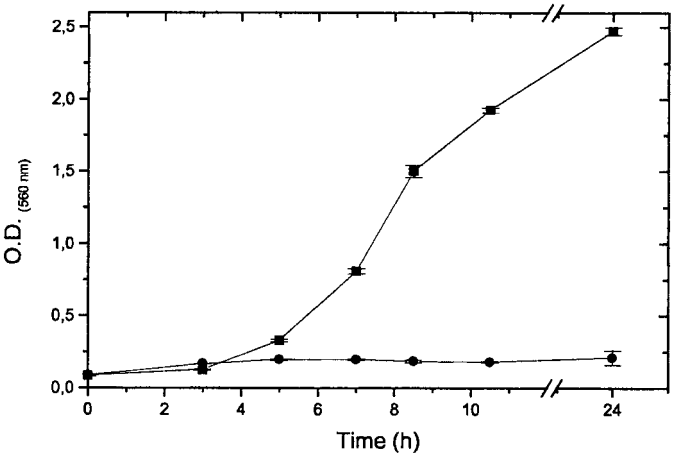

Figure 4. Growth (37°C) kinetic of *Lactobacillus delbrueckii* subsp. *bulgaricus* Ab1 with (●) and without (■) the addition of concentrated cell-free supernatant of *Lactobacillus acidophilus* CNRZ 1881 (mean values ± SD of three experiments).

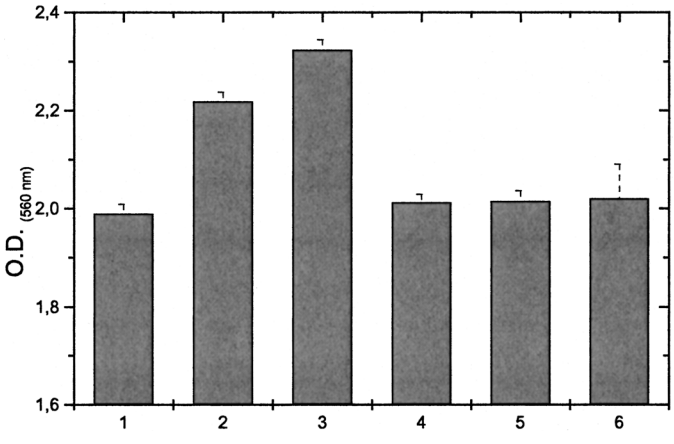

Figure 6. Influence of concentrated cell-free supernatant (MRS broth, 24 h, 37°C) of *Bifidobacterium bifidum* A12 (2) and BBI (3) and 0.05% (4), 0.3% (5) and 1.2% (6) sodium acetate on the growth (after 24 h at 37°C in MRS broth) of *Lactobacillus acidophilus* CNRZ 1881 (1, control) (mean values ± SD of three experiments).

strains of *S. thermophilus* and *Lc. lactis* used, but no inhibition was observed between lactococci and streptococci or when their filtrates were checked on the former. These results are contrary to those previously reported (Babel, 1976), where it was found that some strains of *Lc. lactis* inhibited the growth of *Lb. delbrueckii* subsp. *bulgaricus*. Although nisin is an antibacterial agent produced by certain strains of *Lc. lactis* (Kumar and Prasad, 1992) with a demonstrated antagonistic activity on *Lactobacillus*, including strains of *Lb. acidophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, no inhibition from *Lc. lactis* on lactobacilli strains was observed in our study.

Although a great variety of genera/species/strain combinations of probiotic bacteria are used for probiotic dairy products (fermented or not), the interactions among them were scarcely studied. From our results, *Lb. acidophilus* was the sole species inhibited by the others (*Lc. casei* and *Bifidobacterium*).

Taking into account the inhibitory behavior of some CCF of *Lb. delbrueckii* subsp. *bulgaricus* strains obtained from skim milk towards the growth of some *Lb. acidophilus* strains, we might suppose that one of the factors responsible for the loss of cell viability of *Lb. acidophilus* in different types of yoghurts previously reported (Vinderola et al., 1999) could be the products of the metabolism of *Lb. delbrueckii* subsp. *bulgaricus*. On the other hand, the growth of strains of *Lb. acidophilus* during long-term cold storage of fermented milks at 6°C was reported (Romero et al., 1987). Our results showed an important inhibitory activity of culture filtrates of *Lb. acidophilus* obtained from milk on the growth of lactic acid starter but not on probiotic bacteria. If during the cold storage of a fermented milk *Lb. acidophilus* can grow, its metabolic wastes could jeopardize lactic acid starter bacteria viability. Although lactic acid production is the main factor pointed as growth inhibitor (Juillard et al., 1987), no incompatible effects were found among *Lb. delbrueckii* subsp. *bulgaricus* and bifidobacteria/*Lb. casei* strains. From the point of view of the cell viability these species could be successfully included in the microbiological formulation of fermented dairy products. In general, *S. thermophilus* and *Lc. lactis* strains were weakly inhibited by probiotic bacteria but the contrary effect was not observed.

The study of interactions through growth kinetics allowed the setting of four different kinds of behaviors between species of lactic acid starter and probiotic bacteria (stimulation, delay, and complete inhibition of growth and no effects among them). A bacteriocin was defined as a substance with an antibacterial activity against species closely related to the producer strain, with a peptidic nature inactivated by proteolytic enzymes but not by heat (Juillard et al., 1987). According to this, it was found that the complete inhibition of growth of *Lb. delbrueckii* subsp. *bulgaricus* Ab1 by CCFS of *L. acidophilus* CNRZ 1881 was due to the production by the latter of a bacteriocin-like substance. The inhibitory behavior among strains of lactobacilli was previously reported (Vignolo et al., 1993; Giraffa et al., 1996). Although it was stated that acetate enhanced the growth of *Lb. acidophilus* (Marshall, 1991), the addition of sodium acetate did not reproduce the stimulation on growth of *Lb. acidophilus* CNRZ 1881 and A3 observed when CFS of *B. bifidum* A12 and BBI were added.

CONCLUSIONS

The results of this work showed that a variety of interactions could occur when lactic acid starters and probiotic bacteria are mixed for the manufacture of dairy products. Several types of interactions were detected among lactic acid starter bacteria. *Lb. acidophilus* was the sole probiotic species that was inhibited by the others (*Lc. casei* and *Bifidobacterium*). In general, probiotic bacteria proved to be more inhibitory toward lactic acid bacteria than vice versa since the latter did not exert any effect on the growth of the former, with some exceptions. The study of interactions through growth kinetics allowed us to set four different kinds of behaviors between species of lactic acid starter and probiotic bacteria (stimulation, delay, complete inhibition of growth, and no effects between them).

The possible interactions among the strains selected to manufacture a dairy product, when grown in milk, should be taken into account to select the best combination(s) to optimize their technological performance in the process and their survival in the products during cold storage.

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