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Temperature effect on the biological activity of *Bifidobacterium longum* CRL 849 and *Lactobacillus fermentum* CRL 251 in pure and mixed cultures grown in soymilk

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Abstract

The influence of temperature on the growth and biological activity of two probiotic strains (*Bifidobacterium longum* CRL 849 and *Lactobacillus fermentum* CRL 251) as pure and mixed cultures in soymilk (SM) were evaluated. Maximum growth was observed at 37°C in both mixed and pure cultures. In a product prepared with the mixed culture (1:1) at 37°C, the amount of lactic acid produced was approximately 55 mmol l^{-1} after 24 h with a slow production rate (2.8 mmol l^{-1} h^{-1}); the formation of acetic acid was higher with respect to pure cultures (82.01 mmol l^{-1} after 24 h), and final pH (24 h) was 5.0. About 85% of the total amount of sugars in SM was reduced, mainly sucrose. Stachyose was reduced (71%) after 4 h of incubation. Maximum activity of alpha-galactosidase (alpha-gal) (13.2 U ml^{-1}) was observed after 6 h. At 37°C the bifidobacterium strain was viable in mixed culture throughout the period assayed. At lower (30°C) or higher (42°C) temperatures, mixed culture showed slower growth and lower acid production in SM but the alpha-gal activity was stimulated at 30°C.

Keywords: Temperature effect; Fermented soymilk; Mixed cultures; Lactic acid bacteria; Bifidobacteria

1. Introduction

The potential of soymilk (SM) as a substitute for cow or human milk has been emphasized over the years, especially in the case of infants or children allergic to cow milk or adults with a low level of lactase in their intestine. SM can also be used as an economical protein beverage when cow milk is not available or expensive. However, the unfavorable beany flavor, flatulence factors and the high contents of indigestible alpha-D-galactosyl oligosaccharides such as raffinose and stachyose limit the consumption of soybeans as raw food material (Steggerda et al., 1966; Thananunkul et al., 1976). To overcome these limitations and to improve the acceptability and the nutritive value of SM, fermentation with various organisms has been attempted (Angeles and Marth, 1971; Mital and Steinkraus, 1975; Garro et al., 1994).

Bifidobacteria are major components of the intestinal flora of healthy humans and they are considered beneficial to all age groups because they help build resistance to infection in the host. Therefore, there exists considerable interest in the use of fermented milk products with bifidobacteria for probiotic purposes.

In previous studies, Garro et al. (1998, 1999a) have reported that *Bifidobacterium* (*B*) *longum* and *Lactobacillus* (*L*) *fermentum* used as pure starter cultures in SM exhibited significant growth and produced substantial amounts of acids under controlled fermentation conditions (at one temperature and free pH-fermentation). These authors demonstrated that 1:1 rate of *B. longum* and *L. fermentum* was the best proportion to improve the growth of each strain in mixed culture (data not published). The survival of these pure cultures in fermented SM during storage was also examined and high levels of viable organisms were obtained even after 28 days of storage (Garro et al., 1999b).

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The aim of this work was to evaluate the effect of different temperatures on the bacterial population, alpha-galactosidase (alpha-gal) activity, residual sugars and organic acids produced in fermented SM with pure and mixed cultures (*B. longum* CRL 849 and *L. fermentum* CRL 251) in order to improve their performance in probiotic foods.

2. Materials and methods

2.1. Microorganisms and growth conditions

B. longum CRL 849 and *L. fermentum* CRL 251 used in this study were obtained from the Culture Collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA), Tucumán, Argentina. These microorganisms showed the highest alpha-gal activities. *B. longum* CRL 849 is able to grow under aerobic conditions (Garro et al., 1999a, b).

SM was used as growth medium with the following approximate composition: proteins 3.0%, lipids 2.5%, sugars 3.5%, ash 0.5% and water 90.5%. The sugar fraction comprised 2.8% sucrose and 0.7% stachyose. Prior to further use SM was sterilized at 115°C for 20 min. Final pH was 6.8–7.0.

2.2. Culture preparation

Growth experiments were carried out in batch cultures (Erlenmeyer flasks) with 500 ml of SM. Freeze-dried cells (de Valdez et al., 1983) of each strain were used as inoculum at a concentration of 1.0×10^7 colony forming units (cfu) ml^{-1}(final concentration) in pure cultures. Mixed cultures contained a 1:1 proportion of each pure strain (final volume of SM, 500 ml). The cultures were incubated statically in a water bath at three controlled temperatures (30°C, 37°C and 42°C) for 24 h. Samples were taken at different times during 24 h and tested for pH and cell viability. Samples were frozen at −20°C until further analysis: assaying of residual sugars, alpha-gal activity and organic acids concentrations. Non-inoculated SM treated equally was used as control.

Cell populations of pure cultures and total mixed cultures were estimated by the plate dilution method using RCA agar (Barnes and Ingram, 1956) with the addition of 0.0005% hemin and 0.00005% vitamin K. Serial dilutions of each sample were plated out in duplicate and plates were incubated anaerobically at 37°C for 72 h. In order to compare microorganism growth in SM, results were expressed as $\ln(X/X_0)$ versus time, where "X" is the number of cfu ml^{-1} at a given time (t) and "X_0" the initial cfu ml^{-1} at zero time ($t = 0$).

In order to differentiate the cell populations of the mixed culture a phenotypic method for bacterial identification was used. Previously, the enzymatic profile of both microorganisms was examined with the API Zym kit (Bio-Merieux, USA) and can be distinguish by alkaline-phosphate enzyme. Modified RCA agar supplemented with 50 μg/ml of 5-bromo-4-chloro-3-indolyl-phosphate (X-phos) in H_2O. *B. longum* strains that are alkaline phosphatase positive developed blue colonies on RCA agar containing X-phos and *L. fermentum* strains that are phosphatase negative developed uncolored colonies.

2.3. Alpha-gal activity

Cell-free extracts were prepared by suspending washed cell pellets in 1 ml of McIlvaine buffer (100 mM Citrate/Na_2HPO_4), pH 5.8. Cells were disrupted with glass beads (0.10–0.11 mm) on a vortex mixer at maximum speed ten times during 20 min with 1-min intervals on ice. The supernatant was obtained after centrifugation (10,000g for 10 min, 4°C) and then stored at 4°C for alpha-gal assaying. Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as standard protein. Alpha-gal activity was determined in cell-free extracts with McIlvaine buffer according to Church et al. (1980). This technique is based on the measurement of the absorbance at 400 nm of *p*-nitrophenol (pNP) released by the action of the enzyme upon its specific substrate, *p*-nitrophenyl-alpha-D-galactopyranoside (*p*NPG).

The reaction mixture contained: 10 mM *p*NPG, 50 μl; 100mM McIlvaine buffer pH 5.8, 50 μl; cell-free extract, 100 μl; final volume: 200 μl. The mixture was incubated at 37°C for 15 min, and the reaction was stopped by adding 3 ml of sodium carbonate (0.25 M).

One enzyme unit (U) was defined as the amount of enzyme that released 1.0 μmol of *p*NP from its substrate *p*NPG per min under the given assay conditions. The results are expressed as U ml^{-1}.

2.4. Analytical assays

Concentrations of stachyose, sucrose and their hydrolysis products (galactose, glucose and fructose) in SM cultures were determined by high-performance liquid chromatography (HPLC) on a Gilson system (Villers le Bel, France) coupled to a differential refractometer (LKB, model 2142, Bromma, Sweden) and an integrator (Shimadzu, model CR 601, Chromatopac). Samples were deproteinized (Mital et al., 1975) before sugar determination. The supernatants obtained were injected into the column (REZEX RSO Oligosaccharides column (200×10 mm^2) (Phenomenex, Torrance, California, USA) through a 20 μl sample loop (Reodyne, model 9125). Column temperature was 70°C and

HPLC-grade bi-distilled water was used as mobile phase at a flow rate of 0.3 ml min^{-1}.

L(+)-lactate was determined enzymatically according to Gutmann and Walhlefeld (1974), and D(−)-lactate according to Gawehn and Bergmeyer (1974).

Determination of acetic acid was carried out on a gas chromatograph (GC) (Gow Mac Instrument Co.) equipped with a flame ionization detector and a stainless steel capillary column (30 m × 0.32 mm internal diameter) packed with AT-WAX (0.25 μm) (Heliflex capillary, Alltech). Helium was used as carrier gas. The temperature of the column was 90°C (isothermal), the injector 130°C and the detector 190°C. Data were analysed using an integrator (Data Jet, Spectra-Physics) and quantification was achieved by internal standard calibration.

The pH of the samples was measured by potentiometric methods.

2.5. Statistical analysis

All results presented in this study are the mean of three independent experiments with three replicates each. Data were analysed by the general linear model procedures of the Statistical Analysis System (SAS, 1986); $P<0.05$ was considered significant. Means were compared by least significant difference.

3. Results

3.1. Temperature effect on growth kinetic parameters and acidification rate

Growth patterns of mixed (1:1) and pure batch cultures of *B. longum* CRL 849 and *L. fermentum* CRL 251 in SM are illustrated in Fig 1 (a)–(c) and Table 1.

Cultures incubated at 37°C showed a similar growth during the first 8 h (Fig. 1a). Maximum specific growth rates between 2 and 6 h for mixed and pure cultures of *B. longum* were 0.66 and 0.72 h^{-1}, respectively. The pure culture of *L. fermentum* demonstrated a lower specific growth rate ($\mu = 0.44\ h^{-1}$) until 8 h, after which the rate was slow ($\mu = 0.10\ h^{-1}$) reaching a maximum population of 1.95×10^9 cfu ml^{-1} after 24 h. The pure culture of *B. longum* started growth immediately but only lasted about 8 h. Maximum population after 8 h was 1.52×10^9 cfu ml^{-1} (Table 1, Fig. 1a), after which it lost viability (2.80×10^7 cfu ml^{-1} after 24 h). In mixed culture, however, *B. longum* showed a different behavior. Assays in differential medium showed growth kinetics similar to those of the pure culture until 8 h, but afterward it maintained its viability until the fermentation had finished (24 h) (data not shown).

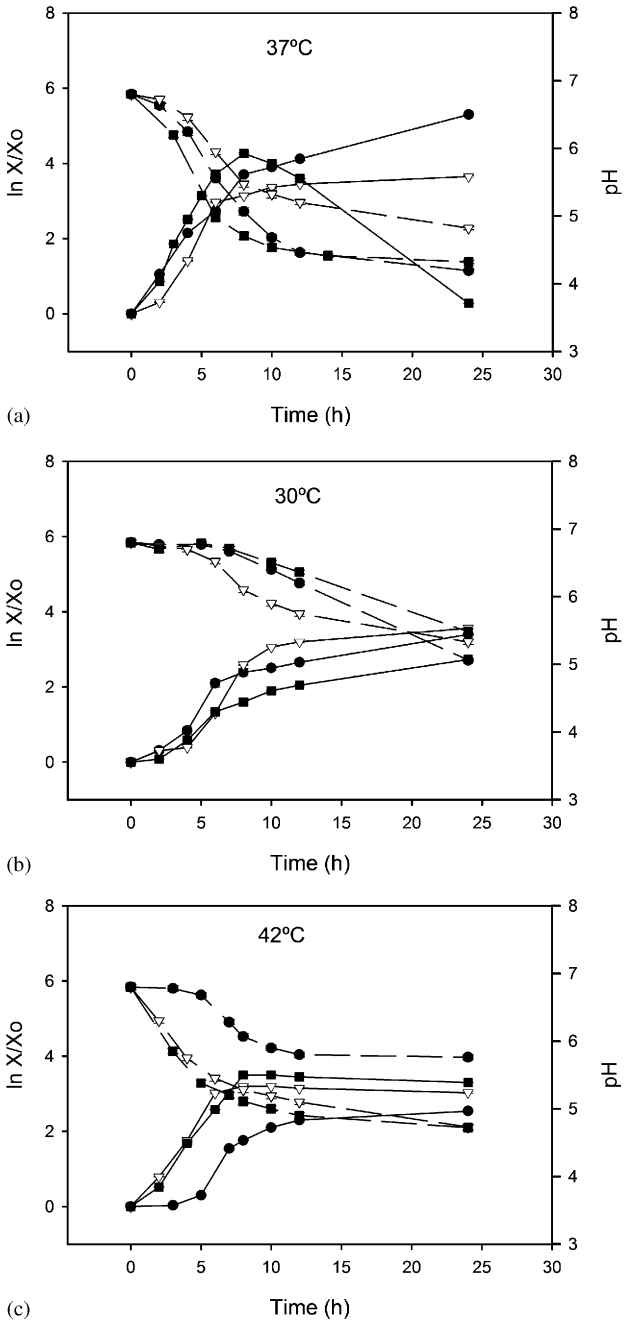

Fig. 1. Growth (—) and pH (- - - - -) of pure cultures of *L. fermentum* CRL 251 (●) and *B. longum* CRL 849 (■) and a 1:1 mixed culture of both strains (△) in SM at (a) 37°C, (b) 30°C, and (c) 42°C.

Maximum pH drop (ΔpH = 2.2) was observed in pure cultures of *B. longum* incubated at 37°C for 8 h. The pH thus reached (pH = 4.7) was close to the isoelectric point of soy proteins and therefore protein precipitation of a semi-soft coagulum could be observed. Acidification rate of mixed and pure culture of *L. fermentum* was less (0.29–0.25 pH units h^{-1}) than that observed in pure *B. longum* culture (0.410 pH units h^{-1}). However, final pH of pure *L. fermentum* was similar to that of *B. longum*

Table 1
Temperature effect on growth kinetic parameters of *L. fermentum* CRL 251 and *B. longum* CRL 849, used as starter cultures, in soymilk

Temperature	Pure culture						Mixed culture		
	L. fermentum CRL 251			*B. longum* CRL 849			Lf/Bl (1:1)		
	P_{max} $\times 10^7$ cfu ml^{-1}	Gr (μ) h^{-1}	Ar_{mean} pH unit h^{-1}	P_{max} ($\times 10^7$ cfu ml^{-1})	Gr μ (h^{-1})	Ar_{mean} pH (unit h^{-1})	P_{max} $\times 10^7$ cfu ml^{-1}	Gr (μ) h^{-1}	Ar_{mean} pH unit h^{-1}
30°C	12.2 (10 h)	0.63	0.09	5.5 (8 h)	0.32	0.07	21.0 (10 h)	0.55	0.21
	29.9 (24 h)	(6–4 h)	(24–7 h)	17.2 (24 h)	(6–2 h)	(24–7 h)	34.1 (24 h)	(8–4 h)	(8–6 h)
37°C	39.7 (8 h)	0.44	0.29	152.0 (8 h)	0.72	0.41	22.1 (8 h)	0.66	0.25
	195.0 (24 h)	(8–2 h)	(8–4 h)	2.8 (24 h)	(6–2 h)	(6–3 h)	30.0 (24 h)	(6–2 h)	(8–4 h)
42°C	5.8 (8 h)	0.62	0.20	37.1 (8 h)	0.52	0.28	20.1 (8 h)	0.56	0.27
	12.3 (24 h)	(7–5 h)	(8–5 h)	31.4 (24 h)	(6–2 h)	(5–0 h)	17.0 (24 h)	(6–2 h)	(4–2 h)

Notes: Lf: *L. fermentum*, Bl: *B. longum*, P_{max}: maximum population ($\times 10^7$ cfu ml^{-1}), Gr: growth rate; μ (h^{-1}), Ar_{mean}: mean acidification rate; pH (unit h^{-1}).

after 10 h of incubation (pH = 4.5). Final pH of mixed culture was about 5.0.

When the incubation temperature was diminished to 30°C, all cultures assayed showed a longer lag phase than at the other temperatures (Fig. 1b), approximately 5 h after inoculation. The mixed culture showed good growth with a μ of 0.55 h^{-1} (between 4 and 8 h) and reached a maximum population of 3.41×10^8 cfu ml^{-1} after 24 h. The pure *B. longum* culture showed less growth with a specific growth rate of $\mu = 0.32$ h^{-1} (Table 1). Maximum population was 1.72×10^8 cfu ml^{-1} after 24 h of incubation. The pure culture of *L. fermentum* showed a specific growth rate of 0.63 h^{-1} until 6 h and after this time growth continued slowly (0.070 h^{-1}), reaching a maximum population of 2.99×10^8 cfu ml^{-1}after 24 h. Two exponential growth phases (0.63 ± 0.05 and 0.07 ± 0.01 h^{-1}, respectively, Fig. 1b) were observed for this microorganism. The shift in exponential growth phase occurred at a population level of 1.22×10^8 cfu ml^{-1} (approximately 10 h). Maximum population was reached after 24 h (2.99×10^8 cfu ml^{-1}).

The mixed culture grown at 30°C showed a higher acidification rate (0.21 pH units h^{-1}) than the pure cultures (0.09 pH units h^{-1}) in SM (Fig. 1b, Table 1). Even though the acidification rate was higher in mixed culture, final acidification of fermented SM with all three cultures at 30°C was little (pH values above 5) at the end of the period assayed (24 h) (Fig. 1b).

When the incubation temperature was increased to 42°C, growth kinetics of mixed culture were similar to those of pure culture of *B. longum* ($\mu = 0.56$ and 0.52 h^{-1}, respectively) (Fig. 1c, Table 1). Both cultures reached a stationary phase after 8 h of incubation. Little growth was observed for *L. fermentum* in mixed culture using differential cell count, but it maintained viability throughout the incubation time (24 h) (data not shown). Pure culture of the microorganism showed a lower growth rate compared with both other cultures assayed (lag phase was longer than 5 h, Fig. 1c) and cell growth was less than at the other temperatures assayed. Cell growth was low (5.80×10^7 cfu ml^{-1}) after 8 h of incubation, showing a very short exponential growth phase and an insignificant increase in cells after 24 h of incubation (1.23×10^8 cfu ml^{-1}), whereas the cell number of mixed and pure *B. longum* cultures increased 2–3 times at this temperature.

The acidification rate of mixed culture was similar to that of pure *B. longum* culture (0.27 and 0.28 pH units h^{-1}, respectively) (Table 1) suggesting that acidification in mixed culture was primarily due to the action of *B. longum*. Little acid was produced by the pure culture of *L. fermentum* at 42°C, and the pH decreased only 0.12 units in the first 5 h.

3.2. Temperature effect on the production of organic acids

A temperature effect was also noticed on the production of organic acids (Figs. 2a–c). Mixed culture at 37°C showed a different behavior from that observed in pure cultures (Fig. 2a). During the first 6 h of incubation the main product in pure cultures was lactic acid (production rate between 10.5 and 12.9 mmol l^{-1} h^{-1}), whereas production of this metabolite was low in mixed culture. An increase in lactic acid occurred only after 10 h and at a lower production rate (2.8 mmol l^{-1} h^{-1}). However, the amount of lactic acid at the end of the fermentation period (24 h) in mixed culture was similar to that of pure *L. fermentum* (approx. 55 mmol l^{-1}).

Temperature conditions (37°C) favored formation of acetic acid in mixed culture during the first 10 h with values similar to those of pure *L. fermentum*, but lactic acid synthesis was lower (Fig. 2a). After 10 h, the production of both acids was higher with a molar ratio between acetic and lactic acid of 1.5:1.0. Production rate of acetic acid (3.7 mmol l^{-1} h^{-1}) was highest, reaching maximum concentration after 24 h (82.01 mmol l^{-1}, 3.3 times that of pure cultures). In contrast, acetic

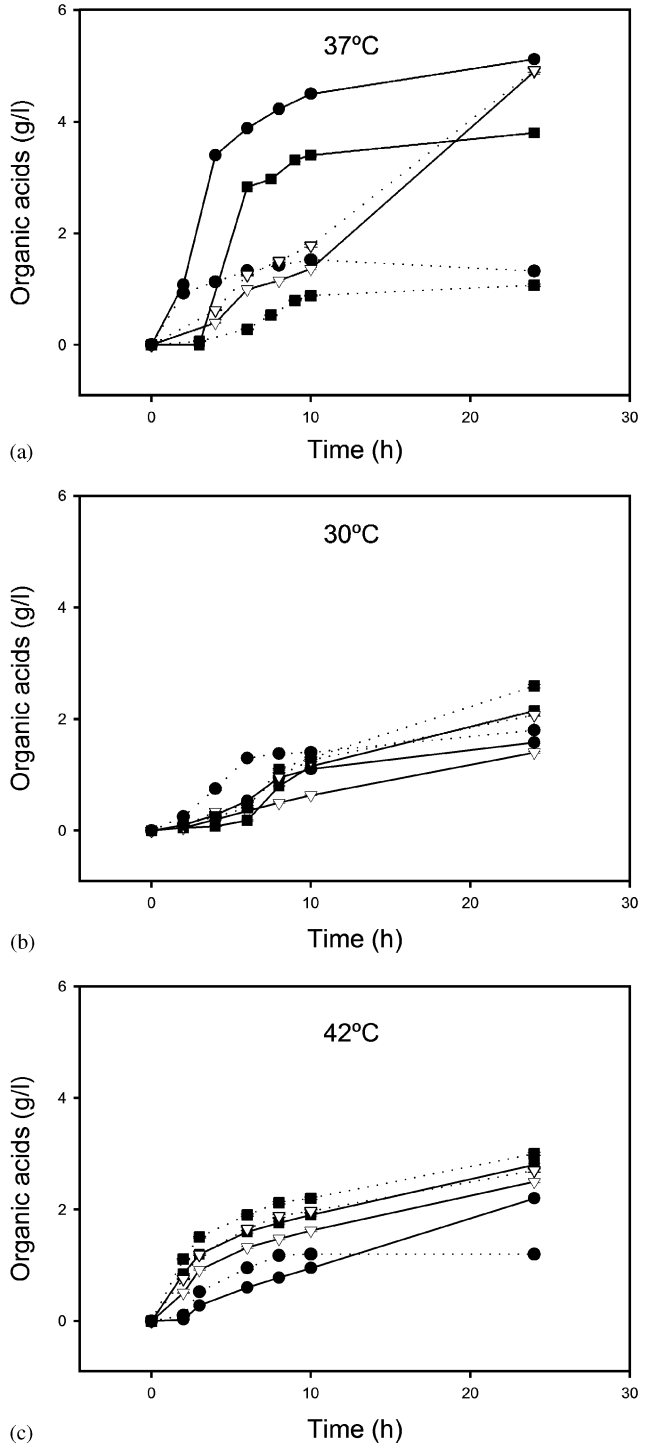

Fig. 2. Lactic acid (—) and acetic acid (.....) production in pure and mixed cultures (△) of *L. fermentum* CRL 251 (●) and *B. longum* CRL 849 (■), grown in SM at (a) 37°C, (b) 30°C, and (c) 42°C.

acid production in pure cultures was much lower (max. 25 mmol l^{-1}), even in *B. longum*, known for its molar acetic acid:lactic acid ratio of 3:2. In our case this molar ratio was about 0.5:1.0, in favor of lactic acid production.

At 30°C, organic acid production was low in all cultures and the patterns were quite similar (Fig. 2b). During the first 6 h of incubation of pure *L. fermentum* acetic acid formation was predominant. This microorganism is heterofermentative and under the given growth conditions acetic acid seemed to be favored (the acetic acid:lactic acid ratio changed from 3.5:1.0 to 4.0:1.0). After 6 h the process was inverse and lactic acid production was higher. Acetic acid production of pure Bifidobacteria culture was higher after 24 h (59.79 mmol l^{-1}), whereas maximum lactic acid production was 34.93 mmol l^{-1}.

At high incubation temperature (42°C) production of both organic acids in mixed culture was similar to that of *B. longum*, which indicates only little influence of *L. fermentum* in the mixture. A pure culture of *B. longum* at this temperature showed its typical metabolism with a higher acetic acid production (2:1 molar ratio). However, maximum acetic acid and lactic acid production after 24 h in both mixed and pure culture of *B. longum* was only 45 and 50 mmol l^{-1} and 28 and 32 mmol l^{-1}, respectively. Organic acid production in a pure culture of *L. fermentum* at 42°C (Fig. 2c) was similar to that at 30°C (Fig. 2b), with 20 mmol l^{-1} for acetic acid and 24.4 mmol l^{-1} for lactic acid.

3.3. Temperature effect on carbohydrates consumption and alpha-gal activity

The amount of metabolized sugars after 24 h of incubation in mixed batch culture at 37°C was similar to that in a pure culture of *L. fermentum* (Table 2). The level of sucrose increased between 2 and 4 h, followed by a high utilization rate with a reduction of ca 80% after 10 h of incubation. A 73% of stachyose was metabolized after 4 h of fermentation. After 10 h the hydrolysis products (glucose, fructose, galactose and raffinose) were only scarcely detected. In pure *B. longum* culture sucrose was metabolized preferentially showing a reduction of 79.30% after 9 h, while the consumption of stachyose was less (52.33% of the original concentration was reduced after 8 h (Table 2)). This behavior is the result of alpha-gal activity of the microorganisms assayed at 37°C (Table 3). *B. longum* used as pure culture reached a maximum activity of 7.7 U ml^{-1} after 8 h. *L. fermentum* showed maximum activity after 6 h of incubation (49.8 U), loosing more than 70% after this time. The production patterns of mixed culture at 37°C and 30°C (see below) were similar, but with less activity (13.2 U) at 37°C. A loss of activity of more than 60% was observed in all cultures after reaching the stationary phase.

The low cell growth observed at 30°C in all the cultures assayed (pure and mixed) was reflected in the low utilization of carbohydrates (sucrose and stachyose): more than 75% remained in the SM after 24 h

Table 2
Residual sugars in soymilk inoculated with *B. longum* CRL 849 and *L. fermentum* CRL 251, used as starter cultures, at 37°C

Fermentation time (h)	Residual sugar (%)					
	B. longum CRL 849		*L. fermentum* CRL 251		Mixed culture (1:1)	
	Stachyose	Sucrose	Stachyose	Sucrose	Stachyose	Sucrose
0	100.00	100.00	100.00	100.00	100.00	100.00
2	78.30	80.20	66.57	59.74	70.10	61.30
4	65.20	62.97	27.15	66.96	29.30	69.20
6	58.60	55.97	27.61	34.40	28.50	37.40
8	47.67	32.00	15.29	22.66	18.90	25.60
24	41.54	18.42	14.09	14.17	15.20	16.10

Table 3
Temperature effect on alpha-gal activity in soymilk fermented with *B. longum* CRL 849 and *L. fermentum* CRL 251, used as starter cultures

Fermentation time (h)	Alpha-gal activity ($U\,ml^{-1}$)		
	30°C	37°C	42°C
B. longum CRL 849			
2	0.150	0.610	0.690
4	0.530	1.800	2.670
6	0.760	6.300	13.820
8	1.200	7.700	8.800
10	3.105	0.310	6.500
24	4.500	0.000	2.810
L. fermentum CRL 251			
2	0.267	0.610	1.600
4	0.590	35.200	3.400
6	0.840	49.880	10.100
8	6.830	4.850	13.670
10	NA	NA	10.100
24	5.530	0.720	2.370
Mixed culture			
2	0.588	0.196	0.570
4	3.370	7.603	8.500
6	14.580	13.169	6.500
8	NA	11.500	5.200
10	17.911	10.020	2.800
24	3.292	1.745	2.460

Notes: NA: not assayed.

of fermentation (data not shown). The enzymatic activity of all cultures showed the maximum at the early stationary phase. Mixed culture showed maximum activity (17.9 U) after 10 h, whereas pure cultures showed minor activity (Table 3).

Total sugar consumption by *B. longum* in pure and mixed culture grown at 42°C was similar. The sugar utilization pattern of this microorganism was the same as that at 37°C, but the amount consumed was less (15%) (data not shown). *L. fermentum* displayed poor sugar consumption similar to growth at 30°C (data not shown). At 42°C, maximum enzyme activity coincided with the beginning of the stationary growth phase in all cultures assayed. Mixed culture showed lower enzyme activity than in pure cultures, which remained constant after 10 h (stationary growth phase) of incubation (Table 3).

4. Discussion

B. longum and *L. fermentum* are commonly used as starter cultures in different fermented foods (dairy products, vegetables, meat, etc.), due to their beneficial probiotic properties (Rasic and Kurmann, 1983; Kurmann and Rasic, 1991; Hammes and Knauf, 1994; Stiles and Holzapfel, 1997; Gardiner et al., 2002). This is the first paper that discusses the behavior of mixed culture of *B. longum* CRL 849 and *L. fermentum* CRL 251 in SM. Several fermentation parameters such as changes in bacterial population and acidification properties of SM fermented with these cultures at different temperatures were assayed in order to improve the performance of these microorganisms in probiotic products.

The mixed culture in SM incubated at 30°C and 42°C demonstrated slow growth rate and a consequent low production of organic acids respect to 37°C.

The growth of *B. longum* in SM as pure culture at 37°C was highest until 8 h then it showed a great loss of viability. The acidification rate was higher than that in the others cultures assayed. A similar phenomenon has been observed previously by Garro et al. (1999a, b) and Turner and Thomas (1975). Various factors such as low pH and a high salt concentration can cause growth inhibition of lactic acid bacteria but without modifying carbohydrate metabolism and product formation. Taniguchi et al. (1987) have observed that initial lactate and acetate concentrations higher than $10\,g\,l^{-1}$ rapidly decreased the growth rate of *B. longum* YT 402, and growth was completely inhibited in media containing more than $18\,g\,l^{-1}$ of both lactate and acetate. Desjardins et al. (1990) have reported that the metabolism of *B. longum* was completely inhibited in milk with concentrations of lactate and acetate higher than $12\,g\,l^{-1}$. In

our experiments production of both organic acids was less ($<6\,g\,l^{-1}$) and consequently the loss of viability is probably due to a low pH reached under the given growth conditions.

On the other hand, behavior of *B. longum* in mixed culture was different from that in pure culture, as viability maintained stable even during the stationary growth phase. Production of organic acids also differed, although final pH values were similar. Furthermore, acetic acid production in mixed culture increased, which suggests that co-existence of both microorganisms would stimulate the natural fermentation pathway of Bifidobacterium. According to our results *B. longum* CRL 849 could be used in this mixed starter culture without encountering difficulties in viability.

Interactions among different strains can have either stimulatory or inhibitory effects on microbial growth and on their metabolic activity. A co-culture with *L. fermentum* could offer nutrients or conditions that somehow maintain the viability of *B. longum* in the mixed culture. Other cases of co-cultures and symbiosis have been reported by Bouquien et al. (1998) and Bellengier et al. (1997). The best-known example is probably the yogurt culture starter, in which growth of *L. bulgaricus* is stimulated by activators produced by *S. thermophilus* (Galesloot et al., 1968; Moon and Reinbold, 1976). Viability of bifidobacterium in dairy products is still an important problem to be solved (Medina and Jordano, 1995).

The SM used in this experiment contains approximately 3.5% fermentable carbohydrates sucrose (2.8%) being the major component which was preferentially hydrolysed by pure and mixed cultures at the different temperatures assayed. *B. longum* CRL 849 and *L. fermentum* CRL 251, both have alpha-gal necessary for stachyose hydrolysis, but their mode of action is different (Garro et al., 1998, 1999a). The type and amount of residual sugars as well as the production of alpha-gal during fermentation at 37°C were characteristic for each microorganism. A pure culture of *L. fermentum* displayed highest activity ($49.8\,U\,ml^{-1}$ after 6 h) under the given conditions, but in mixed culture final activity was similar to that of a pure culture of *B. longum* at 42°C. The co-culture of both microorganisms had no effect on the production of alpha-gal, and neither showed a stimulatory effect on the consumption of stachyose.

The results of this investigation showed that 37°C was the optimal temperature to improve the performance of *B. longum* CRL 849 and *L. fermentum* CRL 251 as mixed culture in SM products. This mixed starter can therefore be considered as a suitable alternative for improving galactosaccharides digestion when bioprocessed soya bean products are included in the human diet.

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References

Angeles, A.G., Marth, E.H., 1971. Growth and activity of lactic acid bacteria in soymilk. I. growth and acid production. J. Milk Food Technol. 34, 30–36.

Barnes, E.M., Ingram, M., 1956. The effect of redox potential on the grown *Clostridium welchii* strain isolated from horse muscle. J. Appl. Bacteriol. 19, 177–178.

Bellengier, P., Richard, J., Foucaud, C., 1997. Associative growth of *Lactococcus lactis* and *Leuconostoc mesenteroides* strains in milk. J. Dairy Sci. 80, 1520–1527.

Bouquien, C.Y., Corrieu, G., Desmazeaud, M.J., 1998. Effect of fermentation conditions on growth of *Streptococcus cremoris* AM2 and *Leuconostoc lactis* CNRZ in pure and mixed cultures. Appl. Environ. Microbiol. 54, 2527–2531.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Church, F.C., Meyers, S.P., Srinivasan, V.R., 1980. Isolation and characterization of alpha-galactosidase from *Pichia guilliermondi*. In: Underkofler, L.A., Wulf, M.L. (Eds.), Developments in Industrial Microbiology, Vol. 21. Society for Industrial Microbiology, Arlington, Virginia, pp. 339–348.

de Valdez, G.F., de Giori, G.S., de Ruiz Holgado, A.P., Oliver, G., 1983. Comparative study on the efficiency of some additives in protecting lactic acid bacteria against freeze drying. Cryobiology 20, 560–566.

Desjardins, M.L., Roy, D., Toupin, C., Goulet, J., 1990. Uncoupling of growth and acids production in *Bifidobacterium* ssp. J. Dairy Sci. 73, 1478–1484.

Galesloot, T.E., Hassing, F., Veringa, H.A., 1968. Symbiosis in yoghurt. I. Stimulation of *Lactobacillus bulgaricus* by a factor produced by *Streptococcus thermophilus*. Neth. Milk Dairy J. 22, 50–63.

Gardiner, G.E., Heinemann, C., Baroja, M.L., Bruce, A.W., Beverman, D., Madrenas, J., Reid, G., 2002. Oral administration of the probiotic combination *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 for human intestinal applications. Int. Dairy J. 12, 191–196.

Garro, M.S., de Giori, G.S., de Valdez, G.F., Oliver, G., 1994. Utilization of soymilk galactosaccharides by lactobacilli. Microbiol.-Aliments-Nutr. 12, 61–66.

Garro, M.S., de Valdez, G.F., Oliver, G., de Giori, G.S., 1998. Growth characteristics and fermentation products of *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus casei* and *L. fermentum* in soymilk. Z. Lebensm. Unters. Forsch. A 206, 72–75.

Garro, M.S., de Valdez, G.F., Oliver, G., de Giori, G.S., 1999a. Hydrolysis of soya milk oligosaccharides by *Bifidobacterium longum* CRL849. Z. Lebensm. Unters. Forsch. A 208, 57–59.

Garro, M.S., de Valdez, G.F., Oliver, G., de Giori, G.S., 1999b. Starter culture activity in refrigerated fermented soymilk. J. Food Prot. 62, 808–810.

Gawehn, K., Bergmeyer, H.U., 1974. D(−)-lactate. In: H. U. Bergmeyer, K. Gawehn, (Eds.), Methods of Enzymatic Analysis, Vol. 3. Academic Press, New York, pp. 1469–1475.

Gutmann, I., Walhlefeld, A.W., 1974. L(+)-lactate. Determination with lactate dehydrogenase and NAD. In: Bergmeyer, H.U., Gawehn, K. (Eds.), Methods of Enzymatic Analysis, Vol. 3. Academic Press, New York, pp. 1464–1468.

Hammes, W.P., Knauf, H.J., 1994. Starters in the processing of meat products. Meat Sci. 36, 155–168.

Kurmann, J.A., Rasic, J.L., 1991. The health potential of products containing Bifidobacteria. In: Robinson, R.K. (Ed.), Therapeutic Properties of Fermented Milks. Elsevier Applied Science Publishers, London, pp. 117–157.

Medina, L., Jordano, R., 1995. Population dynamics of constitutive microbiota in bat type fermented milk products. J. Food Prot. 58, 70–76.

Mital, B.K., Steinkraus, K.H., 1975. Utilization of oligosaccharides by lactic acid bacteria during fermentation of soymilk. J. Food Sci. 40, 114–118.

Moon, N.J., Reinbold, G.W., 1976. Commensalism and competition in mixed cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. J. Milk Food Technol. 39, 337–341.

Rasic, J.L., Kurmann, J.A., 1983. Bifidobacteria and Their Role. Birkhauser Verlag, Basel, Switzerland.

SAS Institute Inc., 1986. User's Guide: Statistics. Version 6. Edition Cary. SAS Institute Inc., North Carolina, pp. 549–630.

Steggerda, F.R., Richards, E.A., Rackis, J.J., 1966. Effects of various soy bean products on flatulence in the adult man. Proc. Soc. Exp. Biol. Med. 121, 1235–1239.

Stiles, M.E., Holzapfel, W.H., 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36, 1–29.

Taniguchi, M., Kotani, N., Kobayashi, T., 1987. High concentration cultivation of *Bifidobacterium longum* in fermenter with cross-flow filtration. Appl. Microbiol. Biotechnol. 25, 438–441.

Thananunkul, D., Tanaka, M., Chichester, C.O., Lee, T.C., 1976. Degradation of raffinose and stachyose in soybean milk by α-galactosidase from *Mortierella vinacea*. Entrapment of α-galactosidase within polyacrylamide gel. J. Food Sci. 41, 173–175.

Turner, K.W., Thomas, T.D., 1975. Uncoupling of growth and acid production in lactic streptococci. N. Z. J. Dairy Sci. Technol. 10, 162–173.