

# Technological Properties of Milks Fermented with Thermophilic Lactic Acid Bacteria at Suboptimal Temperature

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## ABSTRACT

In the present work the synergistic relationship between different strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* was studied at optimal (44°C) and suboptimal temperatures (30°C). Acidification, viscosity, whey syneresis, and bacterial concentration of the final product were evaluated on single-strain and mixed cultures after 24 h at 30°C and 6 h at 44°C.

Three pairs of strains (LBB+CP2, LBP+CP2, and LBR+CP2) showed synergistic effect, which was reflected by the viscosity and syneresis of the coagulum. These results were more significant when cultures were incubated at 30°C, reaching apparent viscosity values of 19 to 28 mPa × s. On the other hand, lactobacilli cultures enhanced the growth of two streptococci strains (CP2 and CP4). These results were confirmed by cultures of streptococci supplemented with supernatants of culture of lactobacilli. Those supernatants stimulate the viscosity produced by CP2 and CP4 strains and reduce the syneresis of all cultures of streptococci. Neither the increase of viscosity nor reduction of syneresis could be attributed to a decrease of pH.

**(Key words:** technological properties, thermophilic lactic acid bacteria, suboptimal temperature)

**Abbreviation key:** EPS = exopolysaccharides, LAB = lactic acid bacteria.

## INTRODUCTION

Lactic acid bacteria play an important role in the production and conservation of foodstuffs, especially in the dairy industry. Mixed "starters cultures" containing selected strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are generally used in the elaboration of yogurts and soft cheese.

Both species present a protocooperative relationship (3, 11). Proteolytic enzymes produced by *L. delbrueckii* subsp. *bulgaricus* degrade casein, releasing low molecular weight peptides and amino acids. These molecules were identified as growth factors for *S. thermophilus* (16). The growth of lactobacilli is stimulated by the carbon dioxide and formic acid produced by the streptococci. These thermophilic cultures are used not only for elaboration of products whose processing involves high temperatures (yogurt, hard cheese), but also for processes where the temperature never exceeds 32°C (soft cheese).

In Argentina, soft cheese production represents 55% of the total cheese production. Statistical data for the last 5 yr indicate that the consumption of this type of cheese is growing (8). Quartirolo cheese, a soft Argentinian cheese (6, 14), is prepared with pasteurized milk (17°D). At 30°C, CaCl<sub>2</sub> (30 g/100 L) and starter are added to the milk; the mixture is incubated approximately 40 to 60 min to increase the acidity in 5°D (2). Then, the rennet is added and temperature is raised to 32 to 34°C for 30 to 40 min until coagulation of milk is produced. The whey is removed; the curd is pressed, salted, and finally packaged in low gas permeability films. The cheeses are ripened at 10°C for 20 d (14).

When formulating mixed starters, it is important to study the antagonistic and synergistic interaction between strains, in order to select those pairs in which the protocooperative effect is observed. However, no synergistic studies have been carried out between streptococci and lactobacilli at suboptimal temperatures (30°C). Moreover, the synergism and rheological properties of the resulting product at 30°C have not been evaluated. These parameters are important in cheesemaking and affect the yield.

An important factor affecting the rheological behavior of the casein coagulum is the production of exopolysaccharides (EPS) by the starter culture. These polymers confer firmness and texture to the final product. Several strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are able to produce high molecular weight polysaccharides with different structures. The fermentation conditions (temperature and time of incu-

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bation) and the composition of the media (availability of nitrogen and carbon) affect both the sugar composition of the polymer and the amount produced (4).

Schellhaass (17), Tegatz (18), and Mozzi et al. (15) demonstrated that single-strain cultures of *S. thermophilus* and *L. bulgaricus* produced more EPS at low temperatures, leading to high viscosity at those temperatures.

Based on these considerations, the aim of this study was to compare the proto cooperative relationship between different thermophilic strains at optimal and suboptimal temperatures as regards the microbial growth and acidification, viscosity, and syneresis of the final product.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

*L. delbrueckii* subsp. *bulgaricus* CIDCA 331 (originally named LBB), CIDCA 332 (LBP), and CIDCA 333 (LBR), *S. thermophilus* CIDCA 321 (CP2), CIDCA 322 (CP3), and CIDCA 323 (CP4) were isolated from a yogurt starter and identified (by growth temperature, cellular morphology, Gram staining, whole-cell soluble proteins, catalase reaction, and sugar fermentation with API system, Appareils et Procédés d' Identification, Montalieu Vercieu, France), in our laboratory (1, 5, 7, 10). All strains were stored at  $-80^{\circ}\text{C}$  in milk and then propagated in UHT skim milk (Parmalat S. A. Buenos Aires 1649, Argentina) at  $37^{\circ}\text{C}$  for 18 h and then in same medium at  $37^{\circ}\text{C}$  until pH 5 (6 to 8 h) to obtain an active inoculum. In all experiments, 10 ml of milk was inoculated with an amount of the previous culture to obtain an initial bacterial concentration of  $10^7$  cfu/ml. Single strains and mixed cultures (with approximately  $10^7$  cfu/ml) were incubated for 24 h at  $30^{\circ}\text{C}$  or 6 h at  $44^{\circ}\text{C}$ .

After the inoculated milks reached stationary phase, bacterial counts, pH, turbidity and viscosity were conducted immediately. Two or three independent experiments were performed for each single-strain and mixed culture at both temperatures.

### Supernatants

Lactobacilli cultures (grown at  $30^{\circ}\text{C}$ ) in stationary phase were centrifuged ( $10,000 \times g$  for 20 min at  $10^{\circ}\text{C}$ ). The supernatants were filtered through  $0.45\text{-}\mu\text{m}$  filters (Millipore Waters Associates). Supernatants were used for testing any stimulus on streptococci cultures.

### Bacterial Counts

Number of viable bacteria was determined by plating appropriate dilutions in 1 g of tryptone/L on 1.1.1. (5)

or Lee's agar plates. The 1.1.1. agar contained 10 g of tryptone/L (Difco, Detroit, MI), 10 g of yeast extract/L (Difco, Detroit, MI), 10 g of lactose/L (Mallinckrodt Chemical Works, NY) and 15 g of agar/L (Britania, Buenos Aires, Argentina). Lee's differential agar (13) was prepared as published previously. Agar plates were incubated at  $37^{\circ}\text{C}$ , aerobically for 48 h.

### pH and Bacterial Cultures Turbidity

The pH of cultures was determined at  $25^{\circ}\text{C}$  with a Cole-Parmer (Chicago, IL) combined glass-calomel microelectrode. Samples were removed from cultures and diluted 1:10 (vol/vol) in 2% wt/vol  $\text{Na}_2\text{EDTA}$  (pH 12), according to Kanasaki et al. (12) and then the turbidity of bacterial cultures was determined at 480 nm in a Shimadzu (Kyoto, Japan) double beam spectrophotometer.

### Viscosity

A rotational viscometer of concentric cylinders (Haake Rotovisco RV2, Germany) with a NV sensor (Nieder Viskositat) was used. Rheological parameters were measured at  $30^{\circ}\text{C}$ . Flow behavior was analyzed plotting shear stress ( $\tau$ ) versus shear rate (D). The following program was performed: an increasing sequence from 0 to  $2769.9\text{ s}^{-1}$  in a period of 3 min, followed by 1 min at maximum value and a corresponding decreasing sequence in 3 min. Apparent viscosity ( $\eta_{\text{ap}}$ ) was calculated at  $256 \times 5.41\text{ s}^{-1}$  and expressed in  $\text{mPa} \times \text{s}$ .

### Whey Syneresis

Spontaneous whey expelled was determined in 10-ml samples of cultures before and after storage at  $10^{\circ}\text{C}$  for 20 d. Each determination was made in tubes of 10 mm diameter. Whey expelled was measured with Eppendorf pipettes (Nether-Hi GmbH, 22331 Hamburg, Germany) calibrated to 0.2 to 0.6% of error.

### Statistical Analysis

Statistical analysis was applied to experimental data using Systat (Systat 5.0, Systat Inc, USA). The ANOVA was conducted to determine significant difference ( $P < 0.05$ ). All significant differences were further analyzed with LSD (least significant difference) test ( $P < 0.05$ ) to determine pairwise differences.

## RESULTS AND DISCUSSION

Tables 1 and 2 show the technological properties of milks fermented with single-strain and mixed cultures

**Table 1.** Technological properties of fermented milk obtained with pure and mixed cultures of streptococci and lactobacilli after 24 h at 30°C.<sup>1</sup>

Starter	OD (480 nm)	Differential enumeration of lactobacilli and streptococci in pure and mixed cultures (10 <sup>7</sup> cfu/ml)		pH	Apparent viscosity (mPa × seg) <sup>2</sup>	Syneresis (μl/10 ml) <sup>3</sup>
		Lactobacilli	Streptococci			
<i>Pure cultures Lactobacillus bulgaricus</i>						
LBB	0.178 ± 0.0018 <sup>a</sup>	3.50 ± 0.70 <sup>a</sup>	—	4.57 ± 0.15 <sup>a</sup>	16.80 ± 1.40 <sup>a</sup>	75 ± 25 <sup>a</sup>
LBP	0.071 ± 0.0025 <sup>b</sup>	3.25 ± 0.35 <sup>ac</sup>	—	4.80 ± 0.07 <sup>b</sup>	11.00 ± 1.00 <sup>b</sup>	<b>1350 ± 150<sup>d</sup></b>
LBR	0.130 ± 0.0026	3.25 ± 0.35 <sup>ac</sup>	—	4.75 ± 0.04 <sup>b</sup>	7.62 ± 0.62 <sup>bc</sup>	<b>2500 ± 500<sup>b</sup></b>
<i>Streptococcus thermophilus</i>						
CP2	0.072 ± 0.0036 <sup>b</sup>	—	13.50 ± 2.12 <sup>a</sup>	4.64 ± 0.07 <sup>ab</sup>	10.20 ± 2.20 <sup>b</sup>	100 ± 50 <sup>ac</sup>
CP3	0.172 ± 0.0029 <sup>a</sup>	—	8.00 ± 2.82 <sup>a</sup>	5.17 ± 0.04 <sup>c</sup>	3.23 ± 0.23 <sup>c</sup>	<b>3500 ± 1500<sup>h</sup></b>
CP4	0.111 ± 0.020	—	45.00 ± 7.07 <sup>b</sup>	5.08 ± 0.18 <sup>c</sup>	5.80 ± 0.80 <sup>c</sup>	150 ± 10 <sup>c</sup>
<i>Mixed cultures</i>						
LBB+CP2	0.382 ± 0.0050	1.75 ± 0.70 <sup>b</sup>	41.00 ± 12.72 <sup>b</sup>	4.03 ± 0.20 <sup>d</sup>	<b>28.00 ± 3.00<sup>e</sup></b>	75 ± 25 <sup>c</sup>
LBB+CP3	0.243 ± 0.0041	2.25 ± 0.35 <sup>abc</sup>	11.00 ± 1.41 <sup>a</sup>	4.15 ± 0.07 <sup>de</sup>	4.80 ± 0.43 <sup>c</sup>	550 ± 50 <sup>c</sup>
LBB+CP4	0.397 ± 0.0022	2.25 ± 0.35 <sup>abc</sup>	93.50 ± 9.19 <sup>c</sup>	3.97 ± 0.16 <sup>de</sup>	7.14 ± 0.29 <sup>bc</sup>	500 ± 50 <sup>c</sup>
LBP+CP2	0.288 ± 0.0013	3.75 ± 0.35 <sup>a</sup>	32.50 ± 10.60 <sup>b</sup>	4.14 ± 0.12 <sup>de</sup>	<b>19.00 ± 2.00<sup>a</sup></b>	75 ± 25 <sup>c</sup>
LBP+CP3	0.259 ± 0.0034 <sup>c</sup>	3.75 ± 1.06 <sup>a</sup>	12.50 ± 3.53 <sup>a</sup>	4.18 ± 0.04 <sup>e</sup>	5.65 ± 0.65 <sup>c</sup>	550 ± 50 <sup>c</sup>
LBP+CP4	0.196 ± 0.0011	1.50 ± 0.70 <sup>bc</sup>	110.00 ± 14.14 <sup>d</sup>	4.10 ± 0.04 <sup>de</sup>	5.90 ± 0.70 <sup>c</sup>	200 ± 20 <sup>c</sup>
LBR+CP2	0.219 ± 0.0019	3.75 ± 1.00 <sup>a</sup>	65.00 ± 21.21 <sup>e</sup>	4.15 ± 0.08 <sup>de</sup>	<b>19.25 ± 2.55<sup>a</sup></b>	50 ± 5.0 <sup>c</sup>
LBR+CP4	0.252 ± 0.0012 <sup>c</sup>	2.50 ± 0.70 <sup>abc</sup>	77.50 ± 17.67 <sup>f</sup>	4.64 ± 0.25 <sup>a</sup>	4.60 ± 0.20 <sup>c</sup>	400 ± 100 <sup>c</sup>

<sup>a,b,c,d,e,f</sup>Numbers with the same letter (for each technological parameter determined) are not significantly different ( $P < 0.05$ ).

<sup>1</sup>Optical density, viable counts, pH, and whey syneresis values are expressed as mean of duplicates of independent cultures. Apparent viscosity values are expressed as mean of triplicates of independent cultures. In the case of mixed culture LBB+CP2 and single-strain LBB apparent viscosity values are expressed as mean of four independent cultures.

<sup>2</sup>Determined at  $\gamma = 256 \times 5.41$  (s<sup>-1</sup>).

<sup>3</sup>After a storage of 20 d at 10°C.

**Table 2.** Technological properties of fermented milk obtained with pure and mixed cultures of streptococci and lactobacilli after 6 h at 44°C.<sup>1</sup>

Starter	OD (480 nm)	pH	Apparent viscosity (mPa × seg) <sup>2</sup>	Syneresis (μl/10 ml) <sup>3</sup>
<i>Pure cultures</i>				
<i>Lactobacillus bulgaricus</i>				
LBB	0.458 ± 0.0020	4.55 ± 0.48 <sup>a</sup>	9.37 ± 0.37 <sup>a</sup>	400 ± 100 <sup>a</sup>
LBP	0.300 ± 0.0017	4.58 ± 0.49 <sup>a</sup>	9.50 ± 0.50 <sup>a</sup>	<b>1000 ± 200<sup>b</sup></b>
LBR	0.268 ± 0.0014	4.58 ± 0.41 <sup>a</sup>	5.88 ± 0.40 <sup>b</sup>	400 ± 150 <sup>a</sup>
<i>Streptococcus thermophilus</i>				
CP2	0.520 ± 0.0026 <sup>a</sup>	4.48 ± 0.42 <sup>a</sup>	8.68 ± 1.66 <sup>a</sup>	100 ± 25 <sup>c</sup>
CP3	0.121 ± 0.0019	4.71 ± 0.48 <sup>a</sup>	7.12 ± 0.37 <sup>ab</sup>	<b>1500 ± 500<sup>d</sup></b>
CP4	0.525 ± 0.042 <sup>ac</sup>	4.40 ± 0.60 <sup>a</sup>	7.96 ± 1.04 <sup>ab</sup>	75 ± 25 <sup>c</sup>
<i>Mixed cultures</i>				
LBB+CP2	0.604 ± 0.0055	4.05 ± 0.22 <sup>a</sup>	<b>15.50 ± 1.40<sup>c</sup></b>	25 ± 25 <sup>c</sup>
LBB+CP3	0.546 ± 0.0031 <sup>b</sup>	4.05 ± 0.15 <sup>a</sup>	7.35 ± 0.15 <sup>ab</sup>	300 ± 50 <sup>ac</sup>
LBB+CP4	0.555 ± 0.0023 <sup>b</sup>	4.13 ± 0.23 <sup>a</sup>	9.30 ± 0.30 <sup>a</sup>	50 ± 25 <sup>c</sup>
LBP+CP2	0.732 ± 0.0019	4.15 ± 0.35 <sup>a</sup>	<b>17.70 ± 1.65<sup>c</sup></b>	20 ± 10 <sup>c</sup>
LBP+CP3	0.430 ± 0.0038	4.13 ± 0.21 <sup>a</sup>	8.41 ± 0.58 <sup>a</sup>	300 ± 50 <sup>ac</sup>
LBP+CP4	0.687 ± 0.0030	4.04 ± 0.27 <sup>a</sup>	8.69 ± 0.90 <sup>a</sup>	150 ± 50 <sup>ac</sup>
LBR+CP2	0.501 ± 0.0017 <sup>ac</sup>	4.11 ± 0.29 <sup>a</sup>	10.00 ± 0.70 <sup>a</sup>	75 ± 25 <sup>c</sup>
LBR+CP4	0.768 ± 0.0032	4.20 ± 0.48 <sup>a</sup>	11.69 ± 0.80 <sup>a</sup>	100 ± 50 <sup>c</sup>

<sup>a,b,c,d</sup>Numbers with the same letter (for each technological parameter determined) are not significantly different ( $P < 0.05$ ).

<sup>1</sup>Optical density, pH, and whey syneresis values are expressed as mean of duplicates of independent cultures. Apparent viscosity values are expressed as mean of triplicates of independent cultures.

<sup>2</sup>Determined at  $\gamma = 256 \times 5.41$  (s<sup>-1</sup>).

<sup>3</sup>After a storage of 20 d at 10°C.

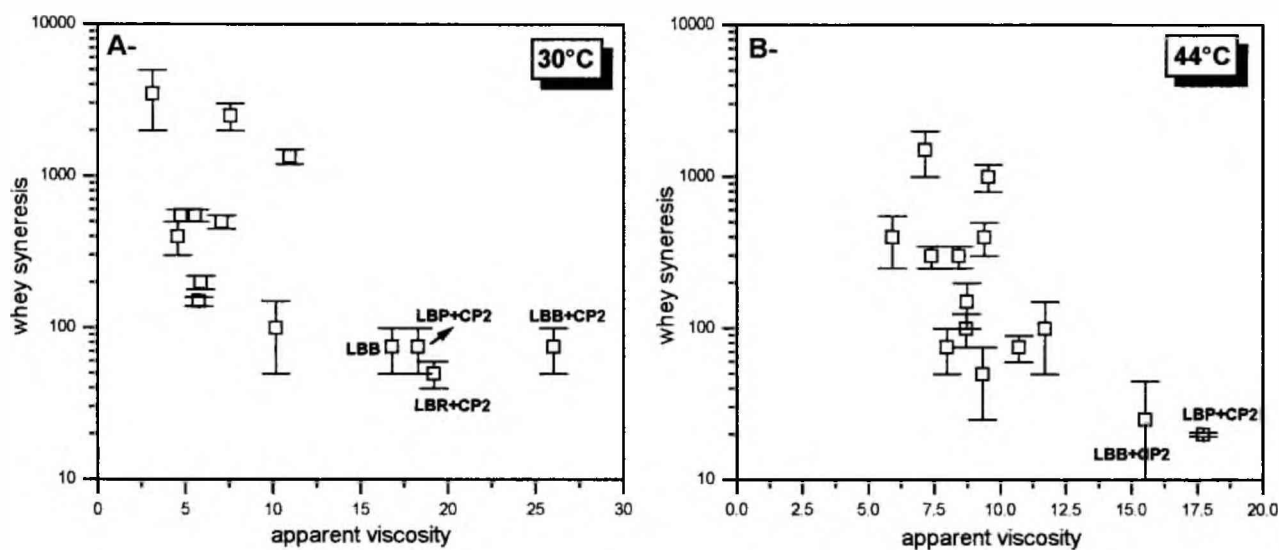
of streptococci and lactobacilli. Although in all cases the inoculum density was the same (approximately  $10^7$  cfu/ml), the optical densities of mixed cultures were higher with respect to the corresponding single-strain cultures after 24 h of incubation at 30°C, ( $P < 0.05$ ). In the pure single-strain cultures, streptococci reached higher values of counts than the lactobacilli. In mixed cultures the streptococci/lactobacilli ratio ranged from approximately 5/1 to 70/1. Counts of lactobacilli in single-strain and mixed cultures were the same ( $P > 0.05$ ), suggesting that lactobacilli grew poorly and were not stimulated by streptococci. Single-strain cultures of streptococci showed lower counts in comparison to the corresponding mixed cultures ( $P < 0.05$ ), except for strain CP3. These data suggest that the growth of strains CP2 and CP4 would be stimulated by lactobacilli, whereas strain CP3 would not. Besides, the acid development was higher in mixed cultures grown at 30°C ( $P < 0.05$ ).

As regards the rheological properties of the coagulum, single-strain cultures of LBB strain gave products of higher viscosity at 30°C than at 44°C ( $P < 0.05$ ). Pure cultures of strain CP2 gave a more viscous product than other *S. thermophilus* strains at 30°C ( $P < 0.05$ ). However, when milks were incubated at 44°C all cultures generated products of similar viscosity (Table 2). Milks fermented with mixed cultures containing LBB+CP2 and LBP+CP2 showed a significant increase ( $P < 0.05$ ) in the apparent viscosity with respect to the corresponding pure cultures at both temperatures, although this synergistic effect was higher at 30°C than at 44°C. At 30°C, the apparent viscosity of cultures

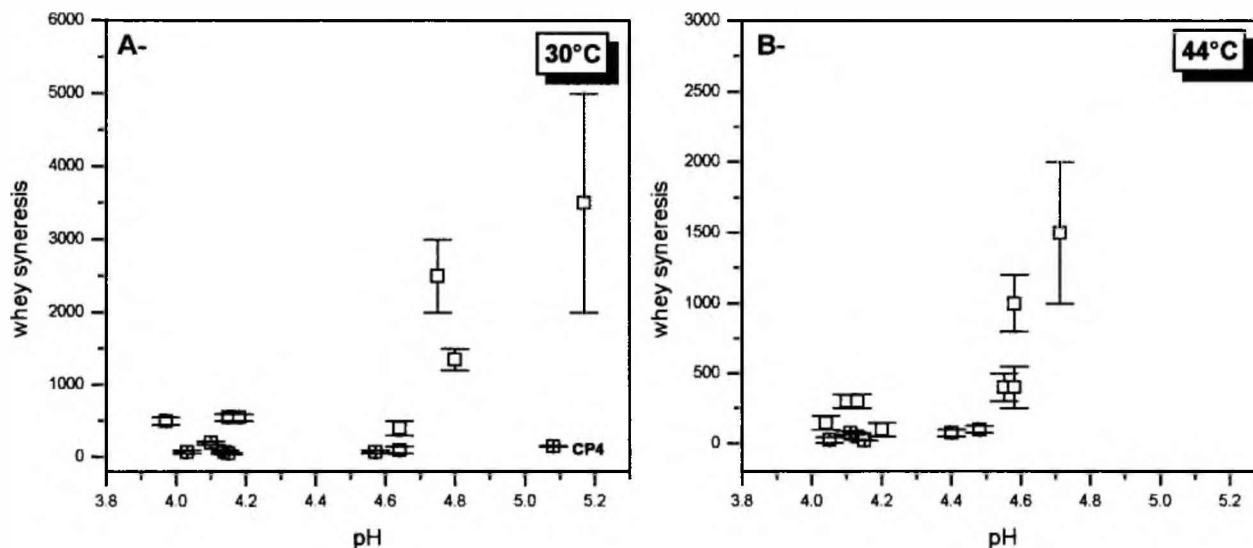
containing LBR+CP2 was higher than the values obtained with the corresponding single-strain cultures ( $P < 0.05$ ). On the other hand, the viscosity of the products obtained with all other mixed cultures did not differ from single-strain culture ( $P > 0.05$ ).

Single-strain cultures of LBP and CP3 at both temperatures as well as LBR at 30°C showed the highest post-storage syneresis ( $P < 0.05$ ). Mixed cultures showed a lower quantity of whey expelled than the corresponding pure cultures at both temperatures. The relationship between viscosity and syneresis of all tested cultures is shown in Figure 1 A and B. No correlation between syneresis and viscosity was found when viscosity values were lower than 15 mPa × s at both temperatures, because for a same viscosity value syneresis varied in a wide range. On the other hand, for cultures having viscosity values higher than 15 mPa × s syneresis was low. These cultures correspond to the mixed cultures prepared with CP2 and each of the three strains of lactobacilli (LBB, LBP, and LBR) and the single-strain culture of LBB at 30°C (Figure 1A). At 44°C these cultures correspond to LBB+CP2 and LBP+CP2 (Figure 1B). Preliminary results indicated that strain CP2 produces polysaccharides (date not shown); suggesting that the high viscosity values achieved could be assigned to this feature.

It is generally accepted that the viscosity of a coagulum depends both on the amount of polysaccharide produced and on the pH. Nevertheless, differences in viscosity values between mixed cultures (LBB, LBP, and LBR with CP2) and the corresponding pure cultures of lactobacilli and streptococci would not be exclusively



**Figure 1.** Relationship between whey syneresis and apparent viscosity. Single-strains and mixed cultures of streptococci and lactobacilli were incubated in milk at 30°C for 24 h (A) and 44°C for 6 h (B).



**Figure 2.** Relationship between whey syneresis and pH. Single-strains and mixed cultures of streptococci and lactobacilli were incubated in milk at 30°C for 24 h (A) and 44°C for 6 h (B).

due to pH since at same pH values, different cultures gave products of different viscosity. The increase in the viscosity values for the mixed cultures with lactobacilli and CP2 with respect to the pure cultures of lactobacilli was higher at 30°C than at 44°C, a finding that could be assigned to a higher production of polysaccharide at lower temperatures. These data agree with results published by other authors who found that every polysaccharide-producing strain would have better production at low temperature (9).

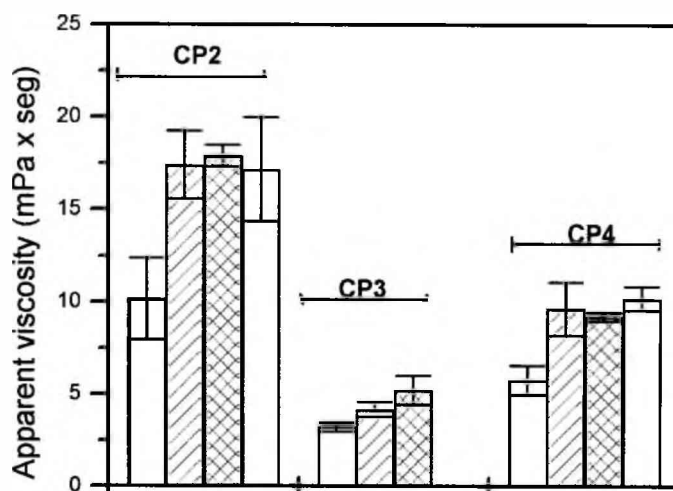
Figure 2 A and B show syneresis as a function of pH. Syneresis seems to correlate exponentially with pH in a range of 4.0 and 5.2 at both temperatures, excepting CP4 at 30°C.

CP2 and CP4 cultures supplemented with LBB, LBP, or LBR supernatants gave high viscous products in comparison with the single-strain cultures of streptococci (CP2 and CP4). The viscosity of the products obtained from CP3 with supernatants of lactobacilli did not vary in comparison to the control ( $P > 0.05$ ) (Figure 3).

There were no differences ( $P > 0.05$ ) on the acid production of the single-strain cultures of streptococci and the same cultures supplemented with lactobacilli supernatants (pH of the coagulum was between 4.2 and 4.9). On the other hand, the addition of lactobacilli supernatant to the streptococci cultures improved syneresis of the coagulum because syneresis was half-reduced (data not shown).

We highlight here that lactobacilli supernatant could replace lactobacilli cultures for the stimulating effect on viscosity and syneresis but not on the acidifying capacity. The supernatants of LBB, LBP, and LBR had

a TCA-soluble nitrogen content between 4.7 and 5.7 mg of Tyr/100 ml, meaning that they provide the culture with a similar amount of peptides and amino acids. However, some streptococci strains were stimulated by these metabolites (CP2 and CP4), whereas others were not. Summing up, the synergistic effect between CP2 and the three strains of lactobacilli tested improved the



**Figure 3.** Effect of the addition of supernatant of lactobacilli on *S. thermophilus* cultures. Cultures of streptococci (CP2, CP3 and CP4) were incubated at 30°C for 24 h and the apparent viscosity was determined as indicated in Materials and Methods. Streptococci cultures: solid, control; striped, plus 150  $\mu$ l of LBB supernatant; hatched, plus 150  $\mu$ l of LBP supernatant; open, plus 150  $\mu$ l of LBR supernatant.

viscosity probably due to the amount of polysaccharide produced.

We consider that when formulating mixed starters for soft cheese, it is important to select the strains at the temperature of the technological process (30 to 32°C).

### CONCLUSIONS

A synergic effect was observed as regards the viscosity of the coagulum when cultures were incubated at 30°C. Besides, there was a decrease in syneresis with mixed cultures of all combination of pairs of strains assayed. Lactobacilli and their supernatants were found to stimulate the growth of streptococci and the viscosity of the final product.

We also observed a synergistic effect at 44°C regarding the viscosity and syneresis of the coagulum. At this temperature the effect was less significant than the one obtained at 30°C.

Products with viscosities higher than 15 mPa × s showed low syneresis. On the other hand, cultures with similar pH showed a different degree of syneresis.

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