Effect of Milk Base and Starter Culture on Acidification, Texture, and Probiotic Cell Counts in Fermented Milk Processing

I. Sodini,* A. Lucas,* M. N. Oliveira,† F. Remeuf,* and G. Corrieu*

*Unité Mixte de Recherche de Génie et Microbiologie des Procédés Alimentaires, Institut National Agronomique de Paris Grignon/Institut National de la Recherche Agronomique, 78 850 Thiverval Grignon, France

†São Paulo University, Department of Biochemical and Pharmaceutical Technology, Av Prof Lineu Prestes, 580, 05508-900, São Paulo, Brazil

ABSTRACT

In the present work, the compared effect of milk base and starter culture on acidification, texture, growth, and stability of probiotic bacteria in fermented milk processing, was studied. Two strains of probiotic bacteria were used, *Lactobacillus acidophilus* LA5 and *L. rhamnosus* LR35, with two starter cultures. One starter culture consisted only of *Streptococcus thermophilus* ST7 (single starter culture); the other was a yogurt mixed culture with *S. thermophilus* ST7 and *L. bulgaricus* LB12 (mixed starter culture). For the milk base preparation, four commercial dairy ingredients were tested (two milk protein concentrates and two casein hydrolysates). The resulting fermented milks were compared to those obtained with control milk (without enrichment) and milk added with skim milk powder. The performance of the two probiotic strains were opposite. *L. acidophilus* LA5 grew well on milk but showed a poor stability during storage. *L. rhamnosus* LR35 grew weakly on milk but was remarkably stable during storage. With the strains tested in this study, the use of the single starter culture and the addition of casein hydrolysate gave the best probiotic cell counts. The fermentation time was of about 11 h, and the probiotic level after five weeks of storage was greater than 10^6 cfu/ml for *L. acidophilus* LA5 and 10^7 cfu/ml for *L. rhamnosus* LR35. However, an optimization of the level of casein hydrolysate added to milk base has to be done, in order to improve texture and flavor when using this dairy ingredient.

(Key words: probiotic bacteria, dairy ingredients, stability, texture)

Abbreviation key: MPC = milk protein concentrate, CH = casein hydrolysate, SSC = single starter culture, MSC = mixed starter culture, SMP = skim milk powder.

INTRODUCTION

Some strains of microorganisms have been used for a long time in animal feeding to improve their zootechnical performances. Over the last few decades, some strains of lactic acid bacteria belonging to bifidobacteria and lactobacilli have been introduced in food products for human consumption, with the aim to improve human health. They are called probiotic bacteria (“for life,” in Greek) and are defined as living microorganisms, which upon ingestion in sufficient quantity, exert health benefits beyond inherent basic nutrition (Guarner and Schaafsma, 1998). Fermented dairy products are the most widely used food vehicle for these bacteria, because dairy products have a healthy image. The first works reporting an effect on human health of the consumption of living bacteria have been criticized and subjected to a lot of controversies because of their lack of convenient design for experimentation. But these last years, a number of well-designed, randomized, and placebo-controlled double blind studies have been done. Some of them showed significant health effects of consumption of living bacteria, including certain strains of lactic acid bacteria as *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shirota, *Lactobacillus acidophilus* NCFB 1748, NCFM, LA5, or *Lactobacillus johnsonii* LA1 (Fonden et al., 2000).

A minimum level of living microorganisms is required to observe a positive effect of their consumption. For instance, a minimum daily dose of 10^9 cfu/d of the strain *L. johnsonii* LA1 is required to modulate certain forms of nonspecific defense mechanisms. With the lower daily dose of 10^8 cfu/d, no significant effect on this function was reported (Donnet-Hughes et al., 1999). More generally, depending on the strains used and the required health effect, this level is usually between 10^8 and 10^{11} cfu/d (Vanderhoof and Young, 1998). Therefore, assuming a daily consumption of fermented dairy products of 100 g, they should contain between 10^6 cfu/g to 10^9 cfu/g of these live bacteria at the time of consumption. This is often not the case in commercial products (Rybka and Fleet, 1997). The first reason for the

Received December 6, 2001.

Accepted January 4, 2001.

Corresponding author: I. Sodini; e-mail: Sodini@grignon.inra.fr.

low levels of probiotic bacteria observed in commercial products is the slow growth of the probiotic strains. They are not competitive with the strains of the starter culture. Secondly, they often have a poor stability during storage. For instance, a 3-log decrease after only 2 wk of storage at 4°C has been reported for the probiotic strain *L. johnsonii* LA1 in a fermented milk (Saxelin et al., 1999).

Several works have been done to improve the growth of probiotic bacteria by acting on milk base. The positive effect of adding nonproteinic nitrogen (Saxena et al., 1994; Dave and Shah, 1998; Oliveira et al., 2002), oxygen scavengers (Dave and Shah, 1998; Shah, 2000), oligosaccharides (Shin et al., 2000), and sugar sources (Saxena et al., 1994), have been reported. For instance, with pure culture of probiotic lactobacilli, Saxena et al. (1994) and Oliveira et al. (2002) noticed that acidification rate was doubled by adding casitone (5 g·kg⁻¹) or tryptone (20 g·L⁻¹). Dave and Shah (1998), using a very small level of tryptone (250 mg·L⁻¹), increased by 3 log the level of bifidobacteria in a fermented milk containing starter culture and probiotic bacteria. The same authors studied the effect of adding cysteine as oxygen scavenger. With 500 mg·L⁻¹ of cysteine, the growth of *L. acidophilus* was slightly better (1.7×10^8 versus 4.5×10^7 cfu/g), and one of the bifidobacteria was greatly improved (4.0×10^5 versus 7.1×10^2 cfu/g). The effect of adding sugar can be positive up to a certain level. Saxena et al. (1994) observed a stimulation of *L. acidophilus* growth when 0.5% of fructose was added to milk (50% increase for acidification rate in pure culture). On the other hand, with high addition level (12 to 16%), Shah and Ravula (2000) noticed a growth inhibition for strains of *L. acidophilus* and bifidobacteria (1 and 2 log less in the final product for both strains, with 12 and 16%, respectively, of sugar compared with 8%). For bifidobacteria, it has been reported that milk supplementation with 5% of fructooligosaccharides can reduce the mean doubling time by almost twice (Shin et al., 2000). The choice of the starter culture seems also to have a positive or negative effect on probiotic growth, depending on involved protocoeoperation, inhibition, or competition phenomena (Dave and Shah, 1997; Saxelin et al., 1999). Dave and Shah (1997), with mixed cultures of starter and probiotic, noticed a probiotic generation number during fermentation comprised between 0.7 and 4 depending on the starter used. Furthermore, for one strain of bifidobacteria, these authors reported an antagonistic relationship with a starter strain, leading to a 3-log decrease of the bifidobacteria during fermentation.

After fermentation and formulation of the dairy product, the level of probiotic bacteria has to remain stable. It has been shown that the product composition as the

content of total nonfat solids (Gardini et al., 1999), and the addition of sugar (Shah and Ravula, 2000), carbon dioxide (Vinderola et al., 2000b), and oligosaccharides (Shin et al., 2000) can have an effect on the stability of probiotic cells. Gardini et al. (1999) noticed a better survival of *L. acidophilus* during storage in milk with low total nonfat solids (10 versus 16%). Vinderola et al. (2000b), studying the effect of CO₂ on growth and viability of *L. acidophilus* cultured with bifidobacteria and starters, showed a significant lowering of *L. acidophilus* in carbonated fermented milk, by comparison to control. According Shah and Ravula (2000), with a sugar level of 12% instead of 8%, the viability of probiotic cultures is lower (loss of 3 log instead of 2 log after 49 d of storage). For two strains of bifidobacteria, Shin et al. (2000) reported a better viability after 4 wk of storage when milk is supplemented with 5% of fructooligosaccharides. They calculated a viability of 44 to 67% when milk is supplemented, instead of 9.3 to 11.6% for control. The activity of the starter culture during fermentation and storage is sometimes negative for the survival of the probiotic strain (Nighswonger et al., 1996; Dave and Shah, 1997; Rybka and Fleet, 1997; Saxelin et al., 1999; Godward et al., 2000). Dave and Shah (1997) compared four starter cultures used in combination with probiotic culture of *L. acidophilus* and bifidobacteria. For *L. acidophilus*, the decrease observed during 5 wk of storage was less pronounced (2 log versus 3 log) when the starter was devoid of *L. bulgaricus*. Saxelin et al. (1999), with seven strains of probiotic bacteria, reported a good stability during 2 wk of storage for three of them, and a decrease comprised between 1 and 3 log for the four other strains. In any case, the higher decreases were noticed with the starter containing *L. bulgaricus*. Nighswonger et al. (1996), comparing seven strains of probiotic lactobacilli, showed that the stability during 4 wk of storage was dependent on probiotic strain (decrease of 3 to 2 log depending on the strain) and starter culture. For instance, with the strain *L. acidophilus* 0 - 16, the decrease was equal to 0.58 log with one starter and to 1.37 log with another.

Thus, milk base formulation and starter culture design seem to have strong effects on growth and stability of probiotic bacteria. The aim of this study was to compare these effects, in order to define the most accurate strategy to improve probiotic counts in fermented milk processing. As the manufacture of fermented milk also requires convenient acidification and texture, these characteristics were evaluated.

MATERIALS AND METHODS

Strains and Dairy Ingredients

Four commercial strains were used: *Streptococcus thermophilus* ST7 and *Lactobacillus bulgaricus* LB12

Table 1. Nitrogen composition of the five dairy ingredients used for milk base formulation: one skim milk powder (SMP), two milk proteins concentrates (MPC1 and MPC2), and two casein hydrolysates (CH1 and CH2). Mean concentrations provided from two replicates are given in % (wt).

Nitrogen	SMP	MPC1	MPC2	CH1	CH2
Total	33.3	47.3	49.1	73.2	74.6
Soluble	7.3	17.1	9.6	73.2	71.1
Nonproteic	2.5	2.7	1.8	73.2	71.1

for starter cultures, and *Lactobacillus acidophilus* LA5 and *Lactobacillus rhamnosus* LR35 for probiotic cultures (Chr. Hansen, Arpajon, France). Pure strain inocula were stored at -70°C as concentrate. They were thawed and diluted 10 times in sterilized milk just before use.

Five dairy ingredients have been tested: a skim milk powder (**SMP** [Elle et Vire, Condé Sur Vire, France]), two milk protein concentrates (**MPC1** and **MPC2** [Promilk C500 A and Promilk C502; Ingredia, Arras, France]), and two casein hydrolysates (**CH1** and **CH2** [Vitalarmor 900LB; Armor Proteines, Saint Brice en Coglès, France, and Tryptone; Difco, Detroit, MI]). Their nitrogen contents are reported in Table 1.

Fermented Milk Preparation

Commercial pasteurized low fat milk ($15\text{ g}\cdot\text{L}^{-1}$ of fat) and whole milk ($36\text{ g}\cdot\text{L}^{-1}$ of fat [Lactel; Lactalis, Laval, France]) were mixed to reach $26\text{ g}\cdot\text{L}^{-1}$ of fat, and standardized with $53\text{ g}\cdot\text{kg}^{-1}$ of dairy ingredient to 13% of nonfat dry matter. Then it was submitted to thermal treatment at 92°C for 5 min, cooled to 4°C in an iced bath, poured into 250-ml Erlenmeyer flasks, and stored 24 h before use. Milk with no added dairy ingredient was used as control.

The flasks were inoculated at the fermentation temperature with a probiotic culture (*L. acidophilus* LA5 or *L. rhamnosus* LR35) and a starter culture. Two starter cultures have been used: a single starter culture (**SSC**) composed only of *S. thermophilus* ST7, and a mixed starter culture (**MSC**) composed of *S. thermophilus* ST7 and *L. bulgaricus* LB12. The inoculation rates were equal to 0.01% for the probiotic culture and 0.02% for the starter culture. All inoculated milks were incubated at 40°C until pH 4.50 was reached. Fermentation was stopped by rapidly cooling the fermented milk in an iced bath, manually stirring it with a stainless steel bored disk by up-and-down movements for almost 2 min, setting the stirred product into 100-ml cups, and storing it at 4°C .

Each of the 24 fermented milks studied (6 milk bases $\times$ 4 cultures) was manufactured in four Erlenmeyer flasks, stirred, and poured at the end of fermentation

into 10 cups. Four cups were used for the texture analysis, and six were kept for the microbiological analysis (one cup analyzed each week over 6 wk).

Acidification Kinetics

Acidification was studied for strains used in pure culture and during fermented milk preparation. Each fermentation, performed in four replicates, was monitored with the Cinac system (Corrieu et al., 1988), which allows a continuous recording of pH. In the case of fermented milk preparation, fermentation time to reach pH 4.55 was chosen as the endpoint to describe acidification. With the pure culture, it was not possible to reach pH 4.55 for all the trials, especially for the one including streptococci. To be able to compare the pure cultures, fermentation time to reach pH 5 was chosen as the endpoint of the fermentation.

Texture Analysis

In order to compare the texture of the fermented milks, two different textural properties were evaluated by using instrumental methods. The measurements were performed three times, after 2 wk of storage at 4°C .

Rheological behavior. The rheological parameters were determined at 10°C , by means of a stress-controlled rheometer model RS1 (Haake, Karlsruhe, Germany), used in harmonic and stationary mode. The rheometer was equipped with a 60-mm diameter cone and plate geometry, with a 2° angle cone and a $117\text{-}\mu\text{m}$ gap. The fermented milk was gently mixed by stirring five times because some phase separation possibly occurred during storage, and 5 ml were deposited on the plate of the rheometer by means of a syringe. The use of the syringe has been proved to avoid the presence of grains in the sampled material, which greatly disturb the measurement. Measurements in dynamic mode were carried out using a shear stress of 0.14 Pa at an oscillatory frequency of 1 Hz for 1 min. The complex viscosity, in Pa·s, was calculated by the determination of the mean of the 20 measures done during the analysis. Measurements in stationary mode were realized by applying a shear rate of 10 s^{-1} . The apparent viscosity was determined after 3 min of shearing.

Graininess evaluation. The determination of the number of grains was made by an automatic image analysis procedure. One sample of fermented milk (1 g) was diluted in 10 ml of distilled water, then poured in a Petri dish placed on an illuminated plate. The diluted sample was visualized by a digital color camera (JAI M2040; Imasys, Soresnes, France) equipped with an optical zoom. The image analysis was performed in

grey level mode with commercial software (Optimas 6.2; Optimas Corporation, Silver Spring, MD). The grains having a perimeter higher than 1 mm were enumerated. The results are given by the number of grains per gram of fermented milk.

Microbiological Analyses

Cell count enumeration. For each run, fermented milks were analyzed each week during a 5-wk storage at 4°C. The first analysis (wk 0) was done just after fermentation at pH 4.50. Fermented milk samples were diluted in sterile tryptone diluent (0.1% wt/vol) and subsequently plated in duplicate onto selective media. The strain of *L. acidophilus* was enumerated according to IDF (1995) on MRS media (Biokar) added with 1.5 g/L of bile (Biokar). The strain *L. rhamnosus* LR35 was enumerated according to Saxelin et al. (1999) on MRS media added with 50 mg/L of vancomycin (Sigma, St. Louis, MO). *S. thermophilus* and *L. bulgaricus* were enumerated on M17 media (Biokar) and MRS, respectively, at pH 5.4. The plate's incubation was done at 37°C for 72 h, in an anaerobic jar containing GENbox (bioMérieux, Lyon, France) for all lactobacilli.

Generation number calculation. The generation number of probiotic during fermentation was determined from cfu enumeration by calculating the number of doublings between the beginning and the end of the fermentation.

Statistical Analyses

Results were submitted to ANOVA procedures using SAS Software (SAS Institute Inc., Cary, NC). For each main effect, a multiple comparison of means was performed, using the Bonferroni test ($P < 0.05$).

RESULTS

Acidification

As a preliminary study, the effect of the dairy ingredients added for milk base preparation was studied with the strains used in pure culture. The results are reported in Figure 1. The strain *L. rhamnosus* LR35 grew poorly in milk, whatever the milk base. This observation was confirmed by a statistical analysis, comprising an ANOVA and a multiple comparison of means (data not shown). It showed that fermentation time was significantly higher for *L. rhamnosus* LR35 than for the three other strains, with a mean value equal to 23.5 h for this strain, by comparison to 11.4 h on average for the other three. The statistical analysis reported also that milk bases enriched with the three dairy ingredients, CH2, MPC1, and CH1 improved significantly the

Figure 1. Effect of milk base on fermentation time required to reach pH 5 for milk inoculated with a single culture of *Streptococcus thermophilus* ST7, *Lactobacillus bulgaricus* LB12, *Lactobacillus acidophilus* LA5, and *Lactobacillus rhamnosus* LR35. Milk base was not supplemented (control), or supplemented with skim milk powder (SMP), milk proteins (MPC1 and MPC2), and casein hydrolysates (CH1 and CH2). Milk bases with no common letter differ according to the Bonferroni test ($P \leq 0.05$).

growth of the strains in pure culture. The mean fermentation times were equal to 10.2 h, 11.4 h, and 15.1 h for milk bases containing CH2, MPC1, and CH1, respectively, by comparison to 16.5 h on average for the other milk bases. A significant interaction between the milk base and the inoculated strain was noticed ($P < 0.01$). The strain *S. thermophilus* ST7 was more stimulated than the other strains by the addition of dairy ingredient, with a fermentation time divided by three when the milk base was added with MPC1, CH1, or CH2, by comparison with the control. For the other strains, the supplementation divided the fermentation time by two, at most.

The fermentation times observed during fermented milk preparation are reported in Figure 2. The starter culture had a great effect on acidification, as fermentation time was, on average, divided by two and three for fermented milk containing *L. acidophilus* LA5 or *L. rhamnosus* LR35, respectively, when the MSC was used instead of the SSC. When the SSC is used, the dairy ingredient added for milk base had a strong effect on fermentation time, with the shortest fermentation time obtained with CH2, and the longest fermentation time obtained with MPC2. The values were, respectively, 5.1 h and 13.2 h for *L. acidophilus* LA5, and 6.1 h and 24 h for *L. rhamnosus* LR35. With the MSC, the milk base had a less important effect than with the SCC. For example, for the strain *L. rhamnosus* LR35, the fermentation time ranged between 4.1 and 7.2 h when the

Figure 2. Effect of starter culture¹ and milk base² on fermentation time required to reach pH 4.55 for milk inoculated with a mixed culture containing *Lactobacillus acidophilus* LA5 or *Lactobacillus rhamnosus* LR35. ¹Starter culture was a mixed culture of *Streptococcus thermophilus* ST7 and *Lactobacillus bulgaricus* LB12 (MSC) or a single culture of *S. thermophilus* ST7 (SSC). ²Milk base was not supplemented (control), or supplemented with skim milk powder (SMP), milk proteins (MPC1 and MPC2), and casein hydrolysates (CH1 and CH2). Milk bases with no common letter differ according to the Bonferroni test ($P \leq 0.05$).

MSC was used, while it varied from 6.1 to 24 h with the SSC.

Texture

The rheological parameters obtained for the fermented milks are given in Figures 3 and 4, showing, respectively, the complex and apparent viscosities. The same results were observed for these two different instrumental descriptors of the texture. The starter culture had a significant effect on rheological parameters, with the higher value obtained with the SSC. The milk

Figure 3. Effect of starter culture¹ and milk base² on complex viscosity of a fermented milk containing *Lactobacillus acidophilus* LA5 or *Lactobacillus rhamnosus* LR35. ¹Starter culture was a mixed culture of *Streptococcus thermophilus* ST7 and *Lactobacillus bulgaricus* LB12 (MSC) or a single culture of *S. thermophilus* ST7 (SSC). ²Milk was not supplemented (control), or supplemented with skim milk powder (SMP), milk proteins (MPC1 and MPC2), and casein hydrolysates (CH1 and CH2). Milk bases with no common letter differ according to the Bonferroni test ($P \leq 0.05$).

base had also a significant effect on texture, with the lowest values obtained when CH1 or CH2 were added, and the highest values obtained with MPC1 and MPC2 (slightly higher for MPC2 than for MPC1 for complex viscosity; see Figure 3). Actually, a lack of convenient coagulation was observed at pH 4.5 when CH1 or CH2 were added to the milk base, which explains the very low values of complex and apparent viscosities reported (less than 10 Pa.s, and 1 Pa.s, respectively, by comparison with 54 to 142 Pa.s, and 3 to 6 Pa.s for the other milk bases). The interaction between the starter culture and the milk base was once more highly significant ($P < 0.01$). The effect of milk base on texture was higher when the SSC was used. For instance, the increase

Figure 4. Effect of starter culture¹ and milk base² on apparent viscosity at 10 s⁻¹ of fermented milk containing *Lactobacillus acidophilus* LA5 or *Lactobacillus rhamnosus* LR35. ¹Starter culture was a mixed culture of *Streptococcus thermophilus* ST7 and *Lactobacillus bulgaricus* LB12 (MSC) or a single culture of *S. thermophilus* ST7 (SSC). ²Milk base was not supplemented (control), or supplemented with skim milk powder (SMP), milk proteins (MPC1 and MPC2), and casein hydrolysates (CH1 and CH2). Milk bases with no common letter differ according to the Bonferroni test ($P \leq 0.05$).

of complex viscosity observed between the fermented milks containing the strain *L. rhamnosus* LR35 and coming from a milk without supplementation (control) or added with MPC2, was equal to 130% and 280%, respectively, with the MSC and the SSC.

In the case of a milk base supplemented with CH1 and CH2, which did not coagulate, the fermented milks were smooth and without any grains. For the other milk bases, whatever the probiotic strain used, the graininess of the fermented milks was negligible (number of grains per gram less than 10) when the MSC was used and high (number of grains per gram was between 40 and 120) with the SSC (data not shown).

Figure 5. Effect of starter culture¹ and milk base² on growth expressed in generation number of *Lactobacillus acidophilus* LA5 or *Lactobacillus rhamnosus* LR35. ¹Starter culture was a mixed culture of *Streptococcus thermophilus* ST7 and *Lactobacillus bulgaricus* LB12 (MSC) or a single culture of *S. thermophilus* ST7 (SSC). ²Milk base was not supplemented (control), or supplemented with skim milk powder (SMP), milk proteins (MPC1 and MPC2), and casein hydrolysates (CH1 and CH2). Milk bases with no common letter differ according to the Bonferroni test ($P \leq 0.05$).

Probiotic Growth

To calculate the probiotic growth during fermentation, the generation number between the beginning and the end of the process was considered. The results are reported in Figure 5. It was between 0 and 6.3 depending on probiotic strain, starter culture, and milk base. It was on average higher for *L. acidophilus* LA5 than for *L. rhamnosus* LR35. The effect of starter culture and milk base was studied by an ANOVA. The effect of starter culture was highly significant ($P < 0.01$), with higher generation numbers when the SSC was used, instead of the MSC (0.6 to 6.3 versus 0 to 3.9). The effect of milk base was also highly significant ($P <$

Table 2. Effect of starter culture¹ and milk base² on the concentration of *Lactobacillus acidophilus* LA5 in fermented milk over a storage period of 5 wk at 4°C. Means are calculated from two duplicates. The comparison between the six milk bases (data in columns) and the six storage periods (data in rows) has been done by a multiple comparison of means using the Bonferroni test (see left and right superscripts, respectively).

Starter culture	Milk base	Storage period (wk)					
		0	1	2	3	4	5
MSC		(log ₁₀ cfu/mL)					
	Control	^c 6.85 ^A	^a 6.64 ^A	^b 6.07 ^B	<3.50	<3.50	<3.50
	SMP	^c 7.05 ^A	^a 7.02 ^A	^c 5.37 ^B	<3.50	<3.50	<3.50
	MPC1	^b 7.33 ^A	^a 7.04 ^A	^d 4.15 ^B	^a 4.37 ^B	<3.50	<3.50
	MPC2	^c 7.06 ^A	^a 7.02 ^A	^c 5.30 ^B	<3.50	<3.50	<3.50
	CH1	^a 7.83 ^A	ND ³	^a 6.77 ^B	^a 4.43 ^C	<3.50	<3.50
	CH2	^a 8.01 ^A	^a 6.87 ^B	^c 4.93 ^C	^b 3.76 ^D	3.72 ^D	<3.50
SSC		(log ₁₀ cfu/mL)					
	Control	^d 7.38 ^A	^d 6.49 ^B	^d 3.84 ^C	<3.50	<3.50	<3.50
	SMP	^{cd} 7.75 ^A	^{bc} 7.84 ^A	^b 6.53 ^B	^c 4.47 ^C	<3.50	<3.50
	MPC1	^{cd} 7.66 ^A	^b 7.99 ^A	^c 4.50 ^B	^c 4.47 ^B	<3.50	<3.50
	MPC2	^{bc} 7.91 ^A	^c 7.53 ^A	^c 5.08 ^B	^d 4.10 ^C	^c 3.83 ^C	<3.50
	CH1	^a 8.73 ^A	^a 8.66 ^A	^a 8.69 ^A	^a 7.08 ^B	^a 6.50 ^C	^a 6.31 ^C
	CH2	^b 8.17 ^A	^c 7.55 ^B	^b 6.81 ^C	^b 6.01 ^D	^b 5.73 ^D	^b 5.13 ^E

¹Starter culture was a mixed culture of *Streptococcus thermophilus* ST7 and *Lactobacillus bulgaricus* LB12 (MSC) or a pure culture of *Streptococcus thermophilus* ST7 (SSC).

²Milk base was not supplemented (control), or supplemented with skim milk powder (SMP), milk protein concentrates (MPC1 and MPC2), and casein hydrolysates (CH1 and CH2).

³Not determined.

^{a, b, c, d}Column means with no common superscripts differ ($P \leq 0.05$).

^{A, B, C, D, E}Row means with no common superscripts differ ($P \leq 0.05$).

0.01). Their ability to stimulate probiotic growth depended on the strain. For *L. acidophilus* LA5, the CH permitted the highest growth (generation number equal to 4 on average). For *L. rhamnosus* LR35, the dairy ingredients MPC2 and CH1 were the most stimulating for the growth of the probiotic (generation number equal to 2 on average). There was a significant interaction between the starter culture and the milk base. For instance, the CH had a positive effect on growth of *L. rhamnosus* LR35 when performed with the MSC, but a negative effect when cultured with the SSC.

Probiotic Stability

The concentration of probiotic bacteria *L. acidophilus* LA5 and *L. rhamnosus* LR35 was followed for 5 wk, and is reported in Tables 2 and 3, respectively. The two probiotic strains did not show the same stability during storage. Whatever the starter culture and the milk base, *L. acidophilus* LA5 concentration decreased significantly during the 5 wk of storage, showing a lowering of at least 2 log to more than 4.5 log. On the other hand, *L. rhamnosus* LR35 did not lower significantly for eight of the 12 conditions tested, and in any case, the lowering was less than 1 log. The effect of starter culture and milk base was not significant for the stability of this strain, which remained at a high concentration in fermented milk (higher than 10⁶ cfu/ml). How-

ever, the starter culture and the milk base influenced the concentration of *L. acidophilus* LA5. The better stability for this strain was observed with the SSC and the milk base enriched in CH, which permitted it to maintain more than 10⁶ cfu/ml after 3 wk of storage, and more than 10⁵ cfu/ml after 5 wk.

DISCUSSION

Acidification

The results obtained with the strains used in pure cultures underlined the poor ability of some strains of probiotic bacteria to grow on milk. For instance, Saxelin et al. (1999) reported for the strain *L. paracasei* F19 a poor milk acidification, with a fermentation time to reach pH 4.5 of more than 20 h. In our study, the strain *L. rhamnosus* LR35 took more than 20 h to reach pH 5.0. Saxelin et al. (1999) and Saxena et al. (1994) reported that the addition of nonproteic nitrogen such as yeast extract or CH could improve greatly the growth of some probiotic strains, as noticed here for strain LA5 with the addition of CH2. Anyway, the most pronounced effect of milk base supplementation was observed with the strain *S. thermophilus* ST7. This could be ascribed to the lower proteolytic activity of *S. thermophilus* than that of lactobacilli (Rajagopal and Sandine, 1990). Thus, *S. thermophilus* should be more sensitive to incor-

Table 3. Effect of starter culture¹ and milk base² on the concentration of *Lactobacillus rhamnosus* LR35 in fermented milk over a storage period of 5 wk at 4°C. Means are calculated from two duplicates. The comparison between the six milk bases (data in columns) and the six storage periods (data in rows) has been done by a multiple comparison of means using the Bonferroni test (see left and right superscripts, respectively).

Starter culture	Milk base	Week					
		0	1	2	3	4	5
MSC		(log ₁₀ cfu/mL)					
	Control	^{cd} 6.72 ^{ABC}	^c 6.90 ^A	^b 6.72 ^{ABC}	^e 6.54 ^C	^c 6.68 ^{BC}	^{bc} 6.79 ^{AB}
	SMP	^d 6.65 ^{AB}	^d 6.57 ^{AB}	^b 6.72 ^{AB}	^d 6.70 ^{AB}	^{cd} 6.84 ^A	^d 6.46 ^B
	MPC1	^{bc} 6.81 ^B	^{bc} 7.02 ^A	^{ab} 6.98 ^A	^{bc} 6.95 ^B	^{ab} 7.08 ^A	^a 7.04 ^A
	MPC2	^b 6.93 ^A	^{bc} 6.92 ^A	^{ab} 6.87 ^A	^b 7.02 ^A	^{bc} 6.90 ^A	^{ab} 6.90 ^A
	CH1	^a 7.16 ^B	^a 7.35 ^A	^a 7.16 ^B	^a 7.23 ^{AB}	^a 7.13 ^B	^{cd} 6.66 ^C
	CH2	^a 7.10 ^A	^b 7.05 ^{AB}	^{ab} 6.98 ^{BC}	^c 6.91 ^C	^{ab} 7.07 ^{AB}	^d 6.56 ^D
SSC		(log ₁₀ cfu/mL)					
	Control	^c 7.75 ^B	^a 8.16 ^A	^{ab} 8.07 ^A	^a 8.05 ^A	^{ab} 8.04 ^A	^c 6.92 ^C
	SMP	^a 8.11 ^A	^a 8.16 ^A	^a 8.23 ^A	^a 8.23 ^A	^{ab} 8.08 ^A	^a 7.85 ^B
	MPC1	^b 7.88 ^B	^a 7.99 ^A	^{ab} 7.89 ^A	^{ab} 7.74 ^B	^b 7.98 ^A	^a 7.99 ^A
	MPC2	^a 8.18 ^A	^a 8.25 ^A	^a 8.22 ^A	^a 8.17 ^A	^a 8.14 ^A	^a 8.03 ^A
	CH1	^c 7.72 ^{AB}	^a 7.81 ^A	^b 7.90 ^A	^{ab} 7.77 ^{AB}	^c 7.76 ^{AB}	^b 7.56 ^B
	CH2	^d 7.10 ^A	^b 6.75 ^A	^c 7.08 ^A	^c 7.04 ^A	^d 7.09 ^A	^c 6.71 ^A

¹Starter culture was a mixed culture of *Streptococcus thermophilus* ST7 and *Lactobacillus bulgaricus* LB12 (MSC) or a single culture of *Streptococcus thermophilus* ST7 (SSC).

²Milk base was not supplemented (control), or supplemented with skim milk powder (SMP), milk protein concentrates (MPC1 and MPC2), and casein hydrolysates (CH1 and CH2).

^{a, b, c, d}Column means with no common superscripts differ ($P \leq 0.05$).

^{A, B, C, D}Row means with no common superscripts differ ($P \leq 0.05$).

poration of potential nitrogen sources brought by dairy ingredients.

The acidification of the fermented milks is strongly related to the composition of the starter culture. The MSC, composed of *S. thermophilus* ST7 and *L. bulgaricus* LB12, presented a high interaction factor (Sodini et al., 2000) and permitted a short fermentation time. Similar results were reported by Kneifel et al. (1993), and Dave and Shah (1997), who observed a decrease in the fermentation time of 20 to 50% when the MSC was used instead of a SSC, as compared with 50 to 70% reduction in our case.

The effect of milk base can be partially explained by the nitrogen composition of the added dairy ingredients reported in Table 1. The CH2, which gave the lowest fermentation time, contained a high level of nonproteic nitrogen (more than 70%), as compared with MPC2 (less than 2%). Besides, the fermentation is not only related to the nonproteic content of the dairy ingredient, because the CH1, which had the same level of nonproteic content than the CH2, did not give the same results. The composition of the nonproteic fraction (content in free amino acids and peptides) should also influence its ability to stimulate bacteria growth.

Texture

The texture was influenced strongly by the starter culture and the milk base. The graininess of fermented

milks, which is higher with the SSC than with the MSC, might be related to the long fermentation time (more than 10 h). It increases the phenomena of coagulation in two steps as described by Lucey and Singh (1998). According to these authors, in a milk base increasingly heated, the aggregation would be expected to start at pH 5.3, which is the isoelectric pH of the β -lactoglobulin, and would continue until pH 4.6, which is the isoelectric pH of the casein. Possibly, some bonds would be broken during this two-step coagulation, when the casein network begins to be built. It should favor grain formation. If the fermentation time is long, the elapsed time between these two pH levels is also long. The number of bonds created during the first stage of coagulation is then higher, so the number of bonds broken is also higher during the second stage, thus leading to an increase of the number of grains at the end of coagulation. The cross-linking density of the matrix, as assessed by viscosity measurements, has been shown to be higher with a slow acidification (Van Marle and Zoon, 1995; Beal et al., 1999), which could explain the generally higher rheological parameters obtained with the SSC (10 to 15 h for fermentation time) than with the MSC (5 h).

The addition of dairy ingredients, which increases the DM content, increased the rheological parameters, except for the CH, which gave products without thickness and with poor viscosity. This effect of CH on fer-

mented milk texture has already been noticed by Saxena et al. (1994). It is possible that the CH, rich in nonproteic material as amino acids or peptides, have a masking effect on milk proteins, hindering the network formation. For the other dairy ingredients, their texturing capacity was directly related to their protein content. It was higher for MPC than for milk powder, with values equal respectively to 30.9, 44.6, and 47.3% for SMP, MPC1, and MPC2, thus giving a final protein content in milk base of 4.66, 5.35, and 5.49%. The positive relationship between the milk base protein content and the yogurt texture has been emphasized by other authors (Rohm and Schmidt, 1993; Lankes et al., 1998).

Probiotic Growth

The probiotic lactobacilli growth during fermentation was generally between 2 and 4 generation number, which is comparable to several values reported in literature, for example 1.3 for Shah and Ravula (2000) and 1 to 3 for Dave and Shah (1997). It was clear that the starter culture chosen had an important effect, and a better growth was observed with the SSC. This effect can be due to a phenomena of competition between the lactobacilli, which could slow the growth of the probiotic lactobacilli when they are cultured with the yogurt one, *L. bulgaricus*. However, these results were in contradiction with those reported by Dave and Shah (1997), who observed no difference between an SSC and an MSC for the growth of strains of *L. acidophilus*.

The milk base had an effect on probiotic growth, as noticed by Dave and Shah (1998), who reported also a high growth when milk was supplemented with CH. This was probably due to the nonproteic nitrogen of the hydrolysate which can stimulate the weakly proteolytic probiotic strains (Shihata and Shah, 2000).

Probiotic Stability

The stabilities of the two strains tested in this study were very different. For *L. rhamnosus* LR35, the decrease in count was less than 1 log in 6 wk, which was comparable to the results obtained by Gardini et al. (1999) with *L. acidophilus* IPVR 224 and by Vinderola et al. (2000b) with *L. acidophilus* LaA3 (decrease of, respectively, 1.7 log in 5 wk, and 0.5 log in 7 wk). For the strain *L. acidophilus* LA5, under the applied experimental conditions, the decrease was much higher, between 2 and 4.5 log after 5 wk of storage, comparable to other strains studied in the literature. For example, Saxelin et al. (1999) with the strain *L. salivarius* UCC118, and Vinderola et al. (2000a) with the strain *L. acidophilus* LA1, reported respectively a decrease of 3 log in 2 wk, and 2.7 to 4.6 log in 4 wk.

The starter culture had a significant effect on the stability of *L. acidophilus* LA5, which was reported for other strains (Dave and Shah, 1997; Saxelin et al., 1999; Vinderola et al., 2000a). Generally, the stability was better with the SSC, devoid of *L. bulgaricus*. The post-acidification and the releasing of hydrogen peroxide caused by *L. bulgaricus* could decrease the stability of the probiotic strains. The works of Vinderola et al. (2000a) and Karagük-Yücer et al. (2000) have shown the sensitivity of probiotic strains to low pH. In our study, the pH of all fermented milks was lowered during the storage period (data not shown), but the final pH after 5 wk of storage was more acidic for the ones containing *L. bulgaricus* (4.12 to 4.15) than for the ones which were devoid of this strain (4.24 to 4.25). On the other hand, Dave and Shah (1997) have noticed a higher production of hydrogen peroxide with the starter culture containing *L. bulgaricus* and suspected it causes a partial injury to the cells of *L. acidophilus*. According to these authors, the partially injured cells might show faster decay in the viable cell counts in fermented milks.

The milk base has also an effect on probiotic stability, in the case of the strain *L. acidophilus* LA5. The milk base enriched with CH reduced the decrease of probiotic counts during storage. Similar results were obtained by Saxena et al. (1994) and Dave and Shah (1998), but they were poorly discussed. Possibly the presence of a fraction of nonproteic nitrogen allows a cellular activity during storage, thus slowing down the reduction of the viable cells.

CONCLUSIONS

This study has shown that the behavior of mixed probiotic cultures is different according to the criteria studied. For a fast acidification and a smooth texture, the best starter culture is the mixed one. For a significant probiotic growth and a high stability, the single one is the most convenient. In this case, the use of casein hydrolysate is useful to lower the fermentation time, and, in addition, it enhances the probiotic stability. The fermentation time is equal to 11 h. After 5 wk of storage, probiotic cell counts exceed 10^6 cfu/ml for *Lactobacillus acidophilus* LA5, and 10^7 cfu/ml for *Lactobacillus rhamnosus* LR35. However, a poor viscosity and a bitter flavor have been observed due to the high level of CH added. A lower level of CH, or the use of whey protein hydrolysates, could help avoid these defects. On the other hand, the change of process parameters such as pH and fermentation temperature, as well as the moment of addition of the starter culture, could improve the growth of the strain *L. rhamnosus* LR35 and the stability of the strain *L. acidophilus* LA5, which both remain low.

ACKNOWLEDGMENTS

Author M. N. Oliveira wishes to thank *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP) for financial support.

REFERENCES

- Beal, C., J. Skokanova, E. Latrille, N. Martin, and G. Corrieu. 1999. Combined effects of culture conditions and storage time on acidification and viscosity of stirred yogurt. *J. Dairy Sci.* 82:673–681.
- Corrieu, G., H. E. Spinnler, D. Picque, and Y. Jomier. 1988. Procédé de mise en évidence et de contrôle de l'activité acidifiante d'agents de fermentation dans des bains de fermentation et dispositif pour sa mise en œuvre. Institut National de la Recherche Agronomique, French Pat. Num. 88 14456.
- Dave, R. I., and N. P. Shah. 1998. Ingredient supplementation effects on viability of probiotic bacteria in yogurt. *J. Dairy Sci.* 81:2804–816.
- Dave, R. I., and N. P. Shah. 1997. Viability of yoghurt and probiotic bacteria in yogurts made from commercial starter cultures. *Int. Dairy J.* 7:31–41.
- Donnet-Hughes, A., F. Rochat, P. Serrant, J. M. Aseschlimann, and E. J. Schiffrin. 1999. Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. *J. Dairy Sci.* 82:863–869.
- Fonden, R., G. Mogensen, R. Tanaka, and S. Salminen. 2000. Culture-containing dairy products: Effects on intestinal microflora, human nutrition and health. Current knowledge and future perspectives. *Bull. Int. Dairy Federation* 352:1–30.
- Gardini, F., R. Lanciotti, M. E. Guerzoni, and S. Torriani. 1999. Evaluation of aroma production and survival of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* in fermented milks. *Int. Dairy J.* 9:125–134.
- Godward, G., K. Sultana, K. Kailasapathy, P. Peiris, R. Arumugaswamy, and N. Reynolds. 2000. The importance of strain selection on the viability and survival of probiotic bacteria in dairy foods. *Milchwissenschaft* 55:441–445.
- Guarner, F., and G. J. Schaafsma. 1998. Probiotics. *Int. J. Food Microbiol.* 39:237–238.
- IDF. 1995. Detection and enumeration of *Lactobacillus acidophilus*. *Bull. IDF.* 306:23–33.
- Karagük-Yücer, Y., J. C. Wilson, and C. H. White. 2000. Formulations and processing of yogurt affect the microbial quality of carbonated yogurt. *J. Dairy Sci.* 84:543–550.
- Kneifel, W., D. Jaros, and F. Erhard. 1993. Microflora and acidification properties of yogurt and yogurt-related products fermented with commercially available starter cultures. *Int. J. Food Microbiol.* 18:179–189.
- Lankes, H., H. B. Ozer, and R. K. Robinson. 1998. The effect of elevated milk solids and incubation temperature on the physical properties of natural yogurt. *Milchwissenschaft* 53:510–513.
- Lucey, J. A., and H. Singh. 1998. Formation and physical properties of acid milk gels: A review. *Food Res. Int.* 30:529–542.
- Nighswonger, B. D., M. M. Brashears, and S. E. Gilliland. 1996. Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in fermented milk products during refrigerated storage. *J. Dairy Sci.* 79:212–219.
- Oliveira, M. N., I. Sodini, F. Remeuf, and G. Corrieu. 2002. Effect of milk supplementation and culture composition on acidification, textural properties, and microbiological stability of fermented milks containing probiotic bacteria. *Int. Dairy J.* 11:939–946.
- Rajagopal, S. N., and W. E. Sandine. 1990. Associative growth and proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in skim milk. *J. Dairy Sci.* 73:894–899.
- Rohm, H., and W. Schmidt. 1993. Influence of dry matter fortification on flow properties of yogurt. 1. Evaluation of flow curves. *Milchwissenschaft* 48:556–560.
- Rybka, S., and G. H. Fleet. 1997. Populations of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium* species in Australian yogurts. *Food Aus.* 49:471–475.
- Saxelin, M., B. Grenov, U. Svensson, R. Fonden, R. Reniero, and T. Mattila-Sandholm. 1999. The technology of probiotics. *Trends Food Sci. Technol.* 10:387–392.
- Saxena, S. N., B. K. Mital, and S. K. Garg. 1994. Effect of casitone and fructose on the growth of *Lactobacillus acidophilus* and its survival during storage. *Int. J. Food Microbiol.* 21:271–272.
- Shah, N. P. 2000. Probiotic bacteria: Selective enumeration and survival in dairy foods. *J. Dairy Sci.* 83:894–907.
- Shah, N. P., and R. R. Ravula. 2000. Influence of water activity on fermentation, organic acids production and viability of yogurt and probiotic bacteria. *Aust. J. Dairy Technol.* 55:127–131.
- Shihata, A., and N. P. Shah. 2000. Proteolytic profiles of yogurt and probiotic bacteria. *Int. Dairy J.* 10:401–408.
- Shin, H. S., J. H. Lee, J. J. Pestka, and Z. Ustunol. 2000. Growth and viability of commercial *Bifidobacterium* ssp in skim milk containing oligosaccharides and inulin. *J. Food Sci.* 65:884–887.
- Sodini, I., G. Corrieu, and E. Latrille. 2000. Identification of interacting mixed cultures of lactic acid bacteria by their exclusion from a model predicting the acidifying activity of non-interacting mixed cultures. *Applied Microbiol. and Biotechnol.* 54:715–718.
- Van Marle, M., and P. Zoon. 1995. Permeability and rheological properties of microbially and chemically acidified skim milk gels. *Neth. Milk Dairy J.* 49:47–65.
- Vanderhoof, J. A., and R. J. Young. 1998. Use of probiotics in childhood gastrointestinal disorders. *J. Pediatric Gastroenterol. and Nutrition* 27:323–332.
- Vinderola, C. G., N. Bailo, and J. A. Reinheimer. 2000a. Survival of probiotic microflora in Argentinian yogurts during refrigerated storage. *Food Res. Int.* 33:97–102.
- Vinderola, C. G., M. Gueimonde, T. Delgado, J. A. Reinheimer, and C. G. de los Reyes-Gavilan. 2000b. Characteristics of carbonated fermented milk and survival of probiotic bacteria. *Int. Dairy J.* 10:213–220.