Development of Probiotic Cheese Manufactured from Goat Milk: Response Surface Analysis via Technological Manipulation

ANA M. P. GOMES and F. XAVIER MALCATA[1]
Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200 Porto, Portugal

ABSTRACT

Production of caprine milk has been rising steadily, partially because of its good nutritional value; the possibility of improving nutritional benefits by adding probiotic species such as *Bifidobacterium lactis* and *Lactobacillus acidophilus* was assessed. The manufacturing process of a traditional semi-hard goat cheese was technologically modified to optimize the process. The amount of starter inoculum, the concentration of salt, the addition of a protein hydrolysate, and the ripening time were varied to improve the microbiological, biochemical, and sensory properties of the cheese. *Bifidobacterium lactis* was able to grow slightly (up to 3×10^8 cfu/g), but growth was dependent on the physicochemical characteristics of the cheese. *Lactobacillus acidophilus* did not grow substantially in any of the experimental cheeses, and maximum numbers did not exceed 6×10^7 cfu/g. Concentrations of lactic acid and acetic acid increased throughout cheese manufacture, indicating that production of these acids was uncoupled from growth. Viability of the probiotic strains during ripening was sufficient to yield numbers that were above the accepted threshold (10^6 cfu/g) for a probiotic effect. Both strains contributed significantly to ripening, especially in the formation of low molecular mass peptides and amino acids, but lipolysis was not greatly affected. Statistical analyses using response surface methodology indicated that the manufacture of goat cheese could be optimized by the addition of 0.30% (vol/wt) milk hydrolysate, 3×10^7 of viable *B. lactis* and 7×10^6 of viable *L. acidophilus* cells/ml of milk, respectively, 3.50% (wt/wt) salt, and ripening for 70 d.

(**Key words**: *Bifidobacterium lactis*, *Lactobacillus acidophilus*, caprine milk, cheese ripening)

Abbreviation key: **BL** = goat cheese manufactured with *Bifidobacterium lactis* and *Lactobacillus acidophilus*, **PTA** = phosphotungstic acid, **R** = reference cheese manufactured with a mesophilic starter, **SDM** = salt in DM, **SN** = soluble N, **TN** = total N, **WSN** = water-soluble N.

INTRODUCTION

Worldwide interest in applications for caprine milk has been growing recently, and the production and manufacture patterns associated with this type of milk have changed considerably. Cheeses manufactured from caprine milk have a social and economic impact in the Mediterranean basin because of their unique organoleptic and textural properties and because of the easy adaptation of goats to poor, dry pastures. Portugal produces large amounts of caprine milk (4), most of which is used in artisanal cheese making. Furthermore, goat cheese is highly nutritious, especially in terms of its high protein content in relation to calorie and fat contents. Caprine milk and dairy products derived from caprine milk might be further improved by the addition of probiotic cultures.

Bifidobacteria have been incorporated into a wide range of milk products (26) to meet a growing consumer demand for these organisms. Previous studies have revealed that bifidobacterial populations may decline very rapidly in a fermented milk product unless specific attention is paid to both strain selection and pH. Hekmat and McMahon (18) and Holcomb et al. (19) proposed ice cream and frozen yogurt, respectively, as effective carriers for *Lactobacillus acidophilus* and *Bifidobacterium bifidum* because survival of these bacteria was good in those products during frozen storage. Nevertheless, delivery systems are needed that improve the viability of bifidobacteria and ensure that reasonable numbers of bacteria are delivered to the host upon consumption after storage at room temperature (20°C) or slightly lower. Recent studies by Gomes et al. (16) have demonstrated that a starter composed entirely of *B. lactis* and *L. acidophilus* could be successfully used for the manufacture of a Gouda-type cheese (**BL**); these species survived well in the experimental cheeses during a 9-wk ripening period. Because such studies sug-

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[1]Corresponding author.

gested that cheese offers an alternative and interesting route for the administration of probiotics, the application of these species as a sole starter to cheeses manufactured with milk from small ruminants is of potential interest. However, the chemical composition of the milk is important to the growth, metabolic activity, and viability of probiotic strains (33). To evaluate further the effectiveness of a cheese as a nutritional food vehicle for *B. lactis* and *L. acidophilus*, cheese-making experiments with these species in caprine milk were conducted in our research facilities.

Queijo de Cabra is a type of semi-hard, lightly pressed goat cheese that is produced on an industrial scale in Portugal. The production process of this semi-hard cheese was chosen for study because the required moisture level is relatively simple to obtain even when the conventional manufacture protocol is modified. The temperature profile essential to promote growth and activity of the starter bacteria was altered, and the cutting time and the agitation speed, which are essential to control the moisture content, were adjusted in this study. Our first objective was to manufacture and examine the physicochemical and biochemical properties and the microbial profiles of a semi-hard goat cheese throughout a 70-d ripening period. For all of the quality factors considered, the second (and principle) objective was to increase the viable numbers of *B. lactis* and *L. acidophilus* to the maximum permissible value and concomitantly to increase the production of organic acids, to increase all ripening indices [ratios of water-soluble N (**WSN**) to total N (**TN**), TCA soluble N (**SN**) to TN and PTA SN to TN], to reduce the firmness of cheese, and to enhance the sensory characteristics. Consequently, it was important to control the process variables that might significantly affect those quality factors. Certain processing variables, namely, the relative concentrations of *B. lactis* and *L. acidophilus* starter inoculum, the salt concentration, the addition of a bifidogenic factor (i.e., a protein hydrolysate), and the duration of the ripening period were manipulated in our study because those variables had been identified as having an important effect on the properties of a BL cheese matrix (16).

MATERIALS AND METHODS

Materials

Caprine milk was obtained from a homogeneous herd of a native Portuguese breed, Alpine. Prior to cheese manufacture, milk was pasteurized for 15 s at 72°C. The starter cultures were *B. lactis* and *L. acidophilus* strain Ki (Coöperatieve Stremsel-en Kleurselfabriek, Leeuwarden, The Netherlands). The reference cheese was manufactured with a mixed-strain starter (Redi-Set 200, a BD-type starter composed of *Streptococcus lactis*, *Streptococcus cremoris*, *Streptococcus diacetylactis* and *Leuconostoc cremoris;* Chr. Hansen's Lab, Horsholm, Denmark). The hydrolysate that was added to the milk used for cheese making was prepared from lowfat bovine milk that had been pretreated according to methods of Gomes et al. (16) using a commercial enzyme preparation from *Aspergillus* sp. (protease 2A™; Amano Pharmaceutical, Nagoya, Japan) with high proteolytic activity.

Cheese Samples

The cheese-manufacturing trials were carried out in a pilot dairy plant (Paços de Ferreira, Portugal) of the Portuguese Governmental Directorate of Agriculture of the Region between the Rivers Douro and Minho (DRAEDM). In each experiment, 100 L of caprine milk were used per cheese vat to produce eight cheeses with a mean weight of 1.48 kg, mean diameter of 12.5 cm, and mean height of 6 cm. Cheese making followed the basic protocol of manufacture of Queijo de Cabra using a mesophilic mixed-strain starter (reference cheese, denoted hereafter as **R** cheese) with several modifications. After pasteurization, the milk was cooled to 32°C (30°C for the R cheese), $CaCl_2$ was added (23 g/100 L of milk), starter concentrates of *B. lactis* and *L. acidophilus* were added at different inoculum levels (Table 1), and double-strength calf rennet (Marshall Products, Rhône-Poulenc, Lyon, France) was added (22 ml/100 L of milk). Curd formed in ca. 25 min (30 min for the R cheese) and was cut with 0.95-cm knives (0.65-cm knives for the R cheese) for 20 min (25 min for the R cheese). The temperature of the curd and whey mixture was raised slowly to 38°C (34°C for the R cheese) within 20 min. Whey (50% of its total volume) was subsequently drained and was replaced with an equal amount of warm water. The resulting curd and whey mixture was maintained at 38°C for an additional 20 min. After the second whey drainage, the curd was placed in hoops and held at 40°C (room temperature for the R cheese) throughout pressing (2-kg weights for 4 h during which cheeses were inverted five times) and holding (3 h) and then brined. Brining conditions varied among the various batches of cheese to attain the desired range for salt in the DM (**SDM**) of 2 to 6% (wt/wt) (Table 1), and the cheeses from the various batches were ripened for 70 d at 6°C and 92% relative humidity. The BL 1, BL 2, BL 3, BL 4, and BL 10 experimental

trials consisted of batches of 8 cheeses that were randomly divided into two groups of 4 cheeses, and one group was removed earlier (level A) from the brine than the other (level B) (Table 1). The remaining experimental trials, namely, BL 5 to BL 9, consisted of batches of 4 cheeses. In each group of 4 cheeses, 1 cheese was picked at random and used for bacteriological examination from 48 h after manufacture throughout the 70-d ripening time to monitor survival of both strains. Of the remaining cheeses, 2 were ripened for 15 and 70 d, respectively; both cheeses were subject to chemical examination, and the cheese that was ripened for 70 d was also subject to organoleptic examination. The fourth cheese was used to carry out the initial (pressed curd up to 48 h-old cheese) microbiological and chemical examinations. These results, except for curd analysis at cutting (which corresponds to 0 d and is denoted as level −1), were not used in the statistical analyses.

Cheese Analysis

Microbiological analysis. The samples used for bacteriological assay were collected aseptically into sterile sample bags according to the methods of Gomes et al. (16). All samples were homogenized with a sterile 2% (wt/vol) sodium citrate (Merck, Frankfurt, Germany) solution at 45°C (1:10, vol/vol, dilution) for 3 min in a stomacher (Lab-Blender 400; Seward Medical, London, United Kingdom). Sequential decimal dilutions of the cheese homogenates were prepared in a sterile solution containing 0.1% (wt/vol) peptone (Oxoid, Basingstoke, United Kingdom) and 0.85% (wt/vol) NaCl (Merck). As described by Gomes et al. (16) the pour plate technique was used for the enumeration of *B. lactis* and *L. acidophilus* on MRS agar (LabM, Bury, United Kingdom) supplemented with 0.05% (wt/vol) L-cysteine·HCl (Merck) and other selective agents and on TGV agar (tryptone, glucose, and meat extract) containing 2% (wt/vol) NaCl, respectively. All determinations were made in duplicate.

Chemical analysis. A section of the cheese was randomly taken and used as a sample for the chemical and organoleptic assays. The rind of the sector samples was removed, and the rindless samples were shredded. The pH was measured with a pH meter (Crison Instruments, Barcelona, Spain) equipped

TABLE 1. Definition of original and coded values of manipulated variables considered in the manufacture of BL cheeses containing *Bifidobacterium lactis* (Bl) and *Lactobacillus acidophilus* (La).

Experimental design[1]	Cheese sample	Original level starter inoculum			Salt in DM		Hydrolysate		Ripening time	
		Bl	La	Code	Original	Code	Original	Code	Original	Code
		(cfu/ml)			(%, wt/wt)		(%, vol/wt)		(d)	
C	BL1A	3.0×10^7	1.0×10^7	+1	2.0	−1	0.00	−1	0	−1
C	BL1A	3.0×10^7	1.0×10^7	+1	2.0	−1	0.00	−1	70	+1
C	BL1B	3.0×10^7	1.0×10^7	+1	6.0	+1	0.00	−1	0	−1
C	BL1B	3.0×10^7	1.0×10^7	+1	6.0	+1	0.00	−1	70	+1
C	BL2A	3.0×10^7	1.0×10^7	+1	2.0	−1	0.30	+1	0	−1
C	BL2A	3.0×10^7	1.0×10^7	+1	2.0	−1	0.30	+1	70	+1
C	BL2B	3.0×10^7	1.0×10^7	+1	6.0	+1	0.30	+1	0	−1
C	BL2B	3.0×10^7	1.0×10^7	+1	6.0	+1	0.30	+1	70	+1
C	BL3A	3.0×10^7	5.0×10^6	−1	2.0	−1	0.00	−1	0	−1
C	BL3A	3.0×10^7	5.0×10^6	−1	2.0	−1	0.00	−1	70	+1
C	BL3B	3.0×10^7	5.0×10^6	−1	6.0	+1	0.00	−1	0	−1
C	BL3B	3.0×10^7	5.0×10^6	−1	6.0	+1	0.00	−1	70	+1
C	BL4A	3.0×10^7	5.0×10^6	−1	2.0	−1	0.30	+1	0	−1
C	BL4A	3.0×10^7	5.0×10^6	−1	2.0	−1	0.30	+1	70	+1
C	BL4B	3.0×10^7	5.0×10^6	−1	6.0	+1	0.30	+1	0	−1
C	BL4B	3.0×10^7	5.0×10^6	−1	6.0	+1	0.30	+1	70	+1
c	BL5	3.0×10^7	7.5×10^6	0	4.0	0	0.15	0	15	−0.6
ax	BL6	3.0×10^7	7.5×10^6	0	4.0	0	0.30	+1	15	−0.6
ax	BL7	3.0×10^7	7.5×10^6	0	4.0	0	0.00	−1	15	−0.6
ax	BL8	3.0×10^7	1.0×10^7	+1	4.0	0	0.15	0	15	−0.6
ax	BL9	3.0×10^7	5.0×10^6	−1	4.0	0	0.15	0	15	−0.6
ax	BL10A	3.0×10^7	7.5×10^6	0	2.0	−1	0.15	0	15	−0.6
ax	BL10B	3.0×10^7	7.5×10^6	0	6.0	+1	0.15	0	15	−0.6

[1]ax = Axial point, C = corner point, and c = center point.

with a combined pH electrode (Ingold, Steinbach, Switzerland). The moisture and fat contents were determined according to method 4 of the International Dairy Federation (21) and van Gulik's butyrometric determination (23), respectively. The NaCl content was measured by the modified Volhard method. For the assays of lactic acid and acetic acid, the samples were prepared and analyzed using the HPLC method that was described previously by Gomes et al. (16), in which separation was accomplished in a chromatograph (Beckman Instruments, San Ramon, CA) with a 300 × 7.8 mm HPX-87H column (Bio-Rad, Richmond, CA); detection was by refractometry (Beckman Instruments), and data acquisition and handling used the System Gold 168 (Beckman Instruments). The lactose content was quantified spectrophotometrically using the phenol-sulfuric acid method developed by Acton (1). Nitrogen contents were determined according to method 20B of the International Dairy Federation (22) adapted to microconditions (i.e., sample size of ca. one-tenth that used for the classical Kjeldahl method) on a Kjeltec System II (with Digestion System 2000 and Distilling Unit 1002; Tecator, Höganäs, Sweden). The procedure by Kuchroo and Fox (28), with modifications, was followed for the extraction of SN in 10-g sample homogenates. Nonprotein N (TCA SN) was estimated in 5 ml of the filtrate obtained after precipitation of the cheese homogenate with 12% TCA (Merck). The extent of secondary proteolysis was assayed as the amount of N that was soluble in 5% phosphotungstic acid (**PTA**) SN (Merck) extracts of 5 ml of the filtrate. The FFA in cheese were determined by titration of a diethyl ether-soluble fat extract with 0.1*N* KOH ethanolic solution to phenolphthalein end point according to Nuñez et al. (37). All determinations were done in duplicate.

Textural analysis. Samples were analyzed for hardness using an Instron Universal Testing Instrument (model 4501; Instron Corp., Canton, MA). Cylindrically shaped samples (diameter of 20 mm; height of 20 mm) were taken randomly from the cheeses but remote from the rind area and were maintained at 20°C (test temperature) for 4 h prior to analysis. The cheese samples were compressed between parallel plates to 80% of their original height using a cell with a pressure capacity of 100 N and a crosshead speed of 10 mm/s. Each sample was analyzed at least three times, and the first three consistent values were averaged to yield a hardness value. Hardness was defined as the force required to compress the cheese to 80% of its original height.

Sensory analysis. A sensory panel was screened for its ability to determine the intensity of basic tastes, odor recognition, and texture rating. The 16 panelists evaluated the experimental cheeses at 70 d of age; the R cheese was similar in age but was produced traditionally. The cheeses were graded for flavor, firmness, and consistency relative to the R cheese. Grade scales were used for flavor (2 = very bad to 6 = very good), consistency (2 = very brittle to 6 = very smooth), and firmness (1 = very soft to 7 = very firm). In addition, the panelists were requested to indicate their overall preference (1 = least preferred to 3 = most preferred).

Statistical Analysis

A 2^3 complete factorial design (including a center point replicated three times as estimator of variance) (5) was initially used to assess the effects of the amount of starter inoculum, the salt level, and the amount of hydrolysate; the effect of ripening time was assessed via a 2^1 design nested in the aforementioned 2^3 design. The statistical effects of these four variables were experimentally evaluated for microbiological, biochemical, sensory, and textural properties of goat cheese. For simplicity, these cheeses are denoted hereafter as BL, and the numerical code indicates the level of the manipulated variable, followed by A or B to indicate 2 or 6% (wt/wt) salt expressed as percentage of cheese DM (Table 1). The linear model to be tentatively fitted by minimum least squares to the experimental data was

$$\hat{y} = \bar{y} + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 \qquad [1]$$

where $\hat{y}$ = fitted response, $\bar{y}$ = mean of all data, b = adjustable parameters, and x = manipulated technological variable in coded, normalized form (Table 1); these variables are defined as $x_1 = (I - 7.5 \times 10^6)/2.5 \times 10^6$, where I = inoculum level of *L. acidophilus* expressed in colony-forming units per gram (under the assumption that the inoculum level of *B. lactis* is 3×10^7 cfu/g); $x_2 = (S - 4)/2$, where S = salt concentration in cheese expressed as a percentage (wt/wt) of DM; $x_3 = (H - 0.15)/0.15$, where H = percentage of addition of milk hydrolysate; and $x_4 = (t - 35)/35$, where t is the ripening time expressed in days. Only one high inoculum level of *B. lactis* but three levels of *L. acidophilus* were used to ensure the accepted threshold (10^6 cfu/g) of *B. lactis* to achieve the probiotic effect. Previous studies have shown the beneficial effect of coculturing with different levels of *L. acidophilus* on growth of *B. lactis* (growth is enhanced to different degrees).

Estimation of the order of magnitude of the sum of the quadratic effects associated with the four factors,

following the method outlined by Box et al. (5), indicated that the linear model did not fit the data set adequately; therefore, the experimental design was expanded accordingly with an extra set of eight experiments as axial points. The quadratic model to be tentatively fitted by minimum least squares to the overall set of experimental data was

$$\hat{y} = \bar{y} + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4. \quad [2]$$

The values estimated for these parameters using the full composite design and their corresponding 95% marginal confidence intervals are depicted in Table 2. Diagnostics of residuals for the quadratic model were performed to check the validity of the regression analyses employed, and a normal distribution of residuals was found. The quadratic model obtained was further utilized to find the analytical expressions of the loci (and type as determined from the sign of the corresponding second derivative) of optima for each technological variable. The results are shown in Table 3.

Statistical assessment of the variability among panelists in the sensory measurements was based on the ANOVA methodology. The measured values for the organoleptic attributes were fitted by linear regression to the following quadratic model

$$\hat{y} = \bar{y} + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad [3]$$

where the three manipulated technological variables were assessed in terms of their effect on both sensory and textural properties of the cheese by 70 d of ripening. The values estimated for the adjustable parameters using the full composite design and their corresponding 95% marginal confidence intervals are tabulated in Table 2. The quadratic model obtained was further utilized to find the analytical expressions of the loci (and type) of optima for each technological variable. The results are shown in Table 3.

RESULTS AND DISCUSSION

Chemical Composition

The BL cheese experiments were performed in such a way that the only intended compositional difference between the batches just after manufacture was that imposed by the experimental design encompassing the manipulated variables. In addition, the previously mentioned modifications to the R cheese-making technology warranted feasible comparisons between the different BL cheeses and the R cheese.

The caprine milk used for cheese manufacture throughout the experimental design had a chemical composition that was typical for goat milk produced in Portugal. Table 4 presents the mean values for composition of the milk of the different BL cheeses and of the R cheese by 70 d of ripening. The fat and protein contents were within the limits acceptable for a high quality R cheese. Moisture contents of experimental cheeses were slightly lower than the R cheese, probably because of the increase of 2°C in the cooking temperature, although the remaining process parameters (cutting procedure and stirring time) were changed to compensate for the temperature difference. The holding temperature of 40°C was used to promote the growth and activity of the thermophilic strains. The inverse relationship between the levels of salt and moisture of the BL cheeses is in agreement with data for other cheese types (17).

The pH decreased slowly during coagulation and manufacture as a result of the slower acid-producing activities of the starter culture. The pH values for the ripened cheeses reached the range of 5.4 to 5.5, which was similar to those values reported for several studies (25, 31) of goat cheeses that underwent moderate proteolysis during ripening. The results of statistical analyses performed, with the salt content expressed as percentage of moisture, were similar to those in which the salt content was expressed as SDM, so we decided to select the latter. The SDM varied among the different BL cheese types as a function of brining time and rose to 2.2 to 5.6% as ripening progressed; higher salt contents in cheese (in either SDM or salt in the moisture phase formats) were coincident with lower pH values and lower water contents at 70 d of ripening. This result is difficult to rationalize considering that a higher salt concentration would be expected to be associated with a lower degree of acid production (owing to the negative effect of salt on growth and lactose fermentation by the starter bacteria) and, therefore, with a higher pH value. Nevertheless, similar trends have also been observed for a white-brined cheese manufactured from pasteurized caprine milk using different starters (44) and for Cheddar-like hard goat cheese (2). The higher degree of proteolysis may have caused an upward shift in pH and possibly accounts for the inverse relationship between pH and salt content by the end of ripening.

Glycolysis

The glycolytic conversion of lactose to lactic acid by the starter bacteria is essential during the manufacture and subsequent early stages of cheese ripening. From an initial lactose content of 1.5 to 2% in the curd of the BL cheeses, lactose depletion occurred during the early stages of maturation at different rates and to different extents, depending on the process variables (Figure 1). Only trace quantities of lactose could be detected in the R cheese by the end of ripening (Figure 1). Concentrations of lactose were similar in all cheeses at 15 d, even though the composition of the starters differed and *B. lactis* metabolizes hexoses by the fructose-6-phosphate pathway (40). The lactic and acetic acid contents increased during ripening. Lactic acid concentrations compare well with literature values [e.g., 660 mg/100 g of cheese in Valdeteja cheese (6)], but concentrations in some BL cheeses were lower than that in the R cheese; the acetic acid contents of all BL cheeses were higher than that usually reported in the literature for goat cheese and that observed for the R cheese (Figure 1). This higher content of acetic acid may have resulted from the greater production of acetic acid by the *B. lactis* species than by the species constituting the mesophilic starter (40), despite its lack of growth in some of the BL cheeses. A similar ability to produce acid has been reported (16) for the same strain of *B. lactis* during the manufacture of a Gouda-type cheese.

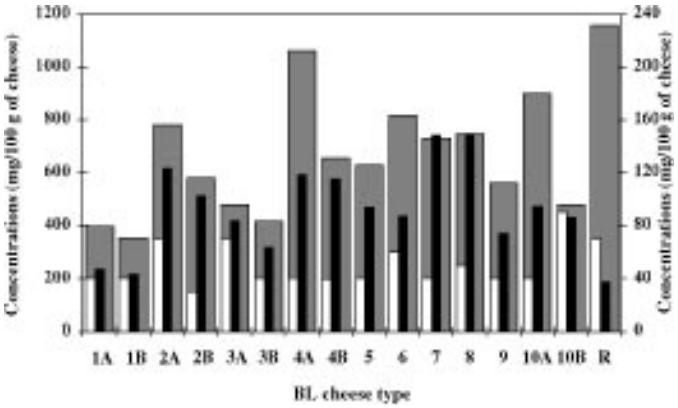

Figure 1. Concentrations of lactose (open bar, right axis), lactic acid (patterned bar, left axis), and acetic acid (solid bar, right axis) of the BL cheeses manufactured with *Bifidobacterium lactis* and *Lactobacillus acidophilus* and the reference cheese (R) after 15 d of ripening at 6°C and 93% relative humidity. Sample designations are given in Table 1.

TABLE 2. Values of main linear effects (x_1, x_2, x_3, and x_4), main quadratic effects (x_1x_1, x_2x_2, x_3x_3, and x_4x_4), and second-order interactions (x_1x_2, x_1x_3, x_1x_4, x_2x_3, x_2x_4, and x_3x_4) for the microbiological (M), biochemical (B), sensory (S), and textural (T) factors.[1]

	MEE					BEE				SEE			TEE
E & I[2]	Bl	La	LC	LAC	AAC	WSN	TCA	PTA SN	FFA	F	Ct	Fl	IH
x_1	1.47×10^7	-2.10×10^6	0.062	−32.840	−5.257	−0.310	−0.592	−0.149	0.216	−0.075	0.022	0.025	−10.374
x_2	-5.60×10^6	-1.89×10^6	0.036	−52.028	−5.219	−1.029	−0.443	0.140	0.000	−0.109	−0.297	0.259	15.272
x_3	-1.96×10^7	1.07×10^6	0.013	105.650	9.648	0.761	0.607	0.435	0.152	0.425	0.141	0.163	−1.548
x_4	-1.08×10^8	-2.21×10^7	−0.650	219.161	19.301	5.713	2.484	1.005	0.607	ND	ND	ND	ND
x_1x_1	7.65×10^7	1.76×10^7	0.287	−112.720	−9.303	−1.060	−0.749	−0.442	−1.762	−0.692	−0.390	0.730	11.101
x_2x_2	2.65×10^7	3.58×10^6	0.602	−202.278	−24.528	−2.247	−1.023	−0.473	−1.522	−0.118	−0.857	0.203	−0.110
x_3x_3	4.65×10^7	1.06×10^7	−0.213	103.265	12.262	0.270	−0.005	0.102	0.833	−0.526	−0.723	0.435	−25.057
x_4x_4	-4.03×10^7	-1.74×10^7	−0.449	204.683	8.940	5.433	2.883	1.092	0.525	ND	ND	ND	ND
x_1x_2	-1.58×10^7	-6.56×10^4	−0.008	18.384	0.795	0.466	0.209	−0.181	−0.003	−0.031	−0.137	0.156	−6.011
x_1x_3	-1.25×10^7	3.15×10^6	−0.398	−14.379	5.664	−0.896	−0.020	0.051	0.171	−0.094	0.043	−0.094	0.346
x_1x_4	-4.60×10^6	7.10×10^5	0.009	−26.134	−2.344	−0.300	−0.550	−0.136	0.007	ND	ND	ND	ND
x_2x_3	3.55×10^6	-7.13×10^6	0.021	−39.017	0.789	0.173	−0.204	−0.081	−0.123	−0.102	−0.215	0.281	−0.996
x_2x_4	-6.38×10^6	-8.77×10^5	−0.056	−35.877	−0.634	−1.136	−0.410	−0.084	−0.353	ND	ND	ND	ND
x_3x_4	1.00×10^7	2.52×10^6	−0.009	73.539	17.720	0.585	0.776	0.366	0.218	ND	ND	ND	ND

[1]AAC = Acetic acid concentration (wt/wt), BEE = biochemical estimated effects, Bl = viable numbers of *Bifidobacterium lactis*, Ct = cheese consistency, E & I = effects (linear and quadratic) and interactions (second order), F = cheese firmness, Fl = cheese flavor, IH = cheese instrumental hardness, La = viable numbers of *Lactobacillus acidophilus*, LAC = lactic acid concentration (wt/wt), LC = lactose content (wt/wt), MEE = microbiological estimated effects, ND = not defined, PTA SN = phosphotungstic acid-soluble N, x_1 = normalized value of starter inoculum defined as $(I - 7.5 \times 10^6)/2.5 \times 10^6$ where I is expressed in colony-forming units per gram, x_2 = normalized salt concentration in the cheese defined as $(S - 4)/2$ where S is expressed in percentage dry matter (wt/wt), x_3 = normalized percentage of milk hydrolyzate addition defined as $(H - 0.15)/0.15$ where H is expressed in percentage (vol/wt), x_4 = normalized ripening time defined as $(t = 35)/35$ where t is expressed in d, TEE = textural estimated effects, TCA = trichloroacetic acid-soluble N, and WSN = water-soluble N.

[2]The 95% confidence intervals associated with the effects (E) and interactions (I) are, respectively, 2.27×10^6 and 2.43×10^6 for Bl, 1.86×10^6 and 1.99×10^6 for La, 0.017 and 0.0186 for LC, 17.090 and 18.291 for LAC, 1.020 and 2.055 for AAC, 0.113 and 0.121 for WSN, 0.010 and 0.011 for TCA, 0.103 and 0.110 for PTA SN, 0.040 and 0.043 for FFA, 0.025 and 0.029 for F, 0.067 and 0.078 for Ct, 0.222 and 0.257 for Fl, and 1.921 and 2.223 for IH.

Based upon Equation [2] and the parameter estimates thereby calculated (Table 2), the most important variable exerting a negative effect on lactose metabolism and the concomitant production of lactic and acetic acids was the salt concentration in the cheese (x_2) in both linear and quadratic forms. Milk hydrolysate addition (x_3) and ripening time (x_4) in their quadratic forms had positive effects. The reduction in water activity effected by the increase in salt concentration accounts for the decreased cellular functions in lactic acid bacteria (41) and is a likely explanation for the effect of salt concentration on the acidification parameters (38, 43). This conclusion is supported by the observation that cheese with higher percentages of SDM exhibited slower lactose utilization and, consequently, lower rates of production of lactic and acetic acids. Other conditions that dictate the rate and extent of bacterial growth and activity in cheese include cheese pH and moisture content, which, as noted, are closely related to the salt concentration. The positive effect of the addition of milk hydrolysate may be explained by its positive effect, via its quadratic form (Table 2), on the viability of both *B. lactis* and *L. acidophilus* (10, 40). The reduced magnitude of the second-order interaction between milk hydrolysate addition and salt concentration (compared with the magnitude of the linear and quadratic effects of salt concentration alone) indicates that the milk hydrolysate reduced the deleterious effect of salt upon the metabolic activity of the strains.

To determine the loci and type of optima for the minimization of residual lactose content and the maximization of the concentration of organic acids, the appropriate value for y in Equation [2] was differentiated with respect to each independent variable at a time, and the corresponding four algebraic expressions were set to 0 (Table 3). No true optima (i.e., either maxima or minima determined from the sign of the second derivative with respect to all manipulated factors simultaneously) existed for any of the three acidification attributes. The loci found were saddle points, indicating that the global maxima (or minima) lie on experimental constraints. However, the type of optima in Table 3 indicates that a true local minimum for the lactose content and true local maxima for the concentration of lactic (and possibly of acetic) acids may be obtained when milk hydrolysate addition (x_3) and ripening time (x_4) are fixed at preset values. Following this rationale and using the equations listed in Table 3, if ripening time is prefixed at 70 d and milk hydrolysate addition is fixed at 0.30%, the optima generated corresponds to 0% (wt/wt), 1149 mg/100 g of cheese and 150 mg/100 g of cheese for the lactose content, lactic acid content, and acetic acid content, respectively (Table 5).

Microbiological Analysis

To obtain the consistent acid production discussed in the previous subsection, the starter bacteria should grow at an approximately steady rate within the cheese and provide suitable conditions in the curd for typical flavor development. Data on the growth of *B. lactis* and *L. acidophilus* in the different BL cheeses are shown in Table 6. Initial numbers of *B. lactis* and *L. acidophilus* inoculated into the milk were 3×10^7 cfu/g and 5 to 10×10^6 cfu/g, respectively, according to the initial experimental design. The growth of *B. lactis* was moderately significant in some of the experimental cheeses (e.g., BL 1 and BL 2 cheeses which reported an increase of 0.5 logarithmic cycle by 1 d of ripening). The addition of milk hydrolysate (Table 1) did not significantly affect the growth of *B. lactis* (data not shown) as might have been expected because peptides have been claimed to stimulate the growth of bifidobacteria (34, 39). Although the loss of milk hydrolysate during whey syneresis may have caused this effect, total solids concentrations of whey (data not shown) of those experimental cheeses with added milk hydrolysate (i.e., BL 2, BL 4 to BL 6, and BL 8 to BL 10 cheeses) were similar to those in whey of experimental cheeses without milk hydrolysate (i.e., BL 1, BL 3, and BL 7 cheeses). *Lactobacillus acidophilus* showed no significant growth in the cheese, although mean maximal bacterial counts (after having deducted an average concentration factor of 7× brought about by shrinkage of curd particles during whey expression for ca. 1.5 h) increased up to a factor of 2 within the first 48 h after cheese manufacture. This result was expected because *L. acidophilus* grows poorly in sterilized caprine milk (Gomes and Malcata, 1997, unpublished data).

A striking contrast was observed between the limited growth rates of *B. lactis* and *L. acidophilus* and the concurrent extensive production of organic acids. Lower viable numbers of either strain did not result in lower degrees of acidification within the cheese matrix during the early stages of ripening (Figure 1 and Table 6). The production of lactic and acetic acids persisted after maximum growth was achieved (24 to 48 h after cheese manufacture), which may reflect organic acid production uncoupled from growth. Similar observations have been reported for other bifidobacterial strains (10, 20).

As indicated earlier, it is important to maintain the viability of *B. lactis* and *L. acidophilus* at above 10^6 cfu/g of cheese (34, 40). Numbers of both species decreased gradually during ripening; *B. lactis* decreased by one to two logarithmic cycles to 0.75 to 10×10^7 cfu/g, and *L. acidophilus* decreased by one

logarithmic cycle to 0.6 to 3×10^7 cfu/g (Figure 2) within 70 d of ripening. Both strains showed a comparable degree of viability loss over the first 8-wk period, but the reduction in viability was more pronounced during the final 2 wk of ripening. Ripening time (x_4) exhibited an effect on survival of both strains (Table 2) in both linear form (largest effect of the four variables studied) and quadratic form

TABLE 3. Loci and type of optima for each normalized operating variable (starter inoculum, x_1; salt concentration in cheese, x_2; level of addition of milk hydrolysate, x_3; and ripening time, x_4) associated with the quadratic models fitted to the experimental data obtained for the microbiological factors (MF), the biochemical factors (BF), the sensory factors (SF), and the textural factors (TF).

Factor type	Quality factor[1]	Variable	Loci of optima	Type of optima
MF	*Bifidobacterium lactis* (cfu/g)	x_1	$-0.096 + 0.103x_2 + 0.082x_3 + 0.030x_4$	Minimum
		x_2	$0.106 + 0.298x_1 - 0.067x_3 + 0.120x_4$	Minimum
		x_3	$0.211 + 0.134x_1 - 0.038x_2 - 0.108x_4$	Minimum
		x_4	$-1.340 - 0.057x_1 - 0.079x_2 + 0.124x_3$	Maximum
MF	*Lactobacillus acidophilus* (cfu/g)	x_1	$0.060 + 0.002x_2 - 0.090x_3 - 0.020x_4$	Minimum
		x_2	$0.264 + 0.009x_1 + 0.996x_3 + 0.123x_4$	Minimum
		x_3	$-0.051 - 0.149x_1 + 0.337x_2 - 0.119x_4$	Minimum
		x_4	$-0.635 + 0.020x_1 - 0.025x_2 + 0.072x_3$	Maximum
MF	Lactose (wt/wt)	x_1	$-0.109 - 0.013x_2 + 0.693x_3 - 0.016x_4$	Minimum
		x_2	$-0.831 + 0.006x_1 - 0.018x_3 + 0.046x_4$	Minimum
		x_3	$0.031 - 0.932x_1 + 0.050x_2 - 0.020x_4$	Maximum
		x_4	$-0.725 + 0.011x_1 - 0.062x_2 - 0.010x_3$	Maximum
MF	Lactic acid (wt/wt)	x_1	$-0.146 + 0.082x_2 - 0.064x_3 - 0.116x_4$	Maximum
		x_2	$-0.129 + 0.045x_1 - 0.096x_3 - 0.089x_4$	Maximum
		x_3	$-0.512 + 0.070x_1 + 0.189x_2 - 0.356x_4$	Minimum
		x_4	$-0.534 + 0.064x_1 + 0.088x_2 - 0.180x_3$	Minimum
MF	Acetic acid (wt/wt)	x_1	$-0.283 + 0.043x_2 + 0.304x_3 - 0.126x_4$	Maximum
		x_2	$0.106 + 0.016x_1 + 0.016x_3 - 0.013x_4$	Maximum
		x_3	$-0.393 - 0.231x_1 - 0.032x_2 - 0.723x_4$	Minimum
		x_4	$-0.393 + 0.131x_1 + 0.036x_2 - 0.991x_3$	Minimum
BF	WSN (% of TN, wt/wt)	x_1	$0.146 + 0.220x_2 - 0.423x_3 - 0.141x_4$	Maximum
		x_2	$-0.229 + 0.104x_1 + 0.039x_3 - 0.253x_4$	Maximum
		x_3	$-1.408 + 1.658x_1 - 0.320x_2 - 1.082x_4$	Minimum
		x_4	$-0.526 + 0.028x_1 + 0.105x_2 - 0.054x_3$	Minimum
BF	TCA SN (% of TN, wt/wt)	x_1	$-0.395 + 0.140x_2 - 0.013x_3 - 0.367x_4$	Maximum
		x_2	$-0.216 + 0.102x_1 - 0.100x_3 - 0.200x_4$	Maximum
		x_3	$56.241 - 1.870x_1 - 18.870x_2 + 71.880x_4$	Maximum
		x_4	$-0.431 + 0.095x_1 + 0.071x_2 - 0.135x_3$	Minimum
BF	PTA SN (% of TN, wt/wt)	x_1	$-0.168 - 0.205x_2 + 0.057x_3 - 0.154x_4$	Maximum
		x_2	$0.148 - 0.192x_1 - 0.085x_3 - 0.089x_4$	Maximum
		x_3	$-2.137 - 0.249x_1 + 0.397x_2 - 1.799x_4$	Minimum
		x_4	$-0.460 + 0.062x_1 + 0.039x_2 - 0.168x_3$	Minimum
BF	FFA (mg of KOH/g of fat)	x_1	$0.061 - 0.001x_2 + 0.048x_3 + 0.002x_4$	Maximum
		x_2	$-0.0001 - 0.001x_1 - 0.040x_3 - 0.116x_4$	Maximum
		x_3	$-0.091 - 0.102x_1 + 0.074x_2 - 0.131x_4$	Minimum
		x_4	$-0.578 - 0.007x_1 + 0.336x_2 - 0.208x_3$	Minimum
SF	Firmness[2]	x_1	$-0.054 - 0.023x_2 - 0.068x_3$	Maximum
		x_2	$-0.463 - 0.132x_1 - 0.430x_3$	Maximum
		x_3	$0.404 - 0.089x_1 - 0.096x_2$	Maximum
SF	Consistency	x_1	$0.028 - 0.175x_2 + 0.055x_3$	Maximum
		x_2	$-0.173 - 0.080x_1 - 0.125x_3$	Maximum
		x_3	$0.097 + 0.030x_1 - 0.148x_2$	Maximum
SF	Flavor	x_1	$-0.017 - 0.107x_2 + 0.064x_3$	Minimum
		x_2	$-0.640 - 0.385x_1 - 0.694x_3$	Minimum
		x_3	$-0.187 + 0.108x_1 - 0.324x_2$	Minimum
TF	Hardness (N)	x_1	$0.467 + 0.271x_2 - 0.016x_3$	Minimum
		x_2	$69.735 - 27.449x_1 - 4.549x_3$	Maximum
		x_3	$-0.031 + 0.007x_1 - 0.020x_2$	Maximum

[1]WSN = Water SN, TN = total N, SN = soluble N, and PTA = phosphotungstic acid.

[2]Grade scales were used for firmness (1 = very soft to 7 = very firm), consistency (2 = very brittle to 6 = very smooth), and flavor (2 = very bad to 6 = very good).

TABLE 4. Chemical composition of the milk, of the different BL cheeses manufactured with *Bifidobacterium lactis* and *Lactobacillus acidophilus*, and of the reference (R) cheese after 70 d of ripening.

	Factor[2]					
Sample[1]	pH	Moisture	Fat	FDM	Protein	SDM	SIM
		(%)					
Milk	6.66	87.26	3.66	28.73	3.95	1.49	0.22
BL 1A	5.49	35.22	26.25	40.52	23.98	2.30	4.23
BL 1B	5.44	34.28	25.25	38.42	24.02	5.73	10.99
BL 2A	5.47	37.65	29.50	47.31	23.43	3.13	5.18
BL 2B	5.45	36.60	30.00	47.32	25.71	5.83	8.18
BL 3A	5.57	38.86	26.00	42.53	23.25	2.22	3.49
BL 3B	5.48	36.50	26.50	41.73	23.12	5.29	9.20
BL 4A	5.55	41.66	25.50	43.71	22.22	2.48	4.87
BL 4B	5.46	39.93	24.25	40.37	22.37	5.64	8.48
BL 5	5.57	43.24	25.50	44.93	23.92	4.15	6.69
BL 6	5.42	39.13	23.00	37.79	24.42	4.97	7.73
BL 7	5.54	39.74	21.25	35.26	25.20	4.17	6.32
BL 8	5.49	37.88	23.20	37.35	25.21	4.61	7.56
BL 9	5.35	36.43	21.50	33.82	25.33	4.32	9.95
BL 10A	5.42	37.82	27.00	43.42	25.13	2.51	4.13
BL 10B	5.33	36.84	25.50	40.37	24.31	5.60	9.60
R	5.41	43.03	19.40	34.05	21.66	4.32	5.72

[1]Mean value of the 10 batches (SEM = 0.11 for pH, 0.56% for moisture, 0.69% for fat, 0.72% for protein, and 0.05% for NaCl). BL5 = Mean value of the three replicates (SEM = 0.09 for pH, 0.61% for moisture, 0.00% for fat, 0.72% for protein, and 0.16% for NaCl). R = Mean value of the reference cheeses (SEM = 0.12 for pH, 3.37% for moisture, 0.35% for fat, 0.56% for protein, and 0.27% for NaCl).

[2]FDM = Fat in the DM, SDM = salt in the DM, and SIM = salt in the moisture phase.

(second to largest effect of the four variables studied). A positive relationship between the two strains is reflected by the increased survival of *B. lactis* throughout ripening when higher numbers of *L. acidophilus* exist (Figure 2a; e.g., BL 1A and BL 5) and justifies the significant effect of x_1 from the magnitude of its linear and quadratic forms (Table 2) on the viability of *B. lactis*. Previous studies (27) have shown the positive contribution of the proteolytic activity of *L. acidophilus* on the growth and maintenance of *B. lactis*. Nevertheless, the greater degree of reduction of total viable counts of *B. lactis* compared with that of *L. acidophilus* (Figure 2, for all cheeses except BL 1A) suggests a competition between the two strains for nutrients and energy sources within the cheese when maximum densities had been achieved. In another study (Gomes and Malcata, 1997, unpublished data) on the effects of controlled environmental conditions on survival of both pure and mixed cultures of these strains in milk, the mechanistic model produced to fit the experimental data predicted a greater loss of viability of *B. lactis* in a mixed culture with *L. acidophilus* than in an independent culture, regardless of experimental conditions. The model predicted a modest dependency of survival of *B. lactis* upon salt concentration in a coculture environment, which may account for the significant negative interaction of *L. acidophilus* (x_1) with salt concentration (x_2) as related to viable numbers of *B. lactis* (Figure 2; e.g., BL 1A compared with BL 1B). As discussed previously, a high salt concentration should inhibit the activity and viability of the starter organisms, which agrees with the experimental data illustrated in Figure 2. In a few experiments, lower bacterial counts of both strains in the high salt cheeses than in their low salt counterparts were observed independently of their initial numbers; other experiments contradict this trend (e.g., BL 3A compared with BL 3B, and BL 4A compared with BL 4B for *B. lactis*; and BL 2A compared with BL 2B and BL 3A compared with BL 3B for *L. acidophilus*). Consequently, the statistical analysis indicates a negative linear effect of salt concentration (x_2) on the viability of *B. lactis* and a linear positive effect of salt concentration on the viability of *L. acidophilus* that is not significant ($P < 0.05$) (Table 2) even though quadratic effects of salt concentration on the viability of both strains were positive. Unlike published results for Gouda cheese (16), higher salt contents did not seem to affect the viability of *L. acidophilus* adversely. These apparent contradictions for the effect of salt may be because variables found to be negligible in a linear model may be significant in a quadratic model, and the entire model should be considered

rather than the linear, quadratic, or interaction factors independently. Positive quadratic effects are, in fact, balanced by negative interaction effects (Table 2). The response surface methodology applied herein is a powerful tool to grasp the performance of several factors from a small number of observations, but the results need to be interpreted with caution in view of the limited range of validity and the empirical nature of the underlying model. The significant positive effect of milk hydrolysate addition on the survival of *B. lactis* and *L. acidophilus*, as observed previously in a Gouda-type cheese (16), indicates the beneficial effect of the milk hydrolysate on the strains. Other studies (39) have also claimed that the growth of bifidobacteria in milk can be enhanced by the addition of bifidogenic factors such as protein hydrolysates.

Table 3 indicates that no true global optimum exists for the maximization of survival of *B. lactis* and *L. acidophilus*. Restrictions imposed on the inoculum level of *L. acidophilus* (x_1), on salt concentration (x_2), and on level of milk hydrolysate (x_3) were necessary before the desired local optimum could be found (Table 5). However, the local maximum on the initial day for *B. lactis* and at 13 d for *L. acidophilus* derived from the best combination of the prefixed variables was beyond reasonable limits, so a restriction of 70 d was imposed on ripening time. Because our main objective was to optimize the viability of both species without impairing the good quality characteristics of the final cheese, taking into account the effects on acid production that were previously discussed, the highest addition of milk hydrolysate (0.30%) together with the lowest salt concentration (2.00%) and the highest addition of *L. acidophilus* (1×10^7 cfu/g) by 70 d of ripening appeared to be the best choice. Although the lowest inoculum, 5×10^6 cfu/g, would achieve viable numbers above 10^6/g of cheese, the positive correlation between inoculum size of *L. acidophilus* and degree of survival of *B. lactis* plus the desirability of shelf-life extension favor the higher inoculum level for *L. acidophilus*.

TABLE 5. Loci of optima and their calculated values (MM) for the microbiological factors (MF), the biochemical factors (BF), the sensory factors (SF), and the textural factors (TF) associated with the operating variables starter inoculum (I), salt concentration in the cheese DM (SDM), level of addition of milk hydrolysate (H), and ripening time (t) for all quality factors.[1,2]

Type of analysis	Parameter	Loci of optima				Estimates		
		I	SDM	H	t	MM	GO	R
		(cfu/g)	(%, wt/wt)	(%)	(d)			
MF	Bl	1.00×10^7*	2.00*	0.15*	70*	1.01×10^7	1.89×10^7	NA[3]
		1.00×10^7*	2.00*	0.30*	70*	3.10×10^7		
MF	La	5.00×10^7*	4.00*	0.15*	70*	1.25×10^7	1.45×10^7	NA
		1.00×10^7*	2.00*	0.30*	70*	2.82×10^7		
MF	LC	7.20×10^6	4.01	0.15*	70*	0	0	0.20
		8.90×10^6	4.01	0.30*	70*	0		
MF	LAC	6.80×10^6	3.54	0.15*	70*	851	1146	1158
		6.62×10^6	3.34	0.30*	70*	1149		
MF	AAC	6.50×10^6	3.75	0.15*	70*	112	150	37
		7.23×10^6	3.79	0.30*	70*	150		
BF	WSN	6.49×10^6	2.95	0.15*	70*	16.84	18.41	18.89
		5.43×10^6	2.94	0.30*	70*	18.92		
BF	TCA SN	1.52×10^7	31.90	0.00	70*	2.41	9.47	8.88
BF	PTA SN	6.84×10^6	4.04	0.30*	70*	4.28	4.25	3.31
BF	FFA	7.66×10^6	3.77	0.15*	70*	7.93	8.97	5.93
		7.78×10^6	3.69	0.30*	70*	9.16		
SF	Fl	7.32×10^6	2.69	0.22	NF[2]	4.41	4.37	4.31
SF	Ct	7.67×10^6	3.61	0.17	NF	5.32	5.28	4.30
SF	F	7.65×10^6	2.61	0.16	NF	4.19	4.32	3.53
TF	IH	7.95×10^6	2.00*	0.15*	70*	36.71	24.49	34.62
		7.99×10^6	2.00*	0.30*	70*	11.17		

[1]AAC = Acetic acid concentration (milligrams per 100 g of cheese), Bl = viable numbers of *Bifidobacterium lactis* (colony-forming units per gram), Ct = cheese consistency, FFA = FFA concentration (milligrams of KOH per gram of fat), F = cheese sensory firmness, Fl = cheese flavor, IH = instrumental hardness, La = viable numbers of *Lactobacillus acidophilus* (colony-forming units per gram), LAC = lactic acid concentration (milligrams per 100 g of cheese), LC = lactose content (percentage, wt/wt), PTA SN = phosphotungstic acid-soluble N (percentage of total N), TCA SN = TCA-soluble N (percentage of total N), and WSN = water-soluble N.

[2]Estimated values (GO) were obtained with $I = 3 \times 10^7$ cfu/g of *Bifidobacterium lactis* and 7×10^6 cfu/g of *Lactobacillus acidophilus*, SDM = 3.50% (wt/wt), H = 0.30% (vol/wt), and t = 70 d; experimental data were obtained for the reference R cheese by 70 d of ripening.

[3]NA = Not applied; NF = operating variable was not fitted to the corresponding quadratic model.

*Prefixed operating variable, used as constraint in the estimation of the local maximum or minimum.

TABLE 6. Changes in the numbers of viable *Bifidobacterium lactis* and *Lactobacillus acidophilus* in the different BL goat cheeses manufactured with *B. lactis* and *L. acidophilus* during manufacture (0.5, 1.5, 24, and 48 h) and at an early stage of ripening (15 d).

Cheese type	*Bifidobacterium lactis*					*Lactobacillus acidophilus*				
	0.5 h	1.5 h	24 h	48 h	15 d	0.5 h	1.5 h	24 h	48 h	15 d
	($\times 10^7$ cfu/ml)									
BL 1A	4.54	12.4	38.5	38.0	30.0	0.95	2.00	6.35	5.80	4.25
BL 1B	4.54	12.4	25.5	24.0	20.0	0.95	2.00	4.25	3.80	3.90
BL 2A	2.41	26.3	27.1	25.4	22.5	1.09	5.92	9.00	7.00	6.00
BL 2B	2.41	26.3	19.5	10.9	10.5	1.09	5.92	4.15	3.83	3.01
BL 3A	2.67	10.5	26.3	12.0	10.1	0.55	5.10	9.95	5.75	2.45
BL 3B	2.67	10.5	26.3	9.10	9.05	0.55	5.10	5.95	8.59	2.45
BL 4A	4.00	12.4	27.5	20.8	18.0	0.63	6.63	9.34	10.4	4.85
BL 4B	4.00	12.4	21.9	15.5	12.6	0.63	6.63	8.80	9.87	4.45
BL 5	4.05	19.2	25.2	23.6	20.2	0.71	3.00	6.35	5.30	3.35

Proteolysis

Proteolysis in the BL and R cheeses over a 70-d ripening period are depicted in Figure 3. The ratio of WSN to TN in the BL cheeses (Figure 3a) increased progressively throughout the 70-d ripening period. No significant differences (evaluated from Student's *t*-tests applied to the sample means at $P < 0.05$) in concentrations of WSN could be detected between the R cheese (mesophilic starter) and the BL cheeses (thermophilic starter) for comparable microbial populations and with comparable cheese composition. These results confirm that primary proteolysis is governed mainly by the activity of chymosin, of plasmin, or of both (45). If the higher pH at brining (38) and the higher cooking temperature (8) of the BL cheeses than those of the R cheese are taken into account in those cheeses with salt concentrations up to ca. 4% SDM, then plasmin probably was as important as chymosin. However, the values for WSN do not provide such information because of the water insolubility of the γ-caseins, the major plasmin-mediated hydrolytic products of β-CN. Further studies are required on the fractionation and characterization of the specific casein fractions to confirm this presumption.

Although the increase in the ratio of WSN to TN (a measure of primary proteolysis) was not strongly dependent on the starter used (BL cheese vs. R cheese), the reverse situation occurred for the ratios of TCA SN to TN and of PTA SN to TN; ratios were higher in cheese made with thermophilic strains than in those made with mesophilic strains (Figure 3c). During the 70 d of ripening, the amount of PTA SN increased progressively, but the increase was more intense after the 15-d initial stage. Apart from some exceptions in the BL cheeses (i.e., those corresponding to higher salt concentrations), an increase of the ratio of PTA SN to WSN was observed as ripening proceeded (data not shown). Results for the R cheese,

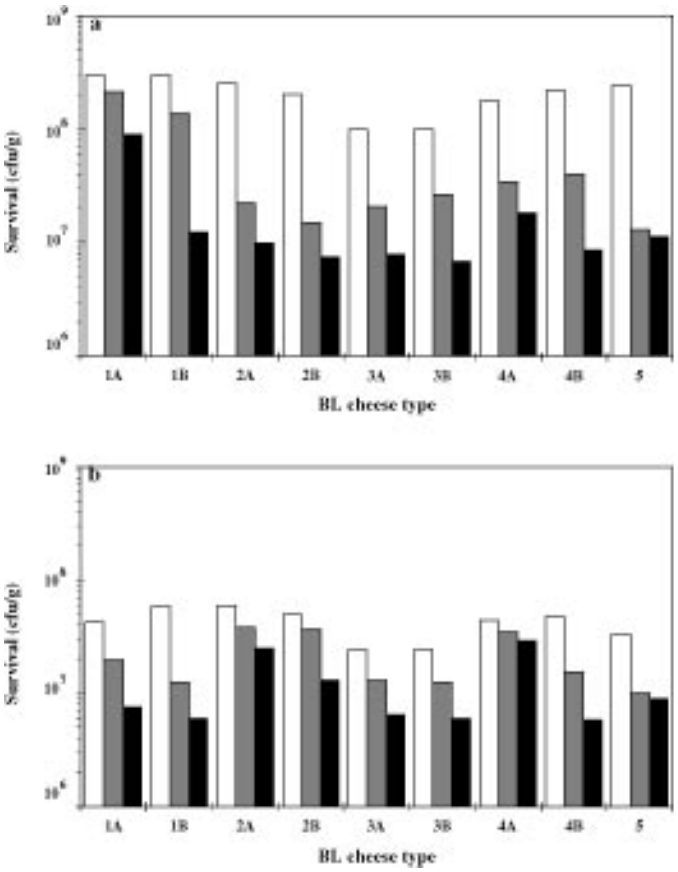

Figure 2. Mean survival rate of a) *Bifidobacterium lactis* and b) *Lactobacillus acidophilus* in the BL cheeses manufactured with *B. lactis* and *L. acidophilus* after 7 d (open bar), 35 d (patterned bar), and 70 d (solid bar) of ripening at 6°C and 93% relative humidity. Sample designations are given in Table 1.

however, do not show that trend; a slight decrease occurred. These variations in behavior between the BL cheeses and the R cheese, presumably from more extensive generation of small peptides in the BL cheeses, reflect the enhanced activity (in terms of conversion of the primary proteolysis products) of peptidases, which become accessible upon limited lysis of the probiotic starter. *Lactobacillus acidophilus* is proteolytic and capable of releasing small peptides (proteinase of 145 kDa) and amino acids (X-prolyl-dipeptidyl-aminopeptidase) (14), but its relative contribution to cheese has not yet been studied in detail. Similarly, knowledge of the function of bifidobacteria during ripening is limited, even though proteolysis has been detected in milk cultures of *B. bifidum* with concomitant release of free amino acids (9). Minagawa et al. (32) described the exopeptidase system of several *Bifidobacterium* strains and demonstrated the presence of aminopeptidase, iminopeptidase, and carboxypeptidase activities, and El Soda et al. (12) reported casein hydrolytic activity among *Bifidobacterium* spp. Further studies are required to assay the qualitative and quantitative nature of individual free amino acids in the BL cheeses to get better insight into the specific contributions of the starters to the proteolytic pattern in the cheese.

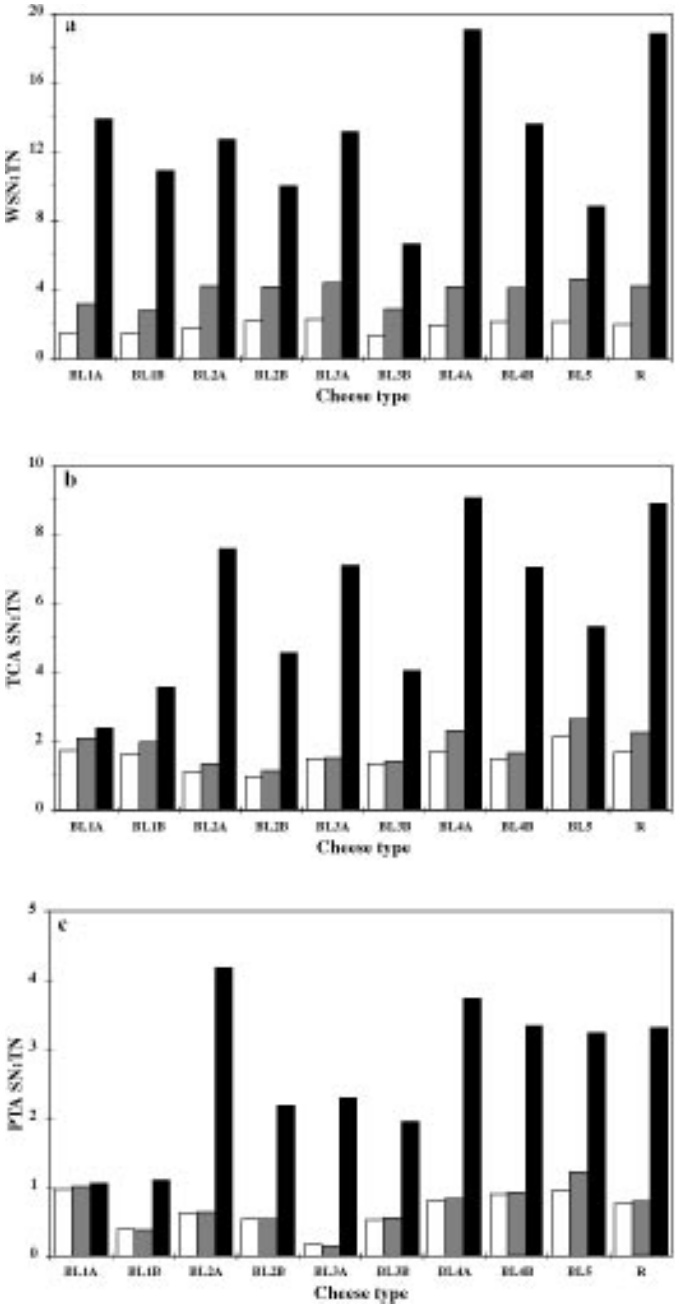

Figure 3. Concentrations (expressed as percentage of total N) of a) water-soluble N (WSN), b) TCA-soluble N (SN), and c) phosphotungstic acid (PTA) SN after 1 d (open bar), 15 d (patterned bar), and 70 d (solid bar) of ripening at 6°C and 93% relative humidity of the BL cheeses manufactured with *Bifidobacterium lactis* and *Lactobacillus acidophilus* and of the R cheese. Sample designations are given in Table 1.

The contribution of *L. acidophilus* and *B. lactis* to casein degradation in cheese was largely determined, in descending order of significance, positively by the ripening time (x_4), negatively by the salt content (x_2), and positively by the milk hydrolysate addition (x_3) (Table 2). Although at lower salt contents a considerable amount of casein was degraded, the opposite appeared to occur at the highest salt level, and this trend became particularly evident as ripening progressed. Moreover, the overall levels of WSN, TCA SN, and PTA SN produced in the highly salted cheeses were lower after the first 2 wk of ripening, probably because of increasing values for SDM and concomitant decreasing values for moisture. Similar results were reported for Cheddar (43) and Gouda (13) cheeses. The less significant negative effect by the SDM on the ratio of PTA SN to TN than on the ratios of WSN to TN and TCA SN to TN (Table 2) is consistent with results obtained by Delacroix-Buchet and Trossat (8). The positive contribution of milk hydrolysate (x_3), via both the linear and quadratic forms, on the development of WSN could have resulted from the presence of higher concentrations of water-soluble peptides. The relationships of the ratios of TCA SN to TN and PTA SN to TN with respect to the effects of milk hydrolysate addition were relatively ill-defined because of the large standard errors associated with those ratios.

Data in Table 3 demonstrate the lack of a true global maximum for all proteolytic parameters considered together. Hence, the possible location of local

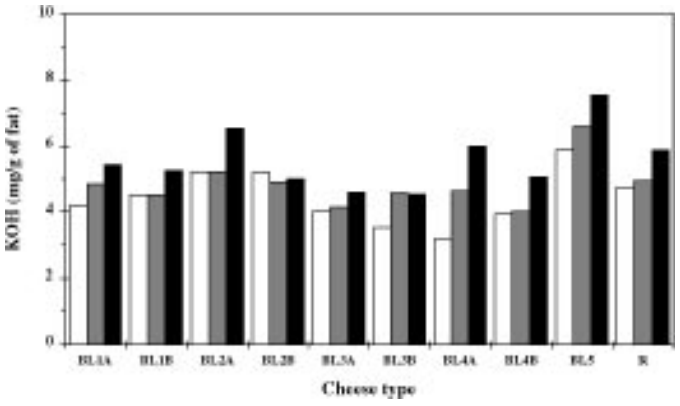

Figure 4. Free fatty acid contents after 1 d (open bar), 15 d (patterned bar), and 70 d (solid bar) of ripening at 6°C and 93% relative humidity in each of the BL cheeses manufactured with *Bifidobacterium lactis* and *Lactobacillus acidophilus*. Sample designations are given in Table 1.

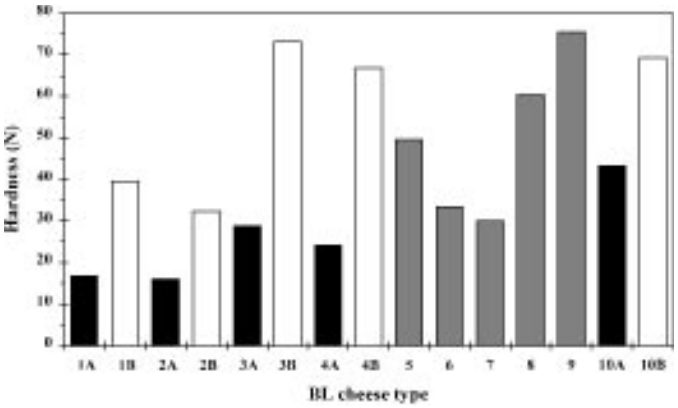

Figure 5. Mean values for hardness in the various BL cheeses manufactured with *Bifidobacterium lactis* and *Lactobacillus acidophilus* with low (solid bar), medium (patterned bar), and high (open bar) salt concentrations by 70 d of ripening. Sample designations are given in Table 1.

maxima has been determined by presetting the milk hydrolysate addition at either 0.15 or 0.30% (positive contribution from its addition) and the ripening time at 70 d (upper limit of our experimental ripening period). All local maxima for the proteolytic parameters associated with the different combinations of such manipulated variables at the preset values were within the experimental range selected except for the ratio of TCA SN to TN (Table 5). The location of such a local maximum is beyond the technological feasibility of the calculated salt level (31.9%), so restrictions to the uppermost limit that is permissible for a good quality cheese would have to be used. Because proper flavor development is essential in cheese making and because such a factor is closely related to the concentration of free amino acids in cheese (which, in turn, results from the action of starter bacteria), a compromise is suggested; a slight loss in the extent of proteolysis from the increase in salt concentration (ca. 4%) does not cause a significant loss in depth of proteolysis in the presence of 0.30% milk hydrolysate (Table 5).

Lipolysis

Concentrations of FFA, measured as the fat acidity index, increased slightly throughout the ripening period in most BL cheeses (Figure 4). The FFA in many of these cheeses were within or above the ranges reported for the R cheese of 6.2 to 6.7 mg of KOH/g of fat. The rate of lipolysis decreased as salt concentration increased beyond the 4% SDM level.

Because the correlation of fat acidity and acetic acid concentration was not significant ($P < 0.01$), the relatively small magnitude of the fat acidity indices reflects a low degree of lipolysis rather than glycolysis in the cheeses. This observation confirms the findings of various researchers (3, 31, 44) for cheeses manufactured with pasteurized caprine milk. The contribution of milk lipase in the present study is unlikely because lipase is only active in cheeses manufactured from raw milk (24). The weak lipolysis may be ascribed to the starter bacteria, which generally have very limited lipolytic activity (7). The contribution of *B. lactis* and *L. acidophilus* to lipolysis has not been studied extensively to date, which precludes conclusions about their impact in this study. However, production of esterases and lipases by *L. acidophilus* was reported by El Soda et al. (11), who showed that selected strains were active on *p*-nitrophenyl derivatives of fatty acids up to C_5. The release of FFA in the BL cheeses may have been accentuated by their high numbers in the cheese.

Salt concentration influenced lipolysis considerably, as is apparent in the low and high salt experimental cheeses (Figure 4). The optimal NaCl concentration for lipolysis was 3.50 to 4.00% SDM; BL 5 to BL 9 cheeses with ca. 4.00% SDM had fat acidity indices above 8.0 mg of KOH/g of fat. This observation, which suggests a stimulatory effect of salt concentration upon lipolytic activity, agrees with the results of Godinho and Fox (15) and Nájera et al. (36). Godinho and Fox (15) concluded that the rate of lipolysis was at maximum at 4 to 6% salt in Blue cheese.

Similar to the analyses of proteolysis, no global maximum was obtained for the FFA content when all four significant ($P < 0.05$, see Table 2) operating

variables were considered simultaneously (Table 3). When the milk hydrolysate addition (x_3) and the ripening time (x_4) were preset at either 0.15 or 0.30% and at 70 d, respectively, the local maxima calculated for both such combinations (0.15% and 70 d or 0.30% and 70 d) were analytically and technologically identical (Table 5). These results suggest a close relationship between the level of starter inoculum and salt concentration and a more important role of these variables than any of the other technological variables in defining maximum values for FFA.

Textural Analysis

The effects of starter inoculum (x_1), salt content (x_2), and milk hydrolysate addition (x_3) on the hardness of the BL cheeses are depicted in Figure 5, and the parameter estimates are tabulated in Table 2. The hardness of the BL cheeses was positively related to salt content via its linear form (Table 2), which suggests that the decrease in the volume of the protein particles in the presence of a high salt concentration increases the hardness of the casein matrix (30). Alternatively, the decreased proteolysis that is associated with the highly salted cheeses (as noted previously) results in a more intact protein network that is more solid-like in character and increases hardness. Because water acts as a lubricant in cheese, a decrease in moisture content (or, equivalently, an increase in salt content) would reduce the hydration of protein and restrict freedom of movement, thereby decreasing the viscous properties and the deformability of the cheese. These results correlate with the chalky and brittle appearance of highly salted cheeses and the smoother appearance of low salt cheeses. The strong dependence of cheese hardness upon salt content has been reported for Mozzarella cheese (35); Mozzarella cheeses brined for a longer period were harder and more brittle. For Gouda cheese (30), high salt contents were associated with higher deformation force at 20% compression as well as lower strain at fracture. For Cheddar-like goat cheese (2), increases in the salt in the moisture phase increased cheese firmness.

Although salt content seems to have a pronounced effect on cheese hardness, other factors (not included in the statistical analysis performed in this study), such as fat, protein, and the ratio of water to protein, are likely to influence the texture of the cheese. There are several examples of these effects. Although cheeses BL 8 and BL 10A had similar moisture contents and similar ratios of water to protein, their salt contents were different (Table 4), which resulted in different hardness (Figure 5) and may reflect the effects of salt on the casein matrix (2). In addition, cheese BL 2B and cheeses BL 3B, BL 4B, and BL 10B had similar salt contents but different percentages of fat in DM and different ratios of water to protein (Table 4), which resulted in different degrees of hardness (Figure 5). Cheese hardness was shown to be affected negatively by the starter inoculum (via its quadratic effect) and positively by the addition of milk hydrolysate (Table 2). The addition of a milk hydrolysate has a modulating effect on cheese hardness, which is due in part to its effect on proteolysis.

No true overall local minimum can be found for cheese hardness (Table 3); however, if salt concentration is preset at 2.00% (wt/wt) and milk hydrolysate at either 0.15 or 0.30% addition, minima are obtained at similar levels of starter inoculum (Table 5). Because the hardness of cheese had a marked effect on its quality and acceptability, the addition of 0.30% milk hydrolysate appears to be preferable because it matches the lowest calculated cheese hardness (threefold decrease in values).

Sensory Analysis

Differences ($P < 0.001$) were observed among the BL cheeses in firmness, consistency, and flavor. Flavor assessment varied considerably among panelists ($P = 0.008$).

Most BL cheeses were firmer than the R cheese, and the firmest cheese was BL 2B. Firmness varied between 4.2 and 6.8, and consistency varied between 4.1 and 5.9 for all 17 BL cheeses; for R cheese, firmness was 3.5, and consistency was 5.2. The lack of relationship between firmness and instrumental hardness of some of the BL cheeses, namely, the BL 2B cheese, is difficult to understand because of the positive correlation that is generally observed between these two quality factors. The high fat in the DM of BL 2B cheese may at least partially account for this contradiction. Alternatively, the strain rates imposed during instrumental compression of the cheese samples may not approximate the complex spectrum of strain rates imposed by teeth during eating, which would weaken the correlation between rheological measurement and consumer perception.

Some of the BL cheeses were preferred over the conventional R cheese. Addition of milk hydrolysate (x_3) and level of starter inoculum (x_1), via their quadratic forms, were significant variables relative to flavor (Table 2). Unlike results pertaining to other cheese types (16), the addition of milk hydrolysate did not impair texture or flavor in cheese. Improved

flavor (flavor scores varied between 3.8 to 6.0 for all 17 BL cheeses and was 4.1 for the R cheese) appears to be related to the higher contents of PTA SN (38) or free amino acids arising from the starter proteolytic activity. Higher contents of PTA SN in cheese may have also increased the intensity of background taste (29). Salt content (x_2) did not appear to significantly affect flavor, but the overall preference data indicated that cheeses with a final higher salt content of >4.50% SDM were most preferred. Low salt contents (<4.50% SDM) were related to softer, more acidic, and more bitter cheeses (42).

The type of optima found and their location for each quality parameter were within experimental limits (except for the ratio of TCA SN to TN). However, the local optima do not coincide for all quality parameters. Consequently, a good choice for the manufacture of a probiotic cheese from caprine milk would be to set the milk hydrolysate addition at 0.30%, the ripening time to 70 d, the starter inoculum to 3×10^7 cfu/g of *B. lactis* and 7×10^6 cfu/g of *L. acidophilus* and the salt concentration to 3.50% SDM (wt/wt) (Table 5). Although the selection of 70 d as the optimum ripening time may be questioned because no studies were performed at longer ripening times, the normal ripening time of Queijo de Cabra (R cheese) is only 28 to 42 d.

The suggested global optimum is located in the vicinity of the center point of the experiment design in this study with respect to the most important operating variables, starter inoculum and salt concentration, and is relatively similar to conditions employed in the manufacture of the R cheese. Moreover, the manufacture of such a goat cheese under these processing conditions yields a cheese with characteristics that partially overlap those of Queijo de Cabra but that exhibit higher scores for taste, texture, and nutritional value as concluded based on the values tabulated in Table 5.

Statistical Analysis

Because of the nested nature of variable ripening time in the factorial layout encompassing the remaining three processing variables, the error term for testing the ripening time might be different from that for testing the other variables. However, when the three- and four-level interaction terms were split into two groups (those that include ripening time and those that do not), the mean square of the group that included ripening time was not much smaller than the other mean square, and, therefore, direct utilization of results from the whole data set for estimation of all parameters was in order.

The ripening time has two levels, cheese immediately after manufacture (0 d), and cheese by the end of ripening (70 d). The 15-d level (and not 35 d, as would be more logical for a true center point) is the other design point, which was so fixed because of the technological requirements of this type of cheese. Although statistical analyses were performed based only on those data arising from the experimental design (e.g., BL 1A cheese only has data for 0 and 70 d, and BL 5 cheese has data only for 15 d), all cheese samples assayed were considered as the basis for as wide as possible a discussion in microbiological, physicochemical, and biochemical terms. The use of 7.5×10^6 as the center point for *L. acidophilus*, which is the arithmetic mean of 5×10^6 and 1×10^7 cfu/g and not the logarithmic mean, results from cell viability data being expressed in that scale. There was no statistically apparent reason to question the normal distribution of the microbiological data in that form.

CONCLUSIONS

The results of the present study showed that the starter composed of *B. lactis* and *L. acidophilus* could be used for the successful manufacture of a goat cheese with good flavor and texture characteristics. Survival of the probiotic species was dependent on the physicochemical characteristics of the cheese, but final numbers were still above the recommended threshold for a probiotic effect.

Statistical analyses indicated that the local maxima (or minima) did not coincide for all quality factors simultaneously; a reasonable compromise that ensures a cheese with good taste, texture, and nutritional value would be to set the addition of milk hydrolysate at 0.30%, the ripening time at 70 d, the starter inoculum at 3×10^7 cfu/g *B. lactis* and 7×10^6 cfu/g *L. acidophilus*, and the salt concentration at 3.50% SDM (wt/wt).

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