Proteolysis of Hispánico Cheese Manufactured Using Lacticin 481-Producing *Lactococcus lactis* ssp. *lactis* INIA 639

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ABSTRACT

Hispánico cheese was manufactured using lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, or a combination of both strains, as starter cultures. *Lactobacillus helveticus* LH 92, a culture of high aminopeptidase activity sensitive to lacticin 481, was added to all vats. Milk inoculation with the bacteriocin producer promoted early lysis of *Lb. helveticus* cells in cheese. Cell-free aminopeptidase activity in cheese made with the 3 lactic cultures was 1.8 times the level reached in cheese made only with *L. lactis* strain INIA 437 and *Lb. helveticus*, after 15 d of ripening. Proteolysis (as estimated by the *o*-phthaldialdehyde method) in cheese made with the 3 lactic cultures was twice as high, and the level of total free amino acids 2.4 times the level found in cheese made only with *L. lactis* strain INIA 437 and *Lb. helveticus*, after 25 d of ripening. Hydrophobic and hydrophilic peptides and their ratio were at the lowest levels in cheese made with the 3 lactic cultures, which received the lowest scores for bitterness and the highest scores for taste quality.

Key words: lacticin 481, *Lactobacillus helveticus*, proteolysis, Hispánico cheese

INTRODUCTION

Lactic acid bacteria (LAB) are an important source of enzymes such as proteinases, peptidases, amino acid catabolic enzymes and esterases, which transform milk constituents retained in the curd into low molecular weight compounds (Fox et al., 1996). Because most enzymes produced by LAB are intracellular, cell lysis will favor the access of enzymes to their substrates and presumably will accelerate cheese ripening (Garde et al., 1997; Morgan et al., 1997).

Lysis of LAB cells during early ripening may be enhanced by milk inoculation with bacteriocin-producing

(BP) adjunct cultures. Thus, *Lactococcus lactis* ssp. *lactis* DPC3286, a producer of lactococcins A, B, and M (Morgan et al., 1995), had a bacteriolytic effect on sensitive bacteriocin-nonproducing (BNP) lactococci, increased concentrations of free amino acids, and reduced bitterness in 6-mo-old cheese (Morgan et al., 1997). The combination of an enterocin-producing adjunct culture with a BNP mixed-strain starter culture in the manufacture of a semihard cheese accelerated starter cell lysis and flavor development from d 15 (Garde et al., 1997). Moreover, the levels of nonprotein and amino nitrogen increased significantly in 18- to 42-d-old cheeses when a lacticin 3147-producing *L. lactis* strain was used as starter culture (Martínez-Cuesta et al., 2001). Milk inoculation with *L. lactis* ssp. *lactis* INIA 415, a producer of nisin Z and lacticin 481, resulted in accelerated starter cell lysis in Hispánico cheese, which showed higher levels of proteolysis, aminopeptidase activity, free amino acids, and some odor-active volatile compounds, and increased flavor intensity from d 25 of ripening, compared with control cheese (Garde et al., 2002a,b).

The use of highly peptidolytic strains as adjunct cultures in cheese manufacture is another approach for the acceleration of proteolysis during ripening. Strains from some *Lactobacillus* species, normal constituents of thermophilic starters for varieties such as Parmesan, Mozzarella, and Swiss-type cheeses, present a wide range of peptidolytic enzymes, which can influence flavor development. Comparisons between *Lb. helveticus* and other LAB species have demonstrated that *Lb. helveticus* strains possess, by far, the highest aminopeptidase and dipeptidase activity levels (Hickey et al., 1983; Sasaki et al., 1995). The results obtained by inoculating milk with highly autolytic *Lb. helveticus* strains as adjunct cultures for the manufacture of Cheddar cheese ripened for 8 mo (Hanon et al., 2003) and Swiss cheese ripened for 3 mo (Valence et al., 2000) suggest a correlation of adjunct lysis with increased proteolysis and accelerated flavor development.

Addition of a *Lb. casei* strain and a nisin-producing culture in the manufacture of Cheddar cheese, monitored over a 6-mo ripening period, produced a debittering effect and improved flavor quality (Benech et al.,

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2003). Furthermore, a nisin-producing *L. lactis* ssp. *lactis* strain, combined with a highly autolytic and proteolytic *Lb. delbrueckii* ssp. *bulgaricus* strain and with 2 BNP *L. lactis* strains, increased the levels of water-soluble nitrogen, free amino acids, free fatty acids, and flavor in Cheddar cheese ripened for 6 mo with respect to cheese made without BP (Sallami et al., 2004a,b).

In the present work, *L. lactis* ssp. *lactis* INIA 639, a lacticin 481-producing strain, was used in the manufacture of Hispánico cheese together with *L. lactis* ssp. *lactis* INIA 437, a BNP strain, and *Lb. helveticus* LH 92, a culture with high aminopeptidase activity sensitive to lacticin 481 (Ávila et al., 2005b), with the aim of accelerating cheese ripening. The effect of milk inoculation with the BP culture on proteolysis, texture, and taste of Hispánico cheese are reported herein.

MATERIALS AND METHODS

Lactic Cultures and Cheese Manufacture

Lactococcus lactis ssp. *lactis* INIA 639, a lacticin 481-producer from the culture collection of INIA (Madrid, Spain), was used as the BP strain. A BNP mutant of the lacticin 481-producer with acid production, proteolytic activity, and volatile compound profile in curd close to those of the parental strain could not be obtained. Therefore, *L. lactis* ssp. *lactis* INIA 437, a BNP strain with technological characteristics similar to those of the BP, was selected for use in cheese-making experiments. Both strains were kept at -80°C in de Man, Rogosa, and Sharpe broth (Biolife Italiana, Milano, Italy) with 15% glycerol, and grown twice in reconstituted skim milk at 25°C for 16 h before use in cheese manufacture. *Lactobacillus helveticus* LH 92 culture, kindly provided by Rhodia Iberia (Madrid, Spain), was kept at -80°C and grown in reconstituted skim milk at 37°C for 10 h before use in cheese manufacture.

Hispánico cheese, a semihard Spanish variety, was manufactured from pasteurized cows' milk as described by Gómez et al. (1997). Experiments were carried out in duplicate on different days, at the Food Technology Department of INIA. Each experiment consisted of three 50-L vats; *L. lactis* ssp. *lactis* INIA 437 culture, with counts of 1.3×10^9 cfu/mL, was added at 5 mL/L to vat 1 and at 2.5 mL/L to vat 2, resulting in 6.5×10^6 and 3.3×10^6 cfu/mL in inoculated milk, respectively. *Lactococcus lactis* ssp. *lactis* INIA 639 culture, with counts of 8.4×10^8 cfu/mL, was added at 2.5 mL/L to vat 2 and at 5 mL/L to vat 3, resulting in 2.1×10^6 and 4.2×10^6 cfu/mL in inoculated milk, respectively. *Lactobacillus helveticus* LH 92 culture, with counts of 8.1×10^8 cfu/mL, was added at 5 mL/L to all vats, resulting in 4.1×10^6 cfu/mL in inoculated milk. Rennet (6 mL of Maxiren, 1:15,000 strength, Gist Brocades,

Delft, The Netherlands) was added to milk 30 min after lactic culture inoculation. The curds were cut 40 min later into 6- to 8-mm cubes and scalded at 37°C for 15 min. Whey was drained off and curds were distributed into cylindrical molds. Three cheeses, approximately 2 kg each, were obtained from each vat. Cheeses were pressed overnight at 20°C and 1.5 kg/cm^2 pressure, salted at 12°C for 24 h in brine (160 g of NaCl/L), and ripened at 12°C and 85% relative humidity for 50 d. Cheeses were coated on d 7 with 2 layers of polyvinyl acetate containing pimaricine.

To exclude an antagonistic effect of the BNP strain on *Lb. helveticus* LH 92, cheeses were made from milk inoculated with 5 mL/L *Lb. helveticus* LH 92 and 5 mL/L of the BNP strain, and from milk inoculated only with 5 mL/L *Lb. helveticus* LH 92, following the above indicated manufacturing procedures, and ripened at 12°C and 85% relative humidity for 15 d. Also, to ascertain the contribution of the BNP and BP strains to aminopeptidase levels and proteolysis values in cheese, cheeses were made from milk inoculated with 5 mL/L of the BNP strain and 5 mL/L of the BP strain, and from milk inoculated only with 5 mL/L of the BNP strain, following the above indicated manufacturing procedures, and ripened at 12°C and 85% relative humidity for 15 d.

Microbiological Analysis

Viable counts of lactococci were determined in duplicate on M17 agar (already containing 5.0 g/L lactose, Biolife Italiana) using a spiral plater (Interscience, Saint-Nom-La-Bretèche, France), after aerobic incubation at 30°C for 48 h. Bacteriocin-producing lactococci were determined on the surface of double-layer M17 agar plates, with the lower layer inoculated with 1 mL/L of a 16-h culture of *L. lactis* ssp. *cremoris* HP as the indicator microorganism; colonies forming a zone of growth inhibition in the lower layer were considered to be *L. lactis* ssp. *lactis* INIA 639. *Lactobacillus helveticus* counts were determined on de Man, Rogosa, and Sharpe agar plates incubated anaerobically for 48 h at 44°C .

Bacteriocin and Aminopeptidase Activities

For the determination of bacteriocin activity, cheese samples held at -40°C were thawed, and 5-g samples were homogenized in a Stomacher 400 (Seward Laboratory, London, UK) with 10 mL of 0.02 N HCl at 50°C . Homogenates were centrifuged ($12,000 \times g$, 20 min, 4°C), and the pH of fat-free supernatants was adjusted to pH 6 with 1 N NaOH. A volume of 30 μL of each supernatant was placed in triplicate into wells (5 mm

diameter) made in plates of M17 agar inoculated with 1 mL/L of a 16-h culture of *L. lactis* ssp. *cremoris* HP as the indicator microorganism. After incubation at 30°C for 48 h, the diameter of the zone of growth inhibition was measured, and bacteriocin activity was expressed in millimeters.

Aminopeptidase activity released into the cheese was measured on duplicate samples with lysine *p*-nitroanilide (Lys-*p*-NA) as substrate (Garde et al., 1997). One activity unit corresponds to the activity of enzyme(s) producing one nanomole of *p*-nitroaniline per minute per gram of cheese.

Chemical Determinations

Cheese pH was measured in duplicate with a Crison penetration electrode (model 52-3,2; Crison Instruments, Barcelona, Spain) by means of a Crison GPL 22 pH meter (Ávila et al., 2005a). Dry matter was determined after drying to constant weight in vacuum oven at 100°C (AOAC, 1990).

Residual caseins were determined by capillary electrophoresis, using a Beckman P/ACE System 2100 controlled by a System Gold Software data system (Beckman Instruments España, Madrid, Spain). A 5-g aliquot of grated cheese was homogenized with 25 mL of 2% trisodium citrate at 50°C using an Ultra-Turrax T8 homogenizer (IKA, Labortechnik, Staufen, Germany). Sample buffer was as previously described (Recio et al., 1997). Cheese and milk samples were prepared for capillary electrophoresis by mixing 100 μ L of homogenate with 900 μ L of sample buffer. Samples were kept for 60 min at room temperature, filtered through a mixture of cellulose esters 0.45- μ m filter (Teknokroma, Sant Cugat del Vallès, Spain), and injected in duplicate at the anode using N₂ at 0.035 kg/cm² for 15 s. Separation was performed in a hydrophilic coated fused-silica capillary column CElect P150 (Supelco, Bellefonte, PA), 37 cm long (30-cm effective length), with a final applied voltage of 13 kV. Detection of peaks was at 214 nm. Residual caseins in cheese were expressed as percentage of the total amount of the respective casein initially present in milk, taking into account total weights of milk and cheese sampled (Picón et al., 1994).

Cheese proteolysis was determined on duplicate samples by the *o*-phthalaldehyde (OPA) test, based on the reaction of released α -amino groups with this compound and with β -mercaptoethanol to form an adduct that absorbs strongly at 340 nm (Church et al., 1983).

Hydrophilic and hydrophobic peptides in the water-soluble fraction of cheese were determined on duplicate samples by reverse phase-HPLC using a Beckman System Gold chromatograph (Beckman Instruments España) equipped with a diode array detector module 168,

with detection wavelength at 214 nm, as previously described (Lau et al., 1991; Gómez et al., 1997). Peaks with retention times from 8.5 to 14.6 min were considered to correspond to hydrophilic peptides, and those with retention times from 14.6 to 20.5 min to hydrophobic peptides. Results were expressed in arbitrary units of chromatogram area per milligram of cheese DM.

Free amino acids were extracted from duplicate samples of cheese (Krause et al., 1995) and individual amino acids determined by reverse phase-HPLC using a Beckman System Gold chromatograph after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Cohen and Michaud, 1993). Results were expressed as milligrams per gram of cheese DM.

Textural Determinations

Six cylindrical samples (17 mm height $\times$ 17 mm diameter) from each cheese were compressed to 75% of their original height after being held for 2 h at 20 to 22°C, using an Instron Compression Tester 4301 (Instron, High Wycombe, UK) with a crosshead speed of 50 mm/min and 1-kN load cell. Fracturability (expressed in newtons), hardness after 75% compression (expressed in joules), and elasticity (expressed in newtons/mm²) were calculated as previously described (Gaya et al., 1990) from the compression curves obtained.

Sensory Evaluation

Twelve trained panelists scored the cheeses at 25 and 50 d of ripening for quality (overall acceptance) and intensity (overall intensity) of taste on a 10-point scale, using a horizontal line anchored in the middle and at both ends. Taste was defined as the sensation felt by the taste buds. Cheese samples were held for 3 h at 20 to 22°C before sensorial evaluation. After removing the rind, cheeses were cut into wedges and wedges cut into representative triangular slices (15 to 20 g each). Three cheeses per session, one from each of the vats manufactured on the same day, coded with random 3-digit numbers, were presented to panelists in randomized order. Bread and water were used as rinsing agents between cheeses. A descriptive sensory test was developed for Hispánico cheese and panelists were trained following the guidelines for the taste evaluation of hard and semi-hard cheeses recommended by Bérudier et al. (1997). Panelists were asked to assign a score on a 0 to 6 scale, using a horizontal line anchored in the middle and at both ends, to the intensity of the following taste attributes: sour, bitter, sweet, salty, and umami.

Statistical Analyses

Statistics were performed by means of SPSS Win program (version 8.0, SPSS Inc., Chicago, IL). The AN-

Table 1. Lactic acid bacteria (LAB) counts in Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Age	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
Mesophilic LAB (log cfu/g)			
6 h	8.99 ± 0.07 ^a	8.93 ± 0.04 ^a	8.90 ± 0.04 ^a
1 d	9.38 ± 0.01 ^c	9.28 ± 0.02 ^b	9.15 ± 0.07 ^a
7 d	9.35 ± 0.16 ^b	9.21 ± 0.07 ^a	9.13 ± 0.05 ^a
15 d	9.16 ± 0.01 ^b	9.05 ± 0.02 ^a	9.04 ± 0.11 ^a
25 d	9.38 ± 0.03 ^c	8.99 ± 0.04 ^b	8.64 ± 0.30 ^a
50 d	9.32 ± 0.01 ^c	8.65 ± 0.04 ^b	7.44 ± 0.04 ^a
Thermophilic LAB (log cfu/g)			
6 h	7.39 ± 0.01 ^c	6.46 ± 0.10 ^b	5.12 ± 0.21 ^a
1 d	7.62 ± 0.06 ^c	4.75 ± 0.46 ^a	5.35 ± 0.04 ^b
7 d	8.20 ± 0.19 ^c	6.27 ± 0.26 ^b	5.78 ± 0.25 ^a
15 d	7.67 ± 0.07 ^c	5.55 ± 0.21 ^a	5.91 ± 0.09 ^b
25 d	7.20 ± 0.09 ^c	5.51 ± 0.25 ^a	5.89 ± 0.02 ^b
50 d	6.85 ± 0.13 ^c	4.77 ± 0.36 ^a	5.53 ± 0.28 ^b

^{a-c}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 4) of duplicate determinations on 2 cheese-making experiments.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

OVA was carried out with type of mesophilic starter, cheese age, and cheese-making experiment as main effects. Comparison of means was performed using Tukey's test. Principal component analysis with Varimax rotation was carried out on pH, proteolysis parameters, and taste attributes.

RESULTS AND DISCUSSION

LAB and Cheese pH

Mesophilic LAB in BNP cheese corresponded to *L. lactis* ssp. *lactis* INIA 437, in BNP + BP cheese to *L. lactis* ssp. *lactis* INIA 437 plus *L. lactis* ssp. *lactis* INIA 639, and in BP cheese to *L. lactis* ssp. *lactis* INIA 639. The highest mesophilic LAB counts were found in BNP cheese throughout the ripening period (Table 1), with differences in counts with respect to the other cheeses increasing with age. The BP-LAB accounted for 36% of the mesophilic LAB in BNP + BP cheese on d 1, but for only 1% on d 50 (Table 2). Decreases in counts of BP-LAB of 2.05 and 1.74 log units were recorded from d 1 to d 50 in BNP + BP and in BP cheeses, respectively. The higher reduction of BP-LAB counts in BNP + BP cheese than in BP cheese could be due to the fact that

Table 2. Bacteriocin-producer counts and bacteriocin activity in Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Age	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
Bacteriocin-producer count (log cfu/g)			
6 h	ND ^a	8.45 ± 0.06 ^b	8.75 ± 0.05 ^c
1 d	ND ^a	8.83 ± 0.06 ^b	9.17 ± 0.10 ^c
7 d	ND ^a	8.79 ± 0.05 ^b	9.12 ± 0.08 ^c
15 d	ND ^a	8.67 ± 0.04 ^b	9.06 ± 0.12 ^c
25 d	ND ^a	8.43 ± 0.27 ^b	8.70 ± 0.24 ^c
50 d	ND ^a	6.78 ± 0.17 ^b	7.43 ± 0.06 ^c
Bacteriocin (mm inhibition)			
6 h	ND ^a	ND ^a	9.3 ± 0.3 ^b
1 d	ND ^a	8.0 ± 0.3 ^b	10.8 ± 0.3 ^c
7 d	ND ^a	9.3 ± 0.3 ^b	11.8 ± 0.1 ^c
15 d	ND ^a	9.8 ± 0.1 ^b	12.3 ± 0.1 ^c
25 d	ND ^a	ND ^a	10.0 ± 0.0 ^b
50 d	ND ^a	ND ^a	10.3 ± 0.4 ^b

^{a-c}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 4) of duplicate determinations on 2 cheese-making experiments; ND = not detected.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

in the former cheese, the BP strain has to compete for nutrients with the BNP strain. On the other hand, BNP-LAB only decreased by 0.06 log units in BNP cheese and by 0.45 log units in BNP + BP cheese from d 1 to d 50.

The increase in thermophilic LAB counts from inoculated milk to 6-h BNP cheese, considerably lower than that of mesophilic LAB, can be mostly ascribed to bacterial concentration in the curds. Log counts of thermophilic LAB in cheese made from milk inoculated with *Lb. helveticus* LH 92 and the BNP strain did not differ significantly from those in cheese made from milk inoculated only with *Lb. helveticus* LH 92 during the first 15 d of ripening (data not shown), excluding any antagonistic affect of the BNP strain on *Lb. helveticus* LH 92. On the other hand, death of thermophilic LAB occurred in cheeses made with BP culture during the first hours, with counts 0.93 and 2.27 log units lower in BNP + BP and BP cheeses, respectively, than in BNP cheese after 6 h (Table 1). In BNP + BP cheese, death of thermophilic LAB still continued from 6 to 24 h. Recovery and growth of thermophilic LAB was observed in BP cheese from 6 h and in BNP + BP cheese from 24 h (Table 1). Lower

Table 3. Values of pH and DM in Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Age	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
Cheese pH			
6 h	5.15 ± 0.06 ^a	5.18 ± 0.05 ^a	5.13 ± 0.07 ^a
1 d	4.97 ± 0.04 ^a	4.91 ± 0.03 ^a	4.97 ± 0.02 ^a
7 d	4.97 ± 0.08 ^a	4.91 ± 0.06 ^a	4.97 ± 0.04 ^a
15 d	4.88 ± 0.05 ^a	4.97 ± 0.11 ^b	4.99 ± 0.06 ^b
25 d	4.87 ± 0.04 ^a	4.89 ± 0.03 ^a	4.90 ± 0.06 ^a
50 d	4.81 ± 0.05 ^a	4.89 ± 0.02 ^a	4.86 ± 0.01 ^a
DM (%)			
25 d	55.77 ± 0.54 ^a	54.94 ± 0.59 ^a	56.20 ± 0.31 ^a
50 d	58.85 ± 0.59 ^a	59.19 ± 0.27 ^a	58.72 ± 0.64 ^a

^{a,b}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 4) of duplicate determinations on 2 cheese-making experiments.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

LAB counts had been reported in previous works when a BP strain was added in cheese making (Garde et al., 2002a; Benech et al., 2003; Sallami et al., 2004a; Ávila et al., 2005a).

No significant differences in cheese pH during ripening were found, except on d 15, when the pH was higher in cheeses made with BP than in BNP cheeses (Table 3). Retarded acid production in cheese made from milk inoculated with a BP adjunct has been reported by some authors (Garde et al., 1997; Morgan et al., 1997). There were no significant differences in DM between cheeses throughout the ripening period (Table 3).

Bacteriocin Activity and Release of Intracellular Enzymes

Bacteriocin activity in cheeses made with BP increased until d 15, and decreased afterwards (Table 2). Bacteriocin activity was higher in BP cheese than in BNP + BP cheese, in agreement with the higher counts of the BP in the former cheese (Table 2).

Cell-free aminopeptidase activity increased significantly ($P < 0.001$) as cheese aged (Table 4). Aminopeptidase levels remained below 0.5 activity units during the first 15 d of ripening in cheese from milk inoculated only with the BNP strain, and below 2.5 activity units in cheese from milk inoculated only with BNP and BP strains (data not shown), values considerably lower than those found in cheeses from milk inoculated also

Table 4. Cell-free aminopeptidase activity (nmol of lysine *p*-nitroanilide/min × g) in Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Age	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
6 h	9.53 ± 1.30 ^a	12.06 ± 0.35 ^b	11.53 ± 0.50 ^b
1 d	16.08 ± 3.15 ^a	18.30 ± 1.84 ^b	14.79 ± 1.54 ^a
7 d	29.47 ± 6.26 ^a	41.15 ± 2.38 ^b	32.38 ± 2.16 ^a
15 d	32.94 ± 4.78 ^b	57.53 ± 1.05 ^c	26.96 ± 0.86 ^a
25 d	37.56 ± 7.82 ^b	53.67 ± 9.41 ^c	27.58 ± 2.66 ^a
50 d	39.87 ± 6.93 ^a	64.83 ± 7.01 ^b	34.79 ± 0.85 ^a

^{a-c}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 4) of duplicate determinations on 2 cheese-making experiments.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

with *Lb. helveticus* LH 92 (Table 4). Cheese made with BNP + BP showed the highest cell-free aminopeptidase activity values, 1.4- to 1.8-fold higher than those in BNP cheese from d 7 onwards. The increase in cell-free aminopeptidase activity due to bacteriocin-mediated lysis of LAB was in agreement with previous reports (Garde et al., 1997; Morgan et al., 1997; Martínez-Cuesta et al., 2001; Garde et al., 2002a; Ávila et al., 2005a). However, BP cheese showed cell-free aminopeptidase activity values similar to or lower than those of BNP cheese. The considerable bacteriocin production during the first 6 h in BP cheese (Table 2) inhibited growth of *Lb. helveticus*, which, after 6 h, reached counts 1.34 log units lower in BP cheese than in BNP + BP cheese (Table 1), which undoubtedly affected aminopeptidase activity value (Table 4). The retarded bacteriocin production in BNP + BP cheese with respect to BP cheese delayed *Lb. helveticus* inhibition and death. Counts of *Lb. helveticus* decreased by 1.71 log units in BNP + BP cheese from 6 to 24 h (Table 1), and the concomitant cell lysis resulted in high aminopeptidase activity values throughout ripening (Table 4). From d 1 to d 7, *Lb. helveticus* cells recovered in BNP + BP cheese (Table 1). Simultaneously, intracellular aminopeptidases were released, as shown by the 2.2-fold increase in the activity value of this cheese (Table 4). O'Sullivan et al. (2002) observed that some cells in a lactococcal culture exposed to lacticin 481 suffered gradual death and lysis/permeabilization, whereas other cells continued to grow. These authors suggested that the different growth phase, the physiological sta-

Table 5. Overall proteolysis ($A_{340\text{ nm}}$) in Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Age	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
1 d	0.163 ± 0.005 ^b	0.158 ± 0.004 ^a	0.167 ± 0.012 ^b
7 d	0.490 ± 0.105 ^a	0.767 ± 0.120 ^c	0.713 ± 0.089 ^b
15 d	0.665 ± 0.088 ^a	1.246 ± 0.087 ^c	1.127 ± 0.051 ^b
25 d	0.935 ± 0.088 ^a	1.900 ± 0.063 ^c	1.661 ± 0.060 ^b
50 d	2.188 ± 0.317 ^a	3.604 ± 0.414 ^c	3.153 ± 0.293 ^b

^{a-c}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 4) of duplicate determinations on 2 cheese-making experiments.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

tus of individual cells, or a low ratio of lacticin 481 molecules to cells could have been involved in the different sensitivity of lactococcal cells to the bacteriocin. In the present work, the particular mode of action of lacticin 481 would explain growth of *Lb. helveticus* from d 1 to d 7 in BNP + BP cheese with simultaneous release of intracellular aminopeptidases, in agreement with the high aminopeptidase activity values obtained for mixed cultures of *Lb. helveticus* and lacticin 481-producing strains in milk (Ávila et al., 2005b).

Proteolysis

Cheese proteolysis, as determined by the OPA test, which detects released α -amino groups, increased significantly ($P < 0.001$) with cheese age (Table 5). Proteolysis values remained below 0.30 during the first 15 d of ripening in cheese from milk inoculated only with the BNP strain, and below 0.40 in cheese from milk inoculated only with BNP and BP strains (data not shown), levels considerably lower than those found in cheeses from milk inoculated also with *Lb. helveticus* LH 92 (Table 5). The BNP + BP cheese showed the highest proteolysis values throughout ripening, followed by BP cheese. After 25 d of ripening, proteolysis in BNP + BP cheese was 2.0-fold higher than that in BNP cheese. Enhancement of cheese proteolysis was associated with lower levels of viable thermophilic LAB (Table 1) and with a higher aminopeptidase activity (Table 4). These results confirm that early death of LAB cells caused by a bacteriocin increases the release of peptidases and therefore cheese proteolysis (Garde et al., 1997; Martínez-Cuesta et al., 2001; Garde et al.,

Table 6. Residual caseins (%) in Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Age	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
α_S -Casein			
1 d	58.7 ± 2.2 ^a	57.6 ± 3.2 ^a	63.8 ± 1.8 ^a
25 d	22.1 ± 3.9 ^b	15.6 ± 0.4 ^a	16.3 ± 0.4 ^a
50 d	14.7 ± 3.5 ^b	9.7 ± 0.7 ^a	8.8 ± 0.3 ^a
β -Casein			
1 d	83.2 ± 2.8 ^a	79.2 ± 4.3 ^a	85.2 ± 3.8 ^a
25 d	70.7 ± 0.7 ^b	63.0 ± 2.7 ^a	71.0 ± 4.2 ^b
50 d	62.2 ± 2.4 ^a	58.5 ± 5.1 ^a	53.0 ± 2.8 ^a

^{a,b}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 4) of duplicate determinations on 2 cheese-making experiments.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

2002a; Ávila et al., 2005a). A higher release of free NH_2 groups in cheese made with an autolytic *Lb. helveticus* strain than in cheese made with a nonautolytic *Lb. helveticus* strain has also been reported (Valence et al., 2000).

Residual α_S -casein declined considerably during ripening in all cheeses (Table 6), from 58 to 64% on d 1 to 15% on d 50, whereas β -casein was scarcely degraded, with 80 to 85% intact casein on d 1 and 53 to 62% on d 50. Levels of α_S -casein, but not β -casein, were significantly ($P < 0.05$) lower in BP cheeses than BNP cheeses from d 25 onwards. The lower pH value of the latter cheese from d 15 onwards might have retarded the activity of caseinolytic enzymes.

The highest levels of hydrophilic and hydrophobic peptides were found in BNP cheese, followed by BP cheese (Table 7). After 50 d, levels of hydrophilic and hydrophobic peptides in BNP cheese were 2.1- and 1.3-fold higher than in BNP + BP cheese, respectively. The hydrophobic:hydrophilic ratio declined in all cheeses during ripening, with significantly lower values in BNP + BP cheese. Because hydrophobic peptides and the hydrophobic:hydrophilic ratio are associated with cheese bitterness (Lau et al., 1991; Gómez et al., 1997), the lower values of both variables obtained for BNP + BP cheese would be beneficial for flavor quality. Distinct peptide profiles for cheese manufactured with or without a BP adjunct, with a lower proportion of hydrophobic peptides in the cheese made with BP, were obtained by Morgan et al. (1997). Also, lower content of hydrophobic peptides and a lower hydrophobic:hydrophilic ratio

Table 7. Hydrophilic and hydrophobic peptides (arbitrary units) determined at 214 nm, and the hydrophobic:hydrophilic ratio in Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Age	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
Hydrophobic			
25 d	78.9 ± 8.0 ^c	57.1 ± 4.1 ^a	63.3 ± 1.2 ^b
50 d	74.5 ± 15.6 ^c	35.0 ± 6.8 ^a	60.8 ± 0.9 ^b
Hydrophilic			
25 d	124.6 ± 4.4 ^c	113.4 ± 2.4 ^a	119.4 ± 3.0 ^b
50 d	156.5 ± 15.6 ^c	117.3 ± 3.7 ^a	138.6 ± 8.9 ^b
Ratio			
25 d	0.63 ± 0.04 ^c	0.50 ± 0.03 ^a	0.53 ± 0.01 ^b
50 d	0.46 ± 0.05 ^b	0.29 ± 0.05 ^a	0.44 ± 0.03 ^b

^{a-c}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 4) of duplicate determinations on 2 cheese-making experiments.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

were achieved by Garde et al. (2002a) and Ávila et al. (2005a), when BP adjuncts were added together with the starter culture. Higher levels of hydrophilic peptides and a lower hydrophobic:hydrophilic ratio were found in Cheddar cheese made with *Lb. casei* and a nisin Z producer (Benech et al., 2003). In those studies, the lower levels of hydrophobic peptides in experimental cheese were related to the release of intracellular peptidases when the bacteriocins induced the lysis of starter bacteria. More extensive peptide hydrolysis was recorded in cheese made with an autolytic *Lb. helveticus* strain than in cheese made with a nonautolytic *Lb. helveticus* strain (Valence et al., 2000).

Free amino acids increased significantly ($P < 0.001$) with cheese age (Tables 8 and 9). The BNP + BP cheese showed the highest levels of free amino acids, followed by BP cheese. Levels of total free amino acids in BNP + BP cheese on d 25 and 50 were 2.4- and 2.1-fold higher than the respective values in BNP cheese (Tables 8 and 9). After 50 d, the highest increases in individual free amino acids in BNP + BP cheese with respect to BNP cheese were recorded for Ile, His, Glu, and Val (Table 9). Cysteine was not detected in any cheese throughout the ripening period. Higher levels of free amino acids in cheeses made with a BP culture have been previously reported (Morgan et al., 1997; Garde et al., 2002a; Salami et al., 2004a; Ávila et al., 2005a). The high levels of free amino acids in BNP + BP cheese can be explained by a more rapid breakdown of the peptides originating

Table 8. Free amino acids (mg/g of DM) in 25-d-old Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Amino acid	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
Asp	0.18 ± 0.02 ^a	0.41 ± 0.03 ^c	0.30 ± 0.03 ^b
Ser	0.65 ± 0.14 ^a	1.88 ± 0.23 ^c	1.59 ± 0.16 ^b
Glu	0.69 ± 0.12 ^a	2.77 ± 0.25 ^b	2.73 ± 0.13 ^b
Gly	0.13 ± 0.05 ^a	0.49 ± 0.05 ^c	0.38 ± 0.04 ^b
His	0.84 ± 0.19 ^a	3.53 ± 0.43 ^c	2.67 ± 0.27 ^b
Arg	ND ^a	0.06 ± 0.03 ^b	1.06 ± 0.21 ^c
Thr	0.40 ± 0.03 ^a	0.88 ± 0.02 ^c	0.67 ± 0.03 ^b
Ala	0.34 ± 0.06 ^a	0.75 ± 0.07 ^c	0.63 ± 0.07 ^b
Pro	3.05 ± 0.37 ^b	3.23 ± 0.48 ^c	1.65 ± 0.14 ^a
Cys	ND ^a	ND ^a	ND ^a
Tyr	0.30 ± 0.04 ^a	0.75 ± 0.06 ^c	0.60 ± 0.04 ^b
Val	0.47 ± 0.10 ^a	1.57 ± 0.16 ^c	1.21 ± 0.11 ^b
Met	0.24 ± 0.03 ^a	0.52 ± 0.02 ^c	0.41 ± 0.01 ^b
Lys	1.75 ± 0.24 ^a	4.41 ± 0.33 ^c	3.51 ± 0.14 ^b
Ile	0.32 ± 0.04 ^a	1.05 ± 0.15 ^c	0.78 ± 0.09 ^b
Leu	1.56 ± 0.22 ^a	3.76 ± 0.17 ^c	3.20 ± 0.16 ^b
Phe	0.82 ± 0.05 ^a	1.86 ± 0.08 ^c	1.54 ± 0.05 ^b
Total	11.73 ± 1.67 ^a	27.90 ± 1.51 ^c	22.93 ± 1.64 ^b

^{a-c}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 4) of duplicate determinations on 2 cheese-making experiments. ND = not detected.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

from casein when intracellular peptidases are released into the cheese matrix (Wilkinson et al., 1994; Morgan et al., 1997). Higher levels of free amino acids were found in cheese made with an autolytic *Lb. helveticus* strain than in cheese made with a nonautolytic *Lb. helveticus* strain (Valence et al., 2000).

Cheese Texture

Fracturability, hardness, and elasticity, determined from the compression curves obtained with the Instron tester, increased significantly ($P < 0.001$) during cheese ripening (Table 10), probably because the strengthening effect of moisture loss during ripening predominated over the weakening effect of caseinolysis (Picón et al., 1994). The 3 rheological characteristics were higher in BNP cheese than in cheeses made with BP. Residual α_S -casein plays a crucial role in the stability of the cheese protein network (Creamer and Olson, 1982). Degradation of α_S -casein was more extensive in BP cheeses than in BNP cheeses (Table 6). A relationship between lower levels of residual α_S -casein in Hispánico cheese and a softer cheese texture has been previously reported (Mohedano et al., 1998; Ávila et al., 2005a).

Table 9. Free amino acids (mg/g of DM) in 50-d-old Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Amino acid	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
Asp	0.45 ± 0.09 ^a	1.04 ± 0.20 ^c	0.75 ± 0.13 ^b
Ser	1.45 ± 0.32 ^a	3.64 ± 0.54 ^c	2.86 ± 0.30 ^b
Glu	1.51 ± 0.37 ^a	4.61 ± 0.74 ^b	4.52 ± 0.36 ^b
Gly	0.37 ± 0.11 ^a	0.97 ± 0.13 ^c	0.76 ± 0.08 ^b
His	1.96 ± 0.42 ^a	6.15 ± 0.80 ^c	4.65 ± 0.43 ^b
Arg	0.13 ± 0.08 ^a	0.37 ± 0.03 ^b	1.42 ± 0.06 ^c
Thr	0.71 ± 0.07 ^a	1.55 ± 0.21 ^c	1.17 ± 0.05 ^b
Ala	0.58 ± 0.09 ^a	1.36 ± 0.17 ^c	1.06 ± 0.07 ^b
Pro	5.05 ± 0.41 ^b	5.15 ± 1.03 ^b	3.18 ± 0.31 ^a
Cys	ND ^a	ND ^a	ND ^a
Tyr	0.61 ± 0.11 ^a	1.60 ± 0.21 ^c	1.25 ± 0.16 ^b
Val	1.05 ± 0.23 ^a	3.10 ± 0.44 ^c	2.35 ± 0.27 ^b
Met	0.59 ± 0.12 ^a	1.19 ± 0.21 ^c	0.87 ± 0.08 ^b
Lys	3.75 ± 0.60 ^a	7.99 ± 0.94 ^c	6.53 ± 0.48 ^b
Ile	0.74 ± 0.17 ^a	2.40 ± 0.43 ^c	1.71 ± 0.27 ^b
Leu	3.11 ± 0.39 ^a	5.67 ± 0.48 ^c	4.95 ± 0.37 ^b
Phe	1.55 ± 0.09 ^a	3.14 ± 0.32 ^c	2.54 ± 0.20 ^b
Total	23.62 ± 3.65 ^a	49.94 ± 4.74 ^c	40.55 ± 3.62 ^b

^{a-c}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 4) of duplicate determinations on 2 cheese-making experiments. ND = not detected.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

Table 10. Textural characteristics of Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Age	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
Fracturability (N)			
25 d	22.22 ± 1.61 ^b	17.37 ± 0.51 ^a	16.50 ± 0.68 ^a
50 d	36.04 ± 1.70 ^b	30.73 ± 2.30 ^a	28.14 ± 1.66 ^a
Hardness (J)			
25 d	0.27 ± 0.02 ^b	0.22 ± 0.01 ^a	0.20 ± 0.01 ^a
50 d	0.45 ± 0.02 ^b	0.42 ± 0.03 ^{ab}	0.39 ± 0.02 ^a
Elasticity (N/mm ²)			
25 d	0.57 ± 0.06 ^b	0.36 ± 0.02 ^a	0.38 ± 0.02 ^a
50 d	1.51 ± 0.08 ^b	1.17 ± 0.10 ^a	1.12 ± 0.06 ^a

^{a,b}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 12) of 6 determinations on 2 cheese-making experiments.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

Table 11. Sensory evaluation of Hispánico cheese manufactured with lacticin 481-producing (BP) *Lactococcus lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing (BNP) *L. lactis* ssp. *lactis* INIA 437, and a *Lactobacillus helveticus* culture (LH)¹

Age	Experimental cheeses ²		
	BNP + LH	BNP + BP + LH	BP + LH
Taste quality			
25 d	4.30 ± 0.27 ^a	6.28 ± 0.23 ^b	5.69 ± 0.27 ^b
50 d	4.01 ± 0.30 ^a	6.45 ± 0.30 ^b	6.23 ± 0.31 ^b
Taste intensity			
25 d	6.02 ± 0.30 ^a	6.20 ± 0.17 ^a	5.89 ± 0.25 ^a
50 d	6.28 ± 0.24 ^a	6.84 ± 0.24 ^a	6.75 ± 0.20 ^a

^{a,b}Means in the same row with the same superscript do not differ ($P < 0.05$).

¹Mean ± SE (n = 24) of scores from 12 trained panelists on 2 cheese-making experiments using a 10-point scale.

²Cheeses: BNP + LH = 5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and 5 mL/L of a *Lactobacillus helveticus* culture; BNP + BP + LH = 2.5 mL/L of bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, 2.5 mL/L of lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture; BP + LH = 5 mL/L of a lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, and 5 mL/L of a *Lactobacillus helveticus* culture.

Sensory Evaluation

The type of mesophilic starter used in cheese manufacture significantly ($P < 0.05$) affected taste quality, but not taste intensity (Table 11). Both cheeses made with the BP received higher scores for taste quality than did BNP cheese. Taste intensity increased in all cheeses with age, whereas taste quality increased with age in cheese made with the BP, and decreased in BNP cheese. Our results agree with those reported for Cheddar cheese made with a BP adjunct, which showed higher sensory evaluation scores than control cheese (Morgan et al., 1997). Also, addition of a BP adjunct culture in Hispánico cheese manufacture resulted in significantly higher scores of flavor quality and flavor intensity (Garde et al., 2002a). Higher flavor intensity and flavor quality scores were obtained when a nisin-producing *L. lactis* ssp. *lactis* strain was combined with a highly autolytic and proteolytic *Lb. delbrueckii* ssp. *bulgaricus* strain for Cheddar cheese manufacture (Sallami et al., 2004b). Similarly, the addition of *Lb. casei* and liposome-encapsulated nisin Z improved flavor intensity and sensory characteristics of Cheddar cheese (Benech et al., 2003).

Scores for Hispánico cheese taste descriptors (data not shown) were (mean values on a 0 to 6 points scale, considering all ages and all vats): 2.45 for sour, 1.66 for bitter, 0.66 for sweet, 1.04 for salty, and 1.25 for umami. Cheese age had no significant ($P < 0.05$) influence on any of these taste attributes. Bitter taste scores were significantly ($P < 0.05$) higher in BNP cheese (2.39) than in BNP + BP cheese (1.22) or in BP cheese (1.38).

Figure 1. Functions 1 and 2 of principal component analysis with Varimax rotation carried out on pH, proteolysis parameters [caseins, peptides, proteolysis o-phthalaldehyde (OPA) method, free amino acids], and taste attributes (quality, intensity, and individual descriptors).

The use of BP adjuncts had been reported to reduce cheese bitterness in previous works (Benech et al., 2003; Sallami et al., 2004b). On the other hand, umami taste scores were significantly ($P < 0.05$) higher in BNP + BP cheese (1.72) or in BP cheese (1.28) than in BNP cheese (0.74).

Principal component analysis with Varimax rotation was carried out to correlate pH and proteolysis with cheese taste attributes (Figure 1). Functions 1 and 2 explained 47.9 and 17.6% of the variance, respectively. Total free amino acids, Glu, proteolysis (OPA method), umami taste descriptor, taste quality, sweet taste descriptor, and taste intensity correlated positively with function 1, whereas hydrophobic:hydrophilic ratio, hydrophobic peptides, residual β - and α_S -caseins, and bitter taste and sour taste descriptors correlated negatively with function 1. Hydrophilic peptides and taste intensity correlated positively with function 2, whereas pH, residual α_S -casein, and sweet taste descriptor correlated negatively with function 2.

CONCLUSIONS

From the results obtained in the present work, it may be concluded that the combination of bacteriocin-producing *L. lactis* ssp. *lactis* INIA 639, bacteriocin-nonproducing *L. lactis* ssp. *lactis* INIA 437, and a *Lb. helveticus* culture as lactic starter optimized the release of intracellular aminopeptidases during early cheese ripening. A more rapid evolution of proteolysis and higher quality of taste, with a reduction in bitterness scores, were achieved by the combined use of the 3 cultures. The procedure here presented seems a simple and inexpensive method for the acceleration of cheese ripening, with no concomitant risks of bitter flavor defect.

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