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Proteolysis in Hispánico Cheese Manufactured Using a Mesophilic Starter, a Thermophilic Starter, and Bacteriocin-Producing *Lactococcus lactis* Subsp*. lactis* INIA 415 Adjunct Culture

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Lactococcus lactis subsp. *lactis* INIA 415, a strain harboring the structural genes of bacteriocins nisin Z and lacticin 481, was used as adjunct culture in the manufacture of Hispánico cheese with a mesophilic starter and a thermophilic starter of high aminopeptidase activity. Addition of the bacteriocin producer promoted early lysis of mesophilic and thermophilic starter bacteria. Extracellular aminopeptidase activity in 7-day-old cheese made using mesophilic and thermophilic starters plus bacteriocin producer was 3.0-fold the level reached in cheese made without the bacteriocin producer. Proteolysis in cheese made with mesophilic and thermophilic starters plus bacteriocin-producing adjunct culture after 25 days of ripening was 1.5-fold the level reached in cheese made without the bacteriocin producer, and the level of total free amino acids was 2.9-fold the level found in cheese made without the bacteriocin producer. Cheese made with mesophilic and thermophilic starters plus bacteriocin producer received the highest scores for flavor quality and flavor intensity and reached in 25 days the flavor intensity score of a 75-day-old cheese made without the bacteriocin producer.

KEYWORDS: Proteolysis; cheese; flavor; lactic acid bacteria; lysis; bacteriocin

INTRODUCTION

Ripening of hard cheese varieties is a long and costly process because of capital immobilization, large refrigerated storage facilities, weight losses, and spoilage caused by undesirable fermentations. A shortened ripening period would lead to a considerable reduction in manufacturing costs. Enzymes from milk, rennet, starter cultures, and secondary microbiota are responsible for the degradation of milk proteins, fat, lactose, citrate, and lactate, resulting in the formation of a high number of flavor compounds. The type and concentration of flavor compounds confer to each cheese variety its unique characteristics.

Proteolysis is the most complex and perhaps the most important biochemical event during the maturation of most cheese varieties (*1*). Apart from milk plasmin and rennet, lactic acid bacteria are the major source of proteolytic enzymes in a wide variety of cheeses. Their proteinases and peptidases transform caseins into small peptides and free amino acids (*2*, *3*), which contribute to flavor and serve as aroma precursors (*4*, *5*). As peptidases and other enzymes such as esterases and amino acid catabolic enzymes are located in the interior of the cell, the lysis of starter bacteria will favor the access of those enzymes to their substrates and may therefore accelerate the development of cheese flavor and hence cheese ripening (*6*, *7*). Approaches employed to enhance the lysis of starter bacteria during ripening include the selection of autolytic strains of lactic acid bacteria (*8*−*11*), the use of lytic bacteriophages (*12*, *13*), and the inoculation of milk with bacteriocin-producing adjunct cultures (*6*, *7*, *14*, *15*).

Lactococcus lactis subsp. *lactis* DPC3286, a producer of lactococcins A, B, and M, had a bacteriolytic effect on sensitive lactococci, probably due to the concerted action of all three bacteriocins. When this strain was used as adjunct culture in cheese manufacture, concentrations of free amino acids were higher and bitterness was reduced (*6*). *Enterococcus faecalis* INIA 4, a nonvirulent hemolysin-negative enterocin-producing strain (*16*), accelerated cell lysis and flavor development when used as adjunct culture to a commercial mixed-strain LD-type starter in the manufacture of semihard cows' milk cheese (*7*). Similar results were obtained with this strain in the manufacture of semihard cheese from a mixture of cows' and ewes' milk, in which it caused a more rapid loss of viability of starter lactococci (*15*). Proteolysis, total free amino acids, and volatile aroma compounds such as 3-methyl-1-butanal, diacetyl, and acetoin reached their maximum levels in cheese made from milk inoculated with 0.1% bacteriocin-producing culture, which

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exhibited the highest scores for flavor quality and flavor intensity throughout ripening (*15*).

Even though the use of an *Enterococcus faecium* strain as probiotic adjunct culture for Cheddar cheese has been suggested (*17*), the dairy industry is reluctant to employ enterococci as lactic starters in cheese manufacture. *L. lactis* subsp. *lactis* INIA 415 is a strain harboring the structural genes of nisin Z and lacticin 481 (*18*), with a high lytic activity on mesophilic and thermophilic lactic acid bacteria in mixed cultures. For this reason it was used in the present work as bacteriocin-producing adjunct culture in the manufacture of Hispánico cheese, a semihard variety manufactured in Spain from a mixture of cows' and ewes' milk, either raw or pasteurized. Its effects on starter viability, release of intracellular enzymes, proteolysis, and sensory characteristics of the cheese are here reported.

MATERIALS AND METHODS

Lactic Cultures and Cheese Manufacture. All strains used were from the INIA culture collection (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain). *L. lactis* subsp. *lactis* INIA 437 and *L. lactis* subsp. *cremoris* INIA 450, used as mesophilic starter, and *L. lactis* subsp. *lactis* INIA 415, used as bacteriocin-producing adjunct culture, were maintained at −80 °C in MRS broth (Biolife, Milano, Italy) and subcultured twice in reconstituted skim milk at 25 °C for 16 h before use in cheese manufacture. *Streptococcus thermophilus* INIA 463 and INIA 468, used as thermophilic starter of high aminopeptidase activity, were maintained at −80 °C in M17 broth (Biolife) and subcultured twice in reconstituted skim milk at 37 °C for 4 h before use in cheese manufacture.

Hispánico cheese was manufactured in duplicate experiments on different days from a mixture of pasteurized cows' (80%) and ewes' (20%) milk. Each experiment consisted of four 45-L vats. *L. lactis* subsp. *lactis* INIA 437 and *L. lactis* subsp. *cremoris* INIA 450 cultures in milk were added each one at 0.5% to all vats. *S. thermophilus* INIA 463 and INIA 468 cultures in milk were added each one at 0.5% to vats 3 and 4. *L. lactis* subsp. *lactis* INIA 415 culture in milk was added at 0.1% to vats 2 and 4. Rennet (6 mL of Maxiren, 1:15000 strength; Gist Brocades, Delft, The Netherlands) was added to milk 20 min after lactic culture inoculation. The curds were cut 40 min after rennet addition into 6−8 mm cubes and scalded at 37 °C for 15 min. Whey was drained off, and curds were distributed into cylindrical molds. Three cheeses, ∼2 kg in weight, were obtained from each vat. Cheeses were pressed for 18 h at 20 °C, salted in brine (150 g NaCl/L) for 16 h at 12 °C, and ripened at 12 °C for 75 days.

Microbiological Analysis and Cheese pH. Viable counts of lactic acid bacteria were determined in duplicate on M17 agar (Biolife) using a spiral plater (Interscience, Saint-Nom-La-Bretèche, France), after incubation of plates at 37 °C for 48 h. Previous trials had shown that large size colonies corresponded to mesophilic lactic acid bacteria and small size colonies to thermophilic lactic acid bacteria.

Cheese pH was measured in duplicate after homogenization of 10 g of cheese with 20 mL of distilled water at 70 °C by means of a Stomacher 400 (Seward Laboratory, London, U.K.).

Determination of Aminopeptidase Activity and Cheese Proteolysis. Aminopeptidase activity released into the cheese was determined on an extract obtained by homogenizing 10 g of cheese with 20 mL of 10 mM sodium phosphate buffer, pH 7, at 20 °C for 3 min in a Stomacher 400, followed by centrifuging at 10000*g* for 15 min at 4 °C and filtering through Whatman No. 4 paper. It was measured on duplicate samples with lysine *p*-nitroanilide (Lys *p*-NA) and leucine *p*-nitroanilide (Leu *p*-NA) as substrates (*17*). One activity unit corresponded to the amount of enzyme producing 1 nmol of *p*-nitroaniline per minute per gram of cheese.

Cheese proteolysis was determined on duplicate samples by using the *o*-phthaldialdehyde (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 (*19*).

Residual caseins were determined by capillary electrophoresis (CE), using a Beckman P/ACE System 2100 (Beckman Instruments España SA, Madrid, Spain) controlled by a System Gold software data system. 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 (*20*). Cheese samples were prepared for CE by mixing 100 μL of homogenate with 1200 μL of sample buffer. Milk samples were prepared for CE by mixing 100 μL of milk with 900 μL of sample buffer. Samples were kept for 90 min at room temperature, filtered through a methyl ethyl cellulose 0.45 μm filter, and injected in duplicate at the anode using N_2 at 0.5 psi for 15 s. Separation was performed in a hydrophilic coated fused-silica capillary column CElect P150 (Supelco, Bellefonte, PA), 37 cm length (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 (*21*).

Hydrophilic and hydrophobic peptides in the water-soluble fraction of cheese were determined on duplicate samples by RP-HPLC using a Beckman System Gold chromatograph (Beckman Instruments España SA) equipped with a diode array detector module 168, with detection wavelength at 214 nm, as previously described (*22*). 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 as units of chromatogram area per milligram of cheese dry matter.

Free amino acids were extracted from duplicate samples of cheese (*23*) and individual amino acids determined by RP-HPLC using a Beckman System Gold chromatograph, after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (*24*). Results were expressed as milligrams per kilogram of cheese dry matter.

Sensory Evaluation. A representative slice of four cheeses per session, one from each of the vats manufactured on the same day, was presented to panelists. Flavor intensity and flavor quality of 25-, 50-, and 75-day-old cheeses from duplicate experiments were evaluated by 16 trained panelists on a 10-point scale as previously described (*25*).

Statistical Analysis. Analyses of variance with addition of bacteriocin-producing adjunct culture, addition of thermophilic starter, and cheese age as main effects were performed on analytical variables by means of the SPSS Win 5.4 program. Comparison of means was carried out using Tukey's test (*26*).

RESULTS AND DISCUSSION

Cheese pH and Lactic Acid Bacteria. Addition of bacteriocin-producing adjunct culture did not affect significantly cheese pH, although in some previous works (*6*, *7*, *15*) retarded acid production in cheese made from milk inoculated with a bacteriocin producer had been reported. Cheese pH was influenced ($P < 0.001$) by age of cheese, with pH values ranging from 4.95 to 5.04 on day 1, from 5.04 to 5.12 on day 25, and from 5.13 to 5.18 on day 75.

Growth and viability of mesophilic starter lactic acid bacteria were influenced by the addition of bacteriocin-producing adjunct culture (data not shown). Levels of mesophilic lactic acid bacteria on day 1 were on average 0.32 log unit lower ($P < 0.05$) in cheeses manufactured with bacteriocin-producing adjunct culture than in cheeses made without bacteriocin producer, and significant differences persisted until day 15. Counts of thermophilic lactic acid bacteria declined more rapidly from day 1 to day 75 in cheese made with bacteriocin-producing adjunct culture (1.46 log units) than in cheese made without the bacteriocin producer (0.84 log unit), suggesting a bactericidal effect of bacteriocins produced by *L. lactis* subsp. *lactis* INIA 415 on thermophilic bacteria.

Release of Intracellular Enzymes. Addition of bacteriocin-producing adjunct culture, addition of thermophilic starter, and cheese age significantly ($P < 0.001$) affected aminopeptidase activity in cheese (**Table 1**). Cheese made from milk inoculated only with mesophilic starter showed low values of aminopep-

Table 1. Aminopeptidase Activity during Ripening of Cheeses Manufactured with Mesophilic Starter (MS), Thermophilic Starter (TS), and Bacteriocin-Producing *L. lactis* Subsp. *lactis* INIA 415

		nmol of *p*-nitroaniline/min·g[a]			
		1% MS		1% MS + 1% TS	
substrate	age (days)	0% INIA 415	0.1% INIA 415	0% INIA 415	0.1% INIA 415
Lys-*p*-NA	1	0.16 ± 0.01[a]	0.19 ± 0.03[a]	2.10 ± 0.51[b]	5.94 ± 0.68[c]
	7	0.28 ± 0.05[a]	2.79 ± 0.30[b]	5.74 ± 3.04[c]	17.41 ± 3.26[d]
	15	0.24 ± 0.01[a]	2.14 ± 1.47[b]	14.98 ± 0.53[c]	25.24 ± 5.34[d]
	25	0.34 ± 0.05[a]	1.32 ± 0.72[b]	15.12 ± 0.17[c]	26.85 ± 0.69[d]
	50	0.37 ± 0.18[a]	2.20 ± 0.56[b]	11.86 ± 4.26[c]	20.69 ± 1.99[d]
	75	0.37 ± 0.05[a]	1.30 ± 0.14[b]	14.53 ± 2.52[c]	26.99 ± 10.54[d]
Leu-*p*-NA	1	0.15 ± 0.01[a]	0.15 ± 0.05[a]	3.97 ± 0.87[b]	7.71 ± 0.51[c]
	7	0.36 ± 0.06[a]	0.37 ± 0.13[a]	7.57 ± 3.47[b]	20.86 ± 5.33[c]
	15	0.30 ± 0.03[a]	0.43 ± 0.01[a]	22.72 ± 0.64[b]	34.94 ± 6.31[c]
	25	0.28 ± 0.13[a]	1.05 ± 0.22[a]	21.24 ± 1.17[b]	34.47 ± 5.62[c]
	50	0.28 ± 0.07[a]	1.41 ± 0.48[b]	19.18 ± 4.82[c]	30.28 ± 3.06[d]
	75	0.51 ± 0.21[a]	0.62 ± 0.36[a]	21.30 ± 1.47[b]	38.65 ± 12.46[c]

[a] Mean ± SD of duplicate determinations in two cheese-making experiments. Means in a row with different superscript letters are significantly different ($P < 0.05$).

tidase activity. Aminopeptidase activity on Lys-*p*-NA in cheese made with mesophilic starter plus bacteriocin-producing adjunct culture on day 7 was 10-fold that in cheese made without the bacteriocin producer, and still 3.5-fold on day 75 (**Table 1**). Increases in aminopeptidase activity in the presence of bacteriocin-producer were much higher than those recorded in a previous work (*15*).

Cheeses made with thermophilic starter showed considerably higher values of aminopeptidase activity than those made without it. Thus, in the absence of bacteriocin producer, aminopeptidase activity in 1-day-old cheese made with thermophilic starter was 13-fold that in cheese made without thermophilic starter when Lys-*p*-NA was used as substrate and 26-fold with Leu-*p*-NA as substrate (**Table 1**). *S. thermophilus* possesses two additional peptidases (an oligopeptidase and aminopeptidase PepS) with respect to *L. lactis* and shows higher specific activities of PepX, PepN, and PepC (*27*), which would explain the dramatic increases in aminopeptidase activity. Within cheeses made with thermophilic starter, values of aminopeptidase activity on Lys-*p*-NA in cheeses made with bacteriocin producer were 2.8-fold those in cheeses made without the bacteriocin producer on day 1 and 3.0-fold on day 7. The increase in aminopeptidase activity due to bacteriocin-mediated lysis of lactic acid bacteria is in agreement with previous results (*6*, *7*, *15*). Aminopeptidase activity increased mainly during the first 15 days of ripening and afterward remained higher in cheeses made with bacteriocin-producing adjunct culture.

Proteolysis. Cheese proteolysis as determined by the OPA test (**Table 2**) increased significantly ($P < 0.001$) with addition of bacteriocin-producing adjunct culture, with addition of thermophilic starter and with cheese age. After 75 days of ripening, proteolysis in cheese made with mesophilic starter plus bacteriocin producer was 1.8-fold that in cheese only made with mesophilic starter. Within 75-day-old cheeses made with thermophilic starter, proteolysis in cheese made with bacteriocin-producing adjunct culture was 1.6-fold that in cheese made without bacteriocin producer. Enhancement of cheese proteolysis was associated with lower levels of viable lactic acid bacteria and with higher aminopeptidase activity (**Table 1**). These results confirm that early death of starter cells caused by a bacteriocin increases the release of peptidases and enhances cheese proteolysis (*7*, *15*). A similar correlation between cell lysis and proteolysis was reported (*13*) when the extent of phage-induced lysis of a *L. lactis* strain and the proteolysis in Saint-Paulin cheese were monitored.

Table 2. Proteolysis (OPA Test) during Ripening of Cheeses Manufactured with Mesophilic Starter (MS), Thermophilic Starter (TS), and Bacteriocin-Producing *L. lactis* Subsp. *lactis* INIA 415

	A_{340nm}[a]			
	1% MS		1% MS + 1% TS	
age (days)	0% INIA 415	0.1% INIA 415	0% INIA 415	0.1% INIA 415
1	0.147 ± 0.01[ab]	0.154 ± 0.01[b]	0.129 ± 0.01[a]	0.137 ± 0.01[ab]
7	0.251 ± 0.01[a]	0.299 ± 0.02[b]	0.261 ± 0.01[a]	0.304 ± 0.03[b]
15	0.324 ± 0.02[a]	0.436 ± 0.01[c]	0.369 ± 0.01[b]	0.484 ± 0.02[d]
25	0.475 ± 0.03[a]	0.705 ± 0.03[c]	0.584 ± 0.04[b]	0.858 ± 0.03[d]
50	0.645 ± 0.04[a]	0.984 ± 0,17[c]	0.821 ± 0.04[b]	1.364 ± 0.28[d]
75	0.911 ± 0.02[a]	1.635 ± 0.24[c]	1.413 ± 0.13[b]	2.219 ± 0.52[d]

[a] Mean ± SD of duplicate determinations in two cheese-making experiments. Means in a row with different superscript letters are significantly different ($P < 0.05$).

Table 3. Residual α_s- and β-Caseins in Cheeses Manufactured with Mesophilic Starter (MS), Thermophilic Starter (TS), and Bacteriocin-Producing *L. lactis* Subsp. *lactis* INIA 415

		100 casein in cheese/casein in milk[a]			
		1% MS		1% MS + 1% TS	
casein	age (days)	0% INIA 415	0.1% INIA 415	0% INIA 415	0.1% INIA 415
α_s-	25	30.0 ± 0.34[c]	33.7 ± 0.13[d]	23.2 ± 1.29[b]	15.0 ± 0.29[a]
	50	25.9 ± 0.46[c]	26.5 ± 0.65[c]	20.3 ± 0.41[b]	11.9 ± 0.35[a]
	75	23.2 ± 0.01[c]	25.7 ± 2.25[c]	19.5 ± 1.48[b]	11.4 ± 1.51[a]
β-	25	86.8 ± 0.77[b]	86.6 ± 1.72[b]	78.6 ± 4.74[ab]	73.3 ± 0.37[a]
	50	91.7 ± 0.81[b]	89.0 ± 0.87[b]	85.3 ± 0.81[b]	69.3 ± 2.88[a]
	75	90.0 ± 0.69[b]	90.6 ± 2.29[b]	82.5 ± 1.59[b]	67.2 ± 3.20[a]

[a] Mean ± SD of duplicate determinations in two cheese-making experiments. Means in a row with different superscript letters are significantly different ($P < 0.05$).

In cheeses made without thermophilic starter, residual α_s- and β-caseins were not influenced by addition of the bacteriocin producer. However, addition of the thermophilic starter accelerated significantly ($P < 0.001$) the degradation of both α_s- and β-caseins (**Table 3**). Breakdown of caseins in Feta cheese made with *S. thermophilus* as adjunct starter was more extensive than in control cheese, although the proteolytic pattern was similar to that in the control (*28*). The hydrolysis of α_s- and β-caseins was also increased when a thermophilic starter was added

Table 4. Hydrophobic and Hydrophilic Peptides and the Ratio of Hydrophobic Peptides to Hydrophilic Peptides in the Water-Soluble Fraction of Cheeses Manufactured with Mesophilic Starter (MS), Thermophilic Starter (TS), and Bacteriocin-Producing *L. lactis* Subsp. *lactis* INIA 415

		chromatogram units/mg of dry matter[a]			
		1% MS		1% MS + 1% TS	
peptide	age (days)	0% INIA 415	0.1% 'INIA 415	0% INIA 415	0.1% INIA 415
hydrophobic	25	2.94 ± 0.19^{c}	2.57 ± 0.21^{a}	2.71 ± 0.28^{b}	2.96 ± 0.24^{c}
	50	3.57 ± 0.36^{c}	2.90 ± 0.24^{b}	2.74 ± 0.41^{a}	3.00 ± 0.32^{b}
	75	3.12 ± 0.54^{d}	1.94 ± 0.20^{a}	2.68 ± 0.57^{c}	2.21 ± 0.30^{b}
hydrophilic	25	5.97 ± 0.68^{a}	8.18 ± 1.03^{c}	7.73 ± 0.83^{b}	10.49 ± 0.59^{d}
	50	7.50 ± 0.72^{a}	9.81 ± 0.95^{c}	9.54 ± 0.78^{b}	12.47 ± 0.88^{d}
	75	9.08 ± 0.81^{a}	14.31 ± 2.14^{d}	11.42 ± 1.01^{b}	12.79 ± 0.39^{c}
ratio	25	0.49 ± 0.02^{d}	0.31 ± 0.05^{b}	0.35 ± 0.01^{c}	0.28 ± 0.02^{a}
	50	0.48 ± 0.03^{c}	0.30 ± 0.04^{b}	0.29 ± 0.02^{b}	0.24 ± 0.02^{a}
	75	0.34 ± 0.03^{d}	0.14 ± 0.05^{a}	0.23 ± 0.02^{c}	0.17 ± 0.02^{b}

[a] Mean ± SD of duplicate determinations in two cheese-making experiments. Means in a row with different superscript letters are significantly different ($P < 0.05$).

together with the mesophilic starter in the manufacture of a semihard cheese (*29*). Most strains of *S. thermophilus* do not express, or express a very low level of, cell envelope proteinase, and a screening of *S. thermophilus* strains revealed that only 3 of 97 of them possessed a level of proteinase activity close to that of a proteinase-positive lactococcal strain (*30*). Recently, the cell envelope proteinase from *S. thermophilus* CNRZ 385 was characterized, and it presented an intermediate specificity between P_I and P_{III} types of cell envelope proteinases from lactococci (*31*). In the present work, the higher casein hydrolysis in cheeses made with thermophilic starter was most probably due to an additive effect of lactococcal and streptococcal proteinases. In cheeses made with thermophilic starter, addition of the bacteriocin-producing adjunct culture significantly ($P < 0.001$) enhanced α_s- and β-casein degradation (**Table 3**). After 75 days of ripening, residual α_s-casein in cheese made with thermophilic starter was lowered from 19.5 to 11.4% by addition of bacteriocin producer and residual β-casein from 82.5 to 67.2%. This effect could not be attributed to differences in cheese pH, as differences were below 0.05 pH unit during most of the ripening period. Cheese age had a significant ($P < 0.001$) effect on α_s-casein degradation but not on that of β-casein.

Peptides. Levels of hydrophobic and hydrophilic peptides in the water-soluble fraction of cheese, and their ratio, were significantly ($P < 0.001$) affected by addition of bacteriocin-producing adjunct culture, addition of thermophilic starter, and cheese age (**Table 4**). Levels of hydrophilic peptides increased with the addition of bacteriocin-producing adjunct culture, the addition of thermophilic starter, and cheese age. However, levels of hydrophobic peptides decreased with the addition of bacteriocin-producing adjunct culture or the addition of thermophilic starter. The ratio of hydrophobic peptides to hydrophilic peptides was also lower in cheeses made with bacteriocin-producing adjunct culture. This ratio ranged from 0.28 to 0.49 after 25 days and from 0.24 to 0.48 after 50 days, values lower than those recorded for Hispánico cheese made using mesophilic commercial starter CH-N01, which were on average 0.69 after 30 days and 0.53 after 60 days (*22*). As hydrophobic peptides and the ratio of hydrophobic peptides to hydrophilic peptides are associated with cheese bitterness (*22, 32*), the lower values of both variables obtained for cheeses made with bacteriocin-producing adjunct culture would be beneficial for flavor quality. In cheeses made without thermophilic starter addition of bacteriocin producer had no effect on casein degradation, but in cheese made with thermophilic starter casein degradation was higher when the bacteriocin-producing adjunct culture was added (**Table 3**). On day 25 hydrophobic peptides were at higher levels in cheese made with mesophilic and thermophilic starters plus bacteriocin producer (**Table 4**), but afterward they were degraded more rapidly in it than in other cheeses because of the increase in available intracellular peptidases when the bacteriocin induced the lysis of lactic acid bacteria. Lower levels of hydrophobic peptides were also found in experimental Cheddar cheese manufactured with phage-assisted lysis of starter lactococci (*12*).

Table 5. Free Amino Acids in 25-Day-Old Cheeses Manufactured with Mesophilic Starter (MS), Thermophilic Starter (TS), and Bacteriocin-Producing *L. lactis* Subsp. *lactis* INIA 415

	mg/kg of dry matter[a]			
	1% MS		1% MS + 1% TS	
amino acid	0% INIA 415	0.1% INIA 415	0% INIA 415	0.1% INIA 415
Asp	nd[a]	125 ± 6^{b}	70 ± 3^{b}	421 ± 9^{c}
Ser	93 ± 3^{a}	426 ± 4^{b}	225 ± 5^{a}	722 ± 21^{c}
Glu	nd[a]	217 ± 15^{b}	nd[a]	782 ± 41^{c}
Gly	81 ± 0^{a}	164 ± 1^{a}	96 ± 1^{a}	460 ± 16^{b}
His	92 ± 3^{a}	1018 ± 7^{c}	570 ± 24^{b}	1683 ± 26^{d}
Arg	130 ± 3^{a}	258 ± 6^{b}	135 ± 1^{a}	319 ± 5^{c}
Thr	32 ± 2^{a}	177 ± 2^{c}	72 ± 0^{b}	205 ± 5^{d}
Ala	70 ± 1^{a}	223 ± 1^{c}	165 ± 1^{b}	298 ± 6^{d}
Pro	173 ± 1^{a}	379 ± 1^{c}	281 ± 4^{b}	550 ± 9^{d}
Cys	6 ± 1^{ab}	7 ± 1^{b}	3 ± 0^{a}	nd[a]
Tyr	166 ± 2^{a}	424 ± 1^{c}	244 ± 5^{b}	420 ± 6^{c}
Val	120 ± 4^{a}	618 ± 4^{c}	394 ± 10^{b}	901 ± 18^{d}
Met	22 ± 1^{a}	156 ± 2^{c}	45 ± 2^{b}	169 ± 7^{c}
Lys	336 ± 2^{a}	745 ± 5^{c}	441 ± 4^{b}	829 ± 20^{d}
Ile	nd[a]	176 ± 3^{c}	17 ± 1^{b}	228 ± 4^{d}
Leu	266 ± 8^{a}	1383 ± 5^{c}	641 ± 20^{b}	1951 ± 36^{d}
Phe	290 ± 7^{a}	798 ± 11^{c}	433 ± 14^{b}	1069 ± 17^{d}
total	1847 ± 35^{a}	7211 ± 18^{c}	3784 ± 90^{b}	10789 ± 208^{d}

[a] Mean ± SD of duplicate determinations in two cheese-making experiments; nd, below detection limit. Means in a row with different superscript letters are significantly different ($P < 0.05$).

Free Amino Acids. Addition of bacteriocin-producing adjunct culture, addition of thermophilic starter, and cheese age significantly ($P < 0.01$) increased levels of free amino acids in cheese (**Tables 5−7**). Levels of total free amino acids in cheese made with mesophilic starter and bacteriocin producer were 3.9-, 3.4-, and 3.6-fold those in cheese made only with mesophilic starter after 25, 50, and 75 days of ripening, respectively. Those increases were considerably higher than the 22−47% increases reported for Cheddar cheeses manufactured using *L. lactis* subsp. *lactis* DPC3286 as adjunct culture (*6*). Addition of thermophilic starter resulted in levels of total free amino acids 2.0-, 2.3-,

Table 6. Free Amino Acids in 50-Day-Old Cheeses Manufactured with Mesophilic Starter (MS), Thermophilic Starter (TS), and Bacteriocin-Producing *L. lactis* Subsp. *lactis* INIA 415

	mg/kg of dry matter[a]			
	1% MS		1% MS + 1% TS	
amino acid	0% INIA 415	0.1% INIA 415	0% INIA 415	0.1% INIA 415
Asp	84 ± 10[a]	362 ± 26[c]	264 ± 1[b]	492 ± 5[d]
Ser	148 ± 1[a]	586 ± 2[c]	390 ± 8[b]	832 ± 16[d]
Glu	94 ± 11[a]	201 ± 15[b]	70 ± 8[a]	1751 ± 82[c]
Gly	116 ± 1[a]	219 ± 1[a]	120 ± 1[a]	986 ± 11[b]
His	302 ± 15[a]	1449 ± 6[c]	1203 ± 27[b]	2778 ± 85[d]
Arg	212 ± 1[b]	465 ± 1[c]	189 ± 3[a]	468 ± 5[c]
Thr	66 ± 1[a]	234 ± 3[c]	123 ± 1[b]	316 ± 10[d]
Ala	101 ± 2[a]	288 ± 2[c]	240 ± 1[b]	440 ± 13[d]
Pro	185 ± 1[a]	517 ± 4[c]	471 ± 5[b]	824 ± 18[d]
Cys	6 ± 1[a]	4 ± 0[a]	4 ± 0[a]	10 ± 0[b]
Tyr	192 ± 3[a]	525 ± 4[c]	357 ± 5[b]	604 ± 15[d]
Val	224 ± 7[a]	945 ± 10[c]	775 ± 5[b]	1492 ± 34[d]
Met	57 ± 3[a]	254 ± 0[c]	113 ± 2[b]	303 ± 14[b]
Lys	358 ± 4[a]	995 ± 5[c]	630 ± 1[b]	1350 ± 44[d]
Ile	16 ± 2[a]	268 ± 2[c]	72 ± 4[b]	380 ± 11[d]
Leu	519 ± 15[a]	2072 ± 15[c]	1427 ± 11[b]	3027 ± 59[d]
Phe	489 ± 6[a]	1157 ± 12[c]	844 ± 9[b]	1575 ± 28[d]
total	3111 ± 79[a]	10432 ± 95[c]	7232 ± 76[b]	17143 ± 446[d]

[a] Mean ± SD of duplicate determinations in two cheese-making experiments. Means in a row with different superscript letters are significantly different ($P < 0.05$).

Table 7. Free Amino Acids in 75-Day-Old Cheeses Manufactured with Mesophilic Starter (MS), Thermophilic Starter (TS), and Bacteriocin-Producing *L. lactis* Subsp. *lactis* INIA 415

	mg/kg of dry matter[a]			
	1% MS		1% MS + 1% TS	
amino acid	0% INIA 415	0.1% INIA 415	0% INIA 415	0.1% INIA 415
Asp	270 ± 3[a]	468 ± 8[b]	265 ± 25[a]	457 ± 20[b]
Ser	238 ± 7[a]	755 ± 25[c]	589 ± 9[b]	760 ± 23[c]
Glu	74 ± 5[a]	895 ± 36[c]	345 ± 40[b]	1423 ± 34[d]
Gly	319 ± 12[b]	597 ± 5[c]	176 ± 7[a]	1007 ± 15[d]
His	314 ± 6[a]	3084 ± 5[c]	1931 ± 11[b]	3242 ± 7[d]
Arg	345 ± 5[a]	805 ± 2[c]	359 ± 3[a]	757 ± 0[b]
Thr	94 ± 3[a]	380 ± 2[d]	211 ± 1[b]	352 ± 2[c]
Ala	168 ± 3[a]	470 ± 1[c]	346 ± 1[b]	492 ± 4[d]
Pro	258 ± 2[a]	938 ± 3[c]	730 ± 5[b]	1025 ± 14[d]
Cys	13 ± 0[b]	17 ± 0[b]	11 ± 0[a]	54 ± 1[c]
Tyr	261 ± 9[a]	751 ± 1[d]	495 ± 5[b]	734 ± 6[c]
Val	402 ± 13[a]	1639 ± 6[c]	1191 ± 8[b]	1733 ± 27[d]
Met	116 ± 5[a]	448 ± 0[d]	207 ± 3[b]	379 ± 4[c]
Lys	567 ± 10[a]	1794 ± 3[d]	949 ± 5[b]	1740 ± 3[c]
Ile	64 ± 3[a]	502 ± 0[d]	246 ± 1[b]	492 ± 3[c]
Leu	964 ± 29[a]	3260 ± 8[c]	2330 ± 18[b]	3426 ± 42[d]
Phe	728 ± 16[a]	1708 ± 4[c]	1252 ± 10[b]	1719 ± 15[c]
total	5036 ± 111[a]	18216 ± 13[c]	11546 ± 131[b]	19296 ± 150[d]

[a] Mean ± SD of duplicate determinations in two cheese-making experiments. Means in a row with different superscript letters are significantly different ($P < 0.05$).

and 2.3-fold those reached in cheese made only with mesophilic starter after 25, 50, and 75 days of ripening, respectively. Within cheeses made with thermophilic starter, addition of bacteriocin producer resulted in levels of total free amino acids 2.9-, 2.4-, and 1.7-fold those found in cheese made without the bacteriocin producer at days 25, 50, and 75, respectively. From day 25 to day 75 the increase in total free amino acids was 2.7-fold in cheese made only with mesophilic starter, 2.5-fold in cheese made with mesophilic starter and bacteriocin producer, 3.1-fold in cheese made with mesophilic and thermophilic starters, and 1.8-fold in cheese made with both starters and the bacteriocin producer (**Tables 5**−**7**). The highest increases achieved in 75-day-old cheeses through the addition of both thermophilic starter and bacteriocin producer were those of Glu, His, and Ile (19.2-, 10.3-, and 7.7-fold, respectively). The high levels of free amino acids can be explained by a more rapid degradation of the peptides originating from casein when intracellular peptidases are released into the cheese matrix (*6*, *9*, *13*).

Table 8. Sensory Evaluation of Cheeses Manufactured with Mesophilic Starter (MS), Thermophilic Starter (TS), and Bacteriocin-Producing *L. lactis* Subsp. *lactis* INIA 415

		score[a]			
		1% MS		1% MS + 1% TS	
characteristic	age (days)	0% INIA 415	0.1% INIA 415	0% INIA 415	0.1% INIA 415
flavor quality	25	4.92 ± 0.49[a]	4.82 ± 0.98[a]	6.45 ± 0.62[b]	7.58 ± 0.16[c]
	50	4.94 ± 0.24[a]	4.81 ± 0.02[a]	6.01 ± 0.72[b]	7.53 ± 0.53[c]
	75	4.57 ± 0.57[a]	6.03 ± 0.55[b]	5.92 ± 0.55[b]	7.73 ± 0.43[c]
flavor intensity	25	5.09 ± 0.37[a]	5.29 ± 0.45[a]	5.68 ± 0.25[ab]	6.35 ± 0.08[b]
	50	5.42 ± 0.01[a]	5.75 ± 0.34[ab]	6.03 ± 0.02[ab]	6.69 ± 1.22[b]
	75	6.08 ± 0.07[a]	6.64 ± 0.40[ab]	6.31 ± 0.09[a]	7.29 ± 0.17[b]

[a] Mean ± SD from 16 trained panelists on a 0–10 point scale in two cheese-making experiments. Means in a row with different superscript letters are significantly different ($P < 0.05$).

Sensory Evaluation. In cheese made only with mesophilic starter, flavor quality and flavor intensity were not significantly affected by the addition of bacteriocin-producing adjunct culture (**Table 8**). Addition of thermophilic starter significantly enhanced flavor quality ($P < 0.001$) and flavor intensity ($P < 0.05$). Within cheeses made with thermophilic starter, addition of bacteriocin-producing adjunct culture resulted in significantly higher scores of both flavor quality and flavor intensity (**Table 8**). The highest scores for flavor quality and flavor intensity were those of the cheese made with mesophilic and thermophilic starters plus bacteriocin producer, which exhibited the highest levels of free amino acids (**Tables 5**−**7**). The relationship between the increase in flavor quality and flavor intensity scores and the increase in free amino acids has been reported for other cheese varieties (*4*, *6*, *13*, *33*). In our experiments flavor intensity increased as cheese aged, but flavor quality was unaffected.

There was a good correlation between flavor intensity scores and proteolysis, with r values of 0.81, 0.91, and 0.98, respectively, for 25-, 50-, and 75-day-old cheeses. For total free amino acids r values with flavor intensity scores were 0.79, 0.91, and 0.88, respectively, for 25-, 50-, and 75-day-old cheeses. For hydrophilic peptides r values with flavor intensity scores were 0.91, 0.96, and 0.66, respectively, for 25-, 50-, and 75-day-old cheeses. There was a negative correlation between flavor intensity scores and residual α_s-casein, with r values of −0.94, −0.96, and −0.73, respectively, for 25-, 50-, and 75-day-old cheeses, and between flavor intensity scores and residual β-casein, with r values of −0.97, −0.88, and −0.82, respectively, for 25-, 50-, and 75-day-old cheeses. The more pronounced proteolysis may have also influenced the integrity of the cheese matrix, favoring the release of flavor compounds during mastication (*5*).

Flavor quality scores correlated positively with proteolysis, with r values of 0.65, 0.79, and 0.99, respectively, for 25-, 50-, and 75-day-old cheeses. Correlation of flavor quality with total free amino acids had r values of 0.79, 0.91, and 0.88, respectively, for 25-, 50-, and 75-day-old cheeses, whereas with hydrophilic peptides it had r values of 0.79, 0.85, and 0.66,

respectively, for 25-, 50-, and 75-day-old cheeses. There was a negative correlation between flavor quality scores and residual α_s-casein, with r values of −0.99, −0.99, and −0.79, respectively, for 25-, 50-, and 75-day-old cheeses, and between flavor quality and residual β-casein, with r values of −0.99, −0.74, and −0.87, respectively, for 25-, 50-, and 75-day-old cheeses.

Linear regression equations of flavor intensity on days of ripening were calculated for each type of cheese. It was estimated from the respective regression equations that a flavor intensity score of 6 would be reached in 75, 54, 50, and 9 days, respectively, by cheeses made with mesophilic starter, mesophilic starter plus bacteriocin-producing adjunct culture, mesophilic and thermophilic starters, and mesophilic and thermophilic starters plus bacteriocin-producing adjunct culture.

Conclusion. From the results obtained in the present work, we concluded that the use of bacteriocin-producing *L. lactis* subsp. *lactis* INIA 415 as adjunct culture for a more rapid evolution of proteolysis and development of cheese flavor through the lysis of starter lactic acid bacteria and the subsequent release of intracellular enzymes is a simple and noncostly procedure. The bacteriocin producer had also a beneficial effect on flavor quality. The use of *L. lactis* subsp. *lactis* INIA 415 as adjunct culture instead of strains of bacteriocin-producing enterococci offers considerable advantages from the point of view of acceptability by the dairy industry.

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