Proteolysis, Volatile Compounds, and Sensory Evaluation in Hispánico Cheese Manufactured with the Addition of a Thermophilic Adjunct Culture, Nisin, and Calcium Alginate-Nisin Microparticles

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ABSTRACT

Nisin, free or incorporated in calcium alginate microparticles, was added to pasteurized milk (80% cows' and 20% ewes' milk) used for the manufacture of Hispánico cheese with a mesophilic starter and a thermophilic adjunct culture of high aminopeptidase activity. Addition of nisin incorporated in microparticles promoted early lysis of thermophilic adjunct culture bacteria. Extracellular aminopeptidase activity in 1-d-old cheese made with both thermophilic adjunct culture and nisin incorporated in microparticles was 1.8-fold higher than in cheese made with thermophilic adjunct culture and free nisin and 2.0-fold higher than in cheese made only with thermophilic adjunct culture without any addition of nisin. Addition of nisin, free or incorporated in microparticles, did not influence cheese proteolysis measured using hydrophilic or hydrophobic peptides, *o*-phthaldialdehyde ripening index, or free amino acids. Moreover, a total of 37 volatile compounds were identified in the volatile fraction of Hispánico cheese, using a dynamic headspace technique with a purge and trap system followed by a gas chromatography mass spectrometry analysis. The volatile compound profile was not influenced by nisin addition, either free or incorporated in microparticles, but addition of thermophilic adjunct culture enhanced the formation of 2-butanone, diacetyl, 2,3-pentanedione and acetoin and improved the flavor quality (sensory panel) of cheese.

(Key words: proteolysis, volatile compound, nisin microparticle, Hispánico cheese)

Abbreviation key: OPA = *o*-phthaldialdehyde.

INTRODUCTION

Lactic acid bacteria are the main source of enzymes in a wide variety of cheeses. Their proteinases and pep-

tidases transform caseins into small peptides and free amino acids (Kunji et al., 1996; Lane and Fox, 1997), which contribute to cheese flavor and serve as aroma precursors (Engels and Visser, 1996; Fox and Wallace, 1997). Peptidases and other enzymes such as esterases and amino acid catabolic enzymes are located within the cell. Therefore, the lysis of starter bacteria should favor the access of those enzymes to their substrates and may accelerate the development of cheese flavor and hence cheese ripening (Morgan et al., 1997; Garde et al., 1997). Bacteriocin-producing adjunct cultures have already been used to enhance the lysis of starter bacteria during ripening (Morgan et al., 1997; Garde et al., 1997; Martínez-Cuesta et al., 1998; Oumer et al., 2001b; Martínez-Cuesta et al., 2001; Garde et al., 2002a) to accelerate the maturation of cheese.

Addition of a bacteriocin-producing adjunct culture may retard starter growth and consequently lactic acid production and affect cheese sensory characteristics (Morgan et al., 1997; Garde et al., 1997; Oumer et al., 2001b). The delay in lactic acid production would increase when bacteriocins in free form are added to milk, compared with the gradual production of bacteriocins in the curd by adjunct cultures.

An alternative to the addition of free bacteriocins is the use of microencapsulated bacteriocins (Degnan et al., 1993; Wan et al., 1997; Benech et al., 2002). Encapsulation of pediocin in liposomes limited the degree of bacteriocin inactivation in model food systems, but the encapsulation efficiency was relatively low (Degnan et al., 1993). A considerably higher encapsulation efficiency in liposomes, up to 47%, was achieved for nisin Z (Benech et al., 2002). Cheeses made with encapsulated nisin contained after 6 mo less than 10 cfu/g of *Listeria innocua* and 90% of the initial nisin activity, compared with 10⁴ cfu/g and only 12% of initial nisin activity in cheeses made with a nisinogenic starter. Incorporation of nisin in calcium alginate microparticles increased the incorporation efficiency up to 87 to 93% (Wan et al.,

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1997). Nisin incorporated in microparticles was 100% active against an indicator culture in both MRS broth and reconstituted skim milk.

In previous works carried out at our laboratory (Oumer et al., 2001a; Garde et al., 2002a), *Streptococcus thermophilus* INIA 463 and INIA 468 showed a high aminopeptidase activity in milk compared with other strains and higher sensitivity to nisin than to other bacteriocins. On that basis, both strains were selected as a source of aminopeptidases to accelerate cheese ripening. In the present work, the addition of nisin, both free and incorporated in calcium alginate microparticles, to pasteurized milk used for the manufacture of Hispánico cheese was tested. Its effects on the viability of mesophilic starter and thermophilic adjunct culture, release of intracellular enzymes, proteolysis, formation of volatile compounds, and sensory characteristics of the cheese are investigated.

MATERIALS AND METHODS

Nisin Incorporation into Calcium Alginate Microparticles

Nisaplin (Aplin and Barrett Ltd, Beaminster, UK) and sodium alginate at 1:4 ratio (wt/wt) were dry-mixed. Sterile 50 g of CaCl_2/L solution in distilled water was sprayed onto the dry mixture while stirring, until calcium alginate macroparticles were formed. Preparation of microparticles and determination of incorporation efficiency were carried out as described by Wan et al. (1997). Incorporation efficiency was expressed as the percentage of nisin activity remaining in the microparticles compared with the total nisin activity of the original suspension in sterile distilled water. Control calcium alginate microparticles were prepared by the same procedure but without the addition of nisin. Nisin activity was determined in triplicate by the critical dilution method by an agar diffusion assay using *Lactobacillus buchneri* St2A as indicator strain and expressed as IU/ml (Nuñez et al., 1996).

Lactic Cultures and Cheese Manufacture

Lactococcus lactis subsp. *lactis* INIA 437 and *L. lactis* subsp. *cremoris* INIA 450, both from the culture collection of INIA (Madrid, Spain), were used as mesophilic starter. They were stored 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. Two *Streptococcus thermophilus* strains, INIA 463 and INIA 468, were used as thermophilic adjunct culture with a high aminopeptidase activity. They were stored 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 as previously described (Garde et al., 2002a). Each experiment consisted of four 45-L vats. *Lactococcus lactis* subsp. *lactis* INIA 437 and *L. lactis* subsp. *cremoris* INIA 450 cultures were added each at 5 ml/L to all vats. Moreover, *S. thermophilus* INIA 463 and INIA 468 cultures in milk were added each at 5 ml/L to vats 2, 3 and 4 (vat 1 was used as control). Nisin in the form of Nisaplin was added to vat 3 (4 IU/ml) and nisin incorporated in calcium alginate microparticles to vat 4 (8 IU/ml). Rennet was added 20 min after lactic culture inoculation. Cheeses were ripened at 12°C for 75 d.

Microbiological Analysis and Cheese pH Value

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 at 37°C for 48 h aerobically. Previous trials had shown that small size colonies were thermophilic lactic acid bacteria and large size colonies were mesophilic lactic acid bacteria (Garde et al., 2002a).

Cheese pH value was measured in duplicate after homogenizing 10 g of cheese with 20 ml of distilled water at 70°C by means of a Stomacher 400 (Seward Laboratory, London, UK).

Determination of Aminopeptidase Activity and Cheese Proteolysis

Aminopeptidase activity released into the cheese was measured on duplicate samples with lysine *p*-nitroanilide and leucine *p*-nitroanilide as substrates (Garde et al., 1997). One activity unit corresponds to the activity of enzymes producing 1 nmol of *p*-nitroaniline per minute per gram of cheese.

Overall cheese proteolysis (cheese ripening index) was determined on duplicate samples by the *o*-phthaldialdehyde (OPA) method, based on the reaction of released α -amino groups with this compound and with β -mercaptoethanol to form a derivative that absorbs at 340 nm (Church et al., 1983).

Hydrophilic and hydrophobic peptides in the water-soluble fraction of cheese were determined on duplicate samples by reversed-phase-HPLC using a Beckman System Gold chromatograph (Beckman Instruments, Madrid, Spain) equipped with a diode array detector module 168, with detection wavelength at 280 nm, as previously described (Gómez et al., 1997). Results were expressed as units of chromatogram area per milligrams of cheese DM.

Individual free amino acids were extracted from duplicate samples of cheese using the method of Krause et al. (1995) and determined by reversed-phase HPLC using a Beckman System Gold chromatograph, after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as described by Liu et al. (1995). Results were expressed as grams per kilogram of cheese DM.

Analysis of Volatile Compounds

Cheese pieces wrapped in aluminium foil were vacuum packed and frozen at -40°C until analysis. Prior to volatile extraction, frozen pieces were kept overnight at 4°C , and then left stabilized at room temperature. An automatic dynamic headspace apparatus (Purge and Trap, HP 7695, Hewlett-Packard, Palo Alto, CA) connected to a gas chromatograph-mass spectrometer (HP 6890, Hewlett-Packard) was used for volatile compounds analysis. Duplicate 15-g cheese samples were homogenized in an analytical grinder (IKA, Labortechnik, Staufen, Germany), with 20 g of Na_2SO_4 and 75 μl of an aqueous solution containing 0.5 mg/ml cyclohexanone (IS_1) and camphor (IS_2) as internal standards. The analytical procedure and the identification and relative quantification of volatile compounds were carried out as described by Oumer et al. (2000).

Sensory Evaluation

Flavor intensity and flavor quality (preference test) of 25-, 50- and 75-d-old cheeses from duplicate experiments were evaluated by 14 trained panelists on a 10-point scale as previously described (Fernández del Pozo et al., 1988).

Statistical Analysis

Analyses of variance with 1) nisin addition, 2) thermophilic adjunct culture addition, and 3) cheese age as independent variables were performed by means of SPSS Win 8.0 program. Comparison of means between cheeses of the same age was carried out using Tukey's test (Steel and Torrie, 1980).

Two-tailed Pearson correlations between proteolysis (OPA test), peptides, total free amino acids, and flavor intensity or flavor quality were performed by means of SPSS Win 8.0 program. Principal component analysis (PCA) with Varimax rotation was carried out on selected volatile compounds and sensory characteristics by means of SPSS Win 8.0 program.

RESULTS AND DISCUSSION

Efficiency of Nisin Incorporation

Calcium alginate-nisin microparticles (60 mg) suspended in 10 ml of sterile distilled water presented a calculated concentration of 1200 IU/ml. After centrifugation of the suspension, only 75 IU/ml of nisin activity was detected in the supernatant and apparently 100% of nisin activity was retained in the resuspended microparticles. In the supernatant of the free nisin preparation, 100% of nisin activity was detected. Calcium alginate microparticles without addition of nisin did not produce any zone of growth inhibition in the indicator lawn culture. From the results obtained for supernatants, efficiency of nisin incorporation in calcium alginate microparticles was calculated to be 94%. A similar result was obtained by Wan et al. (1997).

Cheese pH Value and Lactic Acid Bacteria

Addition of nisin, either free or incorporated in microparticles, did not significantly affect the pH value of cheese (Table 1). In some previous works (Morgan et al., 1997; Garde et al., 1997; Oumer et al., 2001b), the addition of bacteriocin-producing adjunct culture retarded lactic acid production in cheese. However, Garde et al. (2002a) did not find significant differences between the pH values of cheeses made from milk inoculated with a bacteriocin producer and those of cheeses made without bacteriocin producer. The pH value of cheese was significantly influenced ($P < 0.001$) by ripening (Table 1).

Growth and viability of mesophilic starter lactic acid bacteria were scarcely influenced by the addition of nisin, either free or incorporated in microparticles (Table 2). Inversely, during the first 15 d, counts of thermophilic lactic acid bacteria were significantly ($P < 0.05$) lower in cheeses made with nisin, free or incorporated in microparticles, than in cheese made without nisin (Table 2). Cheese made with nisin incorporated in microparticles exhibited the lowest counts of thermophilic lactic acid bacteria. Garde et al. (2002a) also found lower concentrations of thermophilic lactic acid bacteria in cheese made with *L. lactis* subsp. *lactis* INIA 415, a strain harboring the structural genes of nisin Z and lactacin 481, than in control cheese.

Release of Intracellular Enzymes

Addition of nisin, free or incorporated in microparticles, addition of thermophilic adjunct culture, as well as cheese age significantly ($P < 0.001$) influenced aminopeptidase activity in cheese (Table 3). Cheeses made

Table 1. Changes in pH value during the ripening of cheeses manufactured with a mesophilic starter (MS), a thermophilic adjunct culture (TA), and nisin, in free form or incorporated in microparticles (MP).

	Age (days)	10 ml/L MS	10 ml/L MS + 10 ml/L TA	10 ml/L MS + 10 ml/L TA Free nisin	10 ml/L MS + 10 ml/L TA MP nisin
Cheese pH ¹	1	4.93 ± 0.02	5.04 ± 0.04	4.98 ± 0.01	4.98 ± 0.02
	7	5.07 ± 0.09	5.15 ± 0.03	5.14 ± 0.01	5.16 ± 0.05
	15	5.05 ± 0.11	5.13 ± 0.03	5.11 ± 0.07	5.10 ± 0.03
	25	5.06 ± 0.01	5.08 ± 0.04	5.08 ± 0.01	5.04 ± 0.09
	50	5.06 ± 0.09	5.13 ± 0.02	5.09 ± 0.04	5.02 ± 0.03
	75	5.04 ± 0.01	5.12 ± 0.02	5.10 ± 0.02	5.07 ± 0.01

¹Mean ± SD (n = 4 replicates) of duplicate determinations in two cheese-making experiments.

with thermophilic adjunct culture showed considerably higher values of aminopeptidase activity than cheese made without thermophilic adjunct culture. In the absence of nisin, aminopeptidase activity values on lysine-*p*-nitroanilide and leucine-*p*-nitroanilide in cheese made with thermophilic adjunct culture on d 1 were 14-fold and 21-fold, respectively, those found in cheese made without thermophilic adjunct culture. Similar results were reported by Garde et al. (2002a) for cheese made with a nisin-producing adjunct culture. The presence of two additional peptidases in *S. thermophilus* with respect to *L. lactis* (Rul and Monnet, 1997) should explain the higher aminopeptidase activity. The latter increased during the first 15 d of ripening in cheeses made with nisin and during the first 50 d in cheese made with thermophilic adjunct culture but without nisin. In cheeses made with thermophilic adjunct culture, aminopeptidase activity was higher during the first 15 d of ripening when nisin was added, both free and incorporated in microparticles. Aminopeptidase ac-

tivity in 1-d-old cheese made from milk with nisin incorporated in microparticles almost doubled that found in the respective cheese made from milk with free nisin (Table 3). Nisin incorporated in microparticles probably was retained in the curd in a higher proportion than free nisin but, due to its reduced availability compared with free nisin, had a retarded effect on the thermophilic adjunct culture. This might have allowed the thermophilic adjunct culture to reach a high population in the curd that, when lysed, would have resulted in the higher aminopeptidase activity of the respective 1-d-old cheese. In fact, cheese made with thermophilic adjunct culture and nisin incorporated in microparticles reached on d 1 the same aminopeptidase activity on lysine-*p*-nitroanilide that cheese made with thermophilic adjunct culture but without nisin did on d 15. The increase in aminopeptidase activity due to bacteriocin-mediated lysis of lactic acid bacteria is in agreement with previous reports (Morgan et al., 1997; Garde et al., 1997; Oumer et al., 2001b; Garde et al., 2002a).

Table 2. Levels of mesophilic and thermophilic lactic acid bacteria (LAB) during ripening of cheeses manufactured with a mesophilic starter (MS), a thermophilic adjunct culture (TA), and nisin, in free form or incorporated in microparticles (MP).

	Age (days)	10 ml/L MS	10 ml/L MS + 10 ml/L TA	10 ml/L MS + 10 ml/L TA Free nisin	10 ml/L MS + 10 ml/L TA MP nisin
Mesophilic	1	9.62 ± 0.01 ^b	9.11 ± 0.26 ^a	9.39 ± 0.07 ^{ab}	9.53 ± 0.05 ^b
	7	9.54 ± 0.11 ^b	9.07 ± 0.29 ^a	9.21 ± 0.09 ^{ab}	9.34 ± 0.02 ^{ab}
	15	9.52 ± 0.01 ^b	9.03 ± 0.23 ^a	9.29 ± 0.10 ^{ab}	9.35 ± 0.10 ^b
	25	9.27 ± 0.02 ^b	9.11 ± 0.05 ^a	9.26 ± 0.05 ^b	9.24 ± 0.02 ^b
	50	8.76 ± 0.11 ^a	9.08 ± 0.03 ^{ab}	9.19 ± 0.06 ^b	9.23 ± 0.29 ^b
	75	7.97 ± 0.11	8.64 ± 0.43	8.87 ± 0.41	8.71 ± 0.37
Thermophilic	1	ND	8.70 ± 0.08 ^b	7.18 ± 0.06 ^a	7.03 ± 0.21 ^a
	7	ND	8.97 ± 0.13 ^c	8.18 ± 0.16 ^b	7.83 ± 0.13 ^a
	15	ND	9.01 ± 0.08 ^b	8.46 ± 0.44 ^a	8.22 ± 0.11 ^a
	25	ND	8.24 ± 0.30 ^b	7.91 ± 0.13 ^{ab}	7.76 ± 0.19 ^a
	50	ND	<7	<7	<7
	75	ND	<6	<6	<6

^{a,b,c}Means in the same row with different superscripts differ ($P < 0.05$).

¹Mean ± SD (n = 4 replicates) of duplicate determinations in two cheese-making experiments of lactic acid bacteria, expressed as log cfu/g of cheese. ND = not detected.

Table 3. Aminopeptidase activity during ripening of cheeses manufactured with a mesophilic starter (MS), a thermophilic adjunct culture (TA), and nisin, in free form or incorporated in microparticles (MP).

Substrate	Age (days)	10 ml/L MS	10 ml/L MS + 10 ml/L TA	10 ml/L MS + 10 ml/L TA Free nisin	10 ml/L MS + 10 ml/L TA MP nisin
Lys- <i>p</i> -NA ¹	1	0.45 ± 0.06 ^a	6.50 ± 0.15 ^b	7.15 ± 0.31 ^{bc}	13.00 ± 0.68 ^c
	7	1.11 ± 0.46 ^a	7.76 ± 0.82 ^b	10.62 ± 1.93 ^c	12.36 ± 2.31 ^c
	15	1.87 ± 0.43 ^a	12.75 ± 0.29 ^b	25.78 ± 2.10 ^c	23.15 ± 2.79 ^c
	25	2.19 ± 0.85 ^a	18.31 ± 2.39 ^b	15.92 ± 4.38 ^b	16.00 ± 1.60 ^b
	50	2.10 ± 0.78 ^a	20.85 ± 2.39 ^b	18.87 ± 4.90 ^b	17.14 ± 3.40 ^b
	75	3.34 ± 0.46 ^a	13.53 ± 3.39 ^b	13.53 ± 3.69 ^b	12.60 ± 1.00 ^b
Leu- <i>p</i> -NA ¹	1	0.35 ± 0.06 ^a	7.23 ± 0.66 ^b	9.04 ± 0.76 ^c	13.69 ± 0.67 ^d
	7	0.73 ± 0.30 ^a	8.25 ± 0.22 ^b	14.47 ± 4.44 ^c	18.38 ± 4.36 ^c
	15	1.18 ± 0.18 ^a	16.90 ± 0.50 ^{ab}	35.15 ± 3.15 ^c	33.88 ± 2.68 ^c
	25	1.05 ± 0.30 ^a	27.71 ± 1.81 ^b	23.84 ± 4.31 ^b	24.68 ± 4.57 ^b
	50	0.53 ± 0.18 ^a	38.12 ± 1.92 ^b	36.42 ± 3.57 ^b	35.48 ± 5.67 ^b
	75	1.55 ± 0.56 ^a	29.62 ± 4.20 ^b	28.91 ± 4.02 ^b	26.68 ± 2.06 ^b

^{a,b,c,d}Means in the same row with different superscripts differ ($P < 0.05$).

¹Mean ± SD of ($n = 4$ replicates) duplicate determinations in two cheese-making experiments of aminopeptidase activity, expressed as nmol of *p*-nitroaniline (NA) released per minute per gram of cheese.

Proteolysis

Cheese proteolysis as determined by the OPA test (Table 4) increased significantly ($P < 0.001$) with ripening. Addition of nisin, free or incorporated in microparticles, and addition of thermophilic adjunct culture scarcely influenced cheese proteolysis.

Levels of hydrophobic and hydrophilic peptides in the water-soluble fraction of cheese increased significantly ($P < 0.001$) with cheese age (Table 5), but addition of nisin or addition of thermophilic adjunct culture did not influence hydrophobic or hydrophilic peptides. The level of hydrophobic peptides determined at 280 nm correlates well with mean panel bitterness scores in Hispánico cheese made from pasteurized milk (Gómez et al., 1997). In the present work, the levels of hydrophobic peptides were (mean values from all vats) 0.71 after 25 d, 1.05 after 50 d, and 1.84 after 75 d, whereas the respective values for hydrophilic peptides were 6.63, 7.84, and 10.97 (Table 5). In a previous work on the

same cheese variety (Garde et al., 2002a), mean levels of hydrophobic peptides were higher (2.80, 3.05, and 2.49 after 25, 50, and 75 d, respectively), whereas levels of hydrophilic peptides were similar (6.16, 9.83, and 11.90 after 25, 50, and 75 d, respectively). Aminopeptidase activity was higher on average in the present work (Table 3) than in the work by Garde et al. (2002a), a fact that would account for the low level of hydrophobic peptides (Figure 1) found in the present work.

Ripening significantly ($P < 0.001$) increased cheese content in total free amino acids, independently on the addition of nisin or the addition of thermophilic adjunct culture (Table 6). Concentrations of total free amino acids were (mean values from all vats) 5.40, 8.75, and 12.79 g/kg cheese DM after 25, 50, and 75 d, respectively; values comparable with those recorded for Hispánico cheese (Garde et al., 2002a), which were on average 5.91, 9.48, and 13.52 g/kg cheese DM after the same ripening time. Individual free amino acid concentra-

Table 4. Overall proteolysis (OPA index)¹ during ripening of cheeses manufactured with a mesophilic starter (MS), a thermophilic adjunct culture (TA) and nisin, in free form or incorporated in microparticles (MP).

	Age (days)	10 ml/L MS	10 ml/L MS + 10 ml/L TA	10 ml/L MS + 10 ml/L TA Free nisin	10 ml/L MS + 10 ml/L TA MP nisin
A _{340nm} ²	1	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
	7	0.33 ± 0.01 ^b	0.30 ± 0.02 ^a	0.29 ± 0.01 ^a	0.29 ± 0.01 ^a
	15	0.40 ± 0.04 ^a	0.46 ± 0.01 ^b	0.45 ± 0.01 ^{ab}	0.42 ± 0.03 ^{ab}
	25	0.63 ± 0.14	0.63 ± 0.02	0.62 ± 0.02	0.57 ± 0.05
	50	0.94 ± 0.10 ^{ab}	1.07 ± 0.01 ^b	1.02 ± 0.09 ^{ab}	0.90 ± 0.01 ^a
	75	1.25 ± 0.14	1.37 ± 0.03	1.39 ± 0.07	1.25 ± 0.01

^{a,b}Means in the same row with different superscripts differ ($P < 0.05$).

¹OPA = *ortho*-phthaldialdehyde.

²Mean ± SD ($n = 4$ replicates) of duplicate determinations in two cheese-making experiments of proteolysis, expressed as absorbance at 340 nm.

Table 5. Hydrophilic and hydrophobic peptides determined at 280 nm and the ratio of hydrophobic peptides to hydrophilic peptides in the water-soluble fraction of cheeses manufactured with a mesophilic starter (MS), a thermophilic adjunct culture (TA), and nisin, in free form or incorporated in microparticles (MP).

Peptides ¹	Age (days)	10 ml/L MS	10 ml/L MS + 10 ml/L TA	10 ml/L MS + 10 ml/L TA Free nisin	10 ml/L MS + 10 ml/L TA MP nisin
Hydrophobic	25	0.77 ± 0.01 ^d	0.64 ± 0.01 ^a	0.69 ± 0.01 ^b	0.74 ± 0.01 ^c
	50	1.09 ± 0.02 ^b	1.23 ± 0.01 ^c	1.27 ± 0.01 ^c	0.61 ± 0.01 ^a
	75	1.85 ± 0.01	1.81 ± 0.15	1.70 ± 0.16	1.98 ± 0.01
Hydrophilic	25	7.06 ± 0.04 ^c	6.94 ± 0.04 ^c	6.40 ± 0.04 ^b	6.12 ± 0.03 ^a
	50	8.09 ± 0.01 ^b	8.74 ± 0.04 ^c	8.14 ± 0.08 ^b	6.37 ± 0.01 ^a
	75	10.95 ± 0.04 ^b	12.44 ± 0.17 ^c	10.67 ± 0.09 ^b	9.82 ± 0.03 ^a
Ratio	25	0.11 ± 0.01 ^b	0.10 ± 0.01 ^a	0.11 ± 0.01 ^{ab}	0.12 ± 0.01 ^c
	50	0.13 ± 0.01 ^b	0.14 ± 0.01 ^b	0.16 ± 0.01 ^c	0.10 ± 0.01 ^a
	75	0.17 ± 0.01 ^{ab}	0.15 ± 0.01 ^a	0.16 ± 0.01 ^a	0.20 ± 0.01 ^b

^{a,b,c,d}Means in the same row with different superscripts differ ($P < 0.05$).

¹Mean ± SD (n = 4 replicates) of duplicate determinations in two cheese-making experiments, expressed as units of chromatogram area/mg cheese DM.

tions were not influenced by addition of nisin, free or incorporated in microparticles (data not shown). Alanine was the single amino acid influenced by addition of thermophilic adjunct culture, with a concentration of 0.25 g/kg in 50-d-old cheese made without thermophilic adjunct culture and 0.33 g/kg as the average value for 50-d-old cheeses made with thermophilic adjunct culture.

Volatile Compounds

A total of 37 volatile compounds were identified using the dynamic headspace analysis of Hispánico cheese,

including mainly hydrocarbons, alcohols, ketones, aldehydes, acids, and esters. Eighteen peaks, two of which corresponded to a pair of volatile compounds chromatographically not separated, were not significantly ($P < 0.05$) influenced by the addition of nisin, the addition of thermophilic adjunct culture, or cheese age. Overall mean values of the relative abundances of those compounds (calculated from all ages and all vats) were 0.45 for heptane, 2.13 for cyclohexane, 1.49 for octane, 15.61 for acetone, 2.74 for ethyl acetate, 1.37 for 2-hexanone + 2-hexanal (chromatographically not separated), 0.34 for ethylbenzene, 0.25 for *p*-xylene, 1.01 for *m*-xylene

Figure 1. Reversed-phase HPLC chromatogram of the water-soluble fraction of a 75-d-old Hispánico cheese manufactured with a mesophilic starter and a thermophilic adjunct culture, without any addition of nisin, at a detection wavelength of 280 nm.

Table 6. Total free amino acids during ripening of cheeses manufactured with a mesophilic starter (MS), a thermophilic adjunct culture (TA), and nisin, in free form or incorporated in microparticles (MP).

	Age (days)	10 ml/L MS	10 ml/L MS + 10 ml/L TA	10 ml/L MS + 10 ml/L TA Free nisin	10 ml/L MS + 10 ml/L TA MP nisin
Amino acids ¹	25	5.69 ± 0.05 ^b	5.68 ± 0.40 ^b	5.69 ± 0.46 ^b	4.55 ± 0.04 ^a
	50	8.62 ± 0.13 ^{ab}	9.80 ± 1.48 ^c	8.90 ± 0.53 ^{ab}	7.69 ± 0.03 ^a
	75	12.66 ± 0.22 ^{ab}	14.59 ± 2.06 ^b	13.19 ± 0.60 ^b	10.71 ± 0.13 ^a

^{a,b,c}Means in the same row with different superscripts differ ($P < 0.05$).

¹Mean ± SD (n = 4 replicates) of duplicate determinations in two cheese-making experiments, expressed as g/kg cheese DM.

+ 1-butanol (chromatographically not separated), 3.14 for 2-heptanone, 0.30 for *o*-xylene, 0.72 for 3-methyl-2-buten-1-ol, 0.45 for 2-nonanone, 0.26 for nonanal, 0.10 for 1-heptanol, 0.10 for acetic acid, 0.11 for 1-octanol, and 0.19 for butanoic acid.

Ten further volatile compounds were significantly ($P < 0.05$) influenced by cheese age, but not by the addition of thermophilic adjunct culture or nisin. Their relative abundances (mean values from all vats) at 25 and 75 d, respectively, were 0.95 and 0.59 for acetaldehyde, 4.48 and 7.23 for 2-propanol, 306 and 362 for ethanol, 3.14 and 8.29 for 2-pentanone, 0.13 and 0.41 for ethyl butanoate, not detected and 0.40 for 1-propanol, 8.27 and 2.41 for toluene, 0.35 and 0.54 for 2-methyl-1-pro-

panol, 0.14 and 0.41 for 2-pentanol, and 0.22 and 0.76 for ethyl hexanoate.

As expected from previous results (no influence of nisin on proteolysis and concentration of free amino acids), nisin addition did not significantly change the volatile compound profile of cheese. However, the addition of thermophilic adjunct culture had a significant ($P < 0.05$) effect on the level of seven volatile compounds (Figure 2). Their relative abundances are shown in Table 7. The level of 3-methylbutanal was slightly higher in cheese made without thermophilic starter. This aldehyde originates from Leu by transamination or Strecker degradation (Christensen et al., 1999) and is responsible for unclean and harsh flavors in Cheddar cheese

Figure 2. Gas chromatogram corresponding to the headspace of a 75-d-old Hispánico cheese made with a mesophilic starter and a thermophilic adjunct culture, without any addition of nisin. Peaks were: 7, 2-butanone; 8, 3-methylbutanal; 12, 2,3-butanedione; 16, 2,3-pentanedione; 25, 3-methyl-1-butanol; 27, 3-methyl-3-buten-1-ol; 28, 3-hydroxy-2-butanone; CY, cyclohexanone; CA, camphor. See Materials and Methods for chromatographic conditions.

Table 7. Volatile compounds in cheeses manufactured with a mesophilic starter (MS), a thermophilic adjunct culture (TA), and nisin, in free form or incorporated in microparticles (MP), influenced by TA.

Volatile compound	10 ml/L MS	10 ml/L MS + 10 ml/L TA	10 ml/L MS + 10 ml/L TA Free nisin	10 ml/L MS + 10 ml/L TA MP nisin
3-Methylbutanal	0.76 ± 0.10 ^b	0.53 ± 0.08 ^a	0.52 ± 0.07 ^a	0.52 ± 0.08 ^a
3-Methyl-1-butanol	0.44 ± 0.07 ^a	0.50 ± 0.08 ^{ab}	0.54 ± 0.10 ^b	0.60 ± 0.11 ^b
3-Methyl-3-buten-1-ol	1.75 ± 0.30 ^b	1.38 ± 0.12 ^a	1.43 ± 0.14 ^a	1.46 ± 0.27 ^a
2-Butanone	1.14 ± 0.14 ^a	1.53 ± 0.24 ^b	1.28 ± 0.17 ^{ab}	1.52 ± 0.20 ^b
2,3-Butanedione	7.77 ± 0.89 ^a	19.60 ± 2.51 ^b	19.05 ± 3.19 ^b	13.63 ± 1.21 ^{ab}
2,3-Pentanedione	0.35 ± 0.05 ^a	1.11 ± 0.13 ^b	0.90 ± 0.11 ^b	0.98 ± 0.20 ^b
3-Hydroxy-2-butanone	0.69 ± 0.14 ^a	1.59 ± 0.21 ^{bc}	2.17 ± 0.32 ^c	1.09 ± 0.09 ^{ab}

^{a,b,c}Means in the same row with different superscripts differ ($P < 0.05$).

¹Mean ± SD ($n = 12$ replicates) of duplicate determinations in three ages (25, 50, and 75 d) in two cheese-making experiments, expressed as relative abundance to cyclohexanone.

(Dunn and Lindsay, 1985) and for a spicy chocolate-like flavor in Gouda-type cheese (Ayad et al., 2000), in Proosdij cheese (Engels et al., 1997), in Saint-Paulin cheese (Saboya et al., 2001), in Emmental cheese (Rychlik et al., 1997) as well as in Gruyère cheese (Rychlik and Bosset, 2001a,b). On the contrary, cheeses made with thermophilic adjunct culture showed slightly higher levels of 3-methyl-1-butanol. Formation of this alcohol from 3-methylbutanal through the activity of lactic acid bacteria dehydrogenases should be favored by the strong reducing conditions present in cheese (Molimard and Spinnler, 1996; Engels et al., 1997). 3-Methyl-1-butanol has a fruity and alcohol flavor note (Molimard and Spinnler, 1996). This alcohol has also been associated with unclean and harsh flavor in Cheddar cheese (Dunn and Lindsay, 1985). The level of 3-methyl-3-buten-1-ol was slightly higher in cheese made without thermophilic starter. The relative abundances of 2-butanone, 2,3-butanedione, 2,3-pentanedione and 3-hydroxy-2-butanone were higher in cheeses made with thermophilic adjunct culture. Diacetyl (2,3-butanedione) originates from the unstable precursor α -acetolactate during citrate metabolism and is one of the major aromatic compounds in fermented milk and fresh

cheese (Molimard and Spinnler, 1996). The higher levels of acetoin (3-hydroxy-2-butanone) and 2-butanone in cheeses made with thermophilic adjunct culture were to be expected, since diacetyl reduction generates acetoin, and acetoin reduction generates 2-butanone. 2,3-Pentanedione might be formed from α -aceto- α -hydroxybutyrate, an intermediate of the isoleucine metabolism (Imhof et al., 1995). Some *S. thermophilus* strains have been reported to produce high levels of diacetyl and 2,3-pentanedione (Imhof et al., 1995). Garde et al. (2002b) also recorded higher levels of diacetyl and 2,3-pentanedione in cheeses made with thermophilic adjunct culture than in cheeses made without thermophilic adjunct culture.

Sensory Evaluation

Flavor intensity and flavor quality were not significantly influenced by the addition of nisin, free or incorporated in microparticles (Table 8). Addition of thermophilic adjunct culture significantly enhanced flavor quality ($P < 0.001$), but did not influence flavor intensity. Cheese age influenced flavor intensity ($P < 0.001$), which tended to increase during ripening of cheeses.

Table 8. Sensory evaluation of cheeses manufactured with a mesophilic starter (MS), a thermophilic adjunct culture (TA), and nisin, in free form or incorporated in microparticles (MP).

Characteristic ¹	Age (days)	10 ml/L MS	10 ml/L MS + 10 ml/L TA	10 ml/L MS + 10 ml/L TA Free nisin	10 ml/L MS + 10 ml/L TA MP nisin
Flavor quality	25	5.43 ± 0.94 ^a	6.15 ± 1.09 ^b	6.27 ± 1.06 ^b	6.50 ± 0.80 ^b
	50	5.67 ± 1.32	6.48 ± 1.41	6.49 ± 1.44	6.38 ± 1.33
	75	5.75 ± 0.85 ^a	6.67 ± 1.34 ^b	6.37 ± 1.10 ^{ab}	6.43 ± 0.81 ^{ab}
Flavor intensity	25	5.69 ± 1.24	5.82 ± 1.21	5.71 ± 1.26	5.79 ± 1.50
	50	5.99 ± 0.90	6.17 ± 0.79	6.21 ± 0.85	6.24 ± 1.02
	75	6.04 ± 1.30	6.33 ± 1.22	6.08 ± 1.65	6.16 ± 1.25

^{a,b}Means in the same row with different superscripts differ ($P < 0.05$).

¹Mean ± SD ($n = 28$ replicates) of scores from 14 trained panelists in two cheese-making experiments using a 10-point scale.

Table 9. Correlation coefficients for volatile compounds and sensory characteristics with the functions in the principal component analysis (PCA) with Varimax rotation.

	Components of PCA	
	1	2
Acetaldehyde	-0.281	0.093
2-Butanone	0.231	0.292
3-Methylbutanal	0.796	-0.288
2-Propanol	0.233	-0.433
Ethanol	0.546	0.169
2-Pentanone	0.688	-0.037
2,3-Butanedione	-0.260	0.714
Ethyl butanoate	0.880	0.041
1-Propanol	0.822	-0.079
Toluene	-0.318	-0.352
2,3-Pentanedione	0.101	0.768
2-Methyl-1-propanol	0.676	-0.203
2-Pentanol	0.616	0.144
3-Methyl-1-butanol	0.757	0.124
Ethyl hexanoate	0.717	0.077
3-Methyl-3-buten-1-ol	0.498	-0.406
3-Hydroxy-2-butanone	-0.275	0.686
Flavor intensity	0.625	0.016
Flavor quality	0.197	0.695

The current results do not completely agree with those of Garde et al. (2002a), who recorded a significant enhancement of the flavor quality ($P < 0.001$) and the flavor intensity ($P < 0.05$) of Hispánico cheese by the addition of thermophilic adjunct culture to milk.

There were significant ($P < 0.01$) correlations between flavor intensity scores and proteolysis (OPA test), hydrophilic peptides, and total free amino acids, with r values of 0.458, 0.538, and 0.518, respectively. Flavor quality scores correlated significantly ($P < 0.05$; $r = 0.285$) with proteolysis (OPA test). Similar correlations were recorded by Garde et al. (2002a).

Principal component analysis was carried out to correlate volatile compounds with sensory characteristics. Components 1 and 2 of principal component analysis explained the 31.4 and 14.5% of the variance, respectively. Ethyl butanoate, 1-propanol, 3-methylbutanal, 3-methyl-1-butanol, ethyl hexanoate, 2-pentanone, 2-methyl-1-propanol, 2-pentanol, ethanol, and flavor intensity correlated positively with component 1 (Table 9). Flavor quality and component 1 had a low correlation. 2,3-Pentanedione, 2,3-butanedione, 3-hydroxy-2-butanone, and flavor quality correlated positively with component 2. Flavor intensity and component 2 had a low correlation.

CONCLUSIONS

Calcium alginate was chosen as the incorporation matrix of nisin because of its food-grade additive status, low cost, and simple incorporation process. Addition of nisin, either free or incorporated in calcium alginate

microparticles, to milk promoted early lysis of thermophilic adjunct culture bacteria and increased aminopeptidase activities in cheese during the first days of ripening, but did not influence proteolysis, volatile compound profile, or sensory characteristics of cheese. The use of nisin incorporated in microparticles resulted in a higher aminopeptidase activity in 1-d-old cheese than when free nisin was used, but differences in aminopeptidase activity between cheeses made with free nisin and with nisin incorporated in microparticles disappeared during ripening. Addition of thermophilic adjunct culture did not influence proteolysis. However, it enhanced the formation of some volatiles considered to impact aroma compounds such as 3-methyl-1-butanol, diacetyl, and 2,3-pentanedione and improved flavor quality of cheese.

The performance of nisin, free or incorporated in microparticles, was under those of nisin-producing adjunct cultures used in previous works. A gradual release of the bacteriocin into the cheese matrix seems to be essential for the optimum lysis of lactic acid bacteria and the acceleration of cheese ripening. Further studies using different proportions of nisin-calcium alginate mixtures, which would favor or retard the release of nisin, might contribute to optimize the lysis of lactic acid bacteria.

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