ARTICLES

Effect of Adding *Propionibacterium shermanii* NCDO 853 or *Lactobacillus casei* ssp. *casei* IFPL 731 on Proteolysis and Flavor Development of Cheddar Cheese

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Proteolysis and flavor development were monitored in Cheddar cheese made from milk inoculated with *Lactobacillus casei* ssp. *casei* IFPL 731 (10^4 cfu/mL cheesemilk) or *Propionibacterium shermanii* NCDO 853 at three different levels (10^5, 10^6, and 10^7 cfu/mL cheesemilk). The pH and chemical composition of the experimental cheeses were not affected by the addition of *L. casei* or *Prop. shermanii*. Cheeses inoculated with *L. casei* or *Prop. shermanii* at low and medium levels received the best scores for flavor development and body. However, the addition of *Prop. shermanii* at a high level caused a sweet and nutty flavor. Urea-PAGE, WSN, and RP-HPLC showed minor differences in proteolysis between the control and experimental cheeses. Major differences were found at the amino acid level. In general, the content of amino acid increased as the *Propionibacterium* inoculum increased in the experimental cheeses. Besides, a decrease in the content of hydrophobic peptides was observed in cheeses inoculated with *Prop. shermanii* at medium and high levels. The increase in amino acid content was not apparent when *L. casei* was added to cheese, possibly due to the lack of cell lysis. There is an optimum inoculum level of *Prop. shermanii* above which the flavor of Cheddar cheese resembles that of Swiss-type cheese.

Keywords: *Propionibacterium shermanii; Lactobacillus casei ssp. casei; adjunct cultures; Cheddar cheese; proteolysis*

INTRODUCTION

Methods for acceleration of cheese ripening are based on increased proteolysis or lipolysis in cheese to enhance flavor development. These methods involve the use of the following: exogenous enzymes, modified starters (chemically, physically, or genetically), modification of manufacturing conditions, or addition of adjunct cultures (Fox et al., 1996).

The development of more intense flavor in raw milk cheese than in cheeses made from pasteurized milk has stimulated interest in the study of nonstarter lactic acid bacteria (NSLAB). Raw milk cheeses also ripen faster than pasteurized milk cheeses (Price and Call, 1969; Lau et al., 1990, 1991; McSweeney et al., 1993; Beuvier et al., 1997). Since mesophilic lactobacilli are the predominant species of NSLAB in cheese, numerous studies have been focused on the use of these microorganisms as adjunct cultures (Puchades et al., 1989; Broome et al., 1990; Lee et al., 1990; Lane and Fox, 1996; Lynch et al., 1996). In general, lactobacilli are considered to contribute to flavor development through the action of proteolytic enzymes, which result in an increase in the level of free amino acids in cheese. Not all *Lactobacillus* spp. have a positive effect, and some defects in cheese have been attributed to the action of lactobacilli, e.g., formation of calcium lactate crystals and toxic amines. Results obtained with lactobacilli suggest that acceleration of cheese ripening may be possible if selected suitable organisms are included in the starter as adjunct cultures. Moreover, cheese-related microorganisms other than lactococci and lactobacilli may contribute to the development of distinct flavors in cheese during ripening.

Propionic acid bacteria are important for eye formation and flavor development in Swiss-type cheeses. They grow in cheese after the thermophilic lactic acid bacteria have ceased to grow, reaching high numbers (10^9 cfu/g) during ripening. Although little proteolysis in cheese has been attributed to propionibacteria, proteinase activity was found in all species of dairy *Propionibacterium* studied (Dupuis et al., 1995). Propionibacteria also possess high peptidase activity (Sahlström et al., 1989), especially the ability to release high levels of proline (Langsrud et al., 1977). Until now, only an iminopeptidase, an X-prolyl dipeptidyl aminopeptidase, and an endopeptidase have been purified (Panon, 1990; Ezzat et al., 1994; Tobiassen et al., 1996; Fernández-Esplá and Fox, 1997).

In an attempt to accelerate the ripening of low-fat cheese, El-Soda et al. (1991) used cell-free extracts of some cheese-related microorganisms, including *Propionibacterium shermanii* and *Lactobacillus casei*. Al-

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S0021-8561(97)00535-9 CCC: $15.00 © 1998 American Chemical Society
Published on Web 02/28/1998

though no significant increase in proteolysis was observed in the experimental cheeses, probably due to instability of the free enzymes, a debittering action was observed when these extracts were used. The addition of microbial cells to accelerate cheese ripening or improve cheese flavor offers the possibility of releasing gradually a balanced peptidase system via cell lysis into the cheese matrix, where they may degrade oligopeptides produced by the action of the coagulant and the starter culture. On the other hand, the use of adjunct cells would be feasible only if the strains used did not produce excessive acid at the vat stage.

In this study, the effect of adding *Prop. shermanii* NCDO 853 at three different levels to Cheddar cheese was evaluated and compared with the effect of adding *L. casei* ssp. *casei* IFPL 731.

MATERIALS AND METHODS

Bacterial Strains. *Prop. shermanii* NCDO 853 was obtained from the Department of Microbiology, University College, Cork, Ireland, and *L. casei* ssp. *casei* IFPL 731 was obtained from the culture collection of the Instituto del Frío, Madrid, Spain, and had been isolated from artisanal goats' milk cheese. *Prop. shermanii* and *L. casei* were grown at 30 °C in sodium lactate broth and MRS broth, respectively. The stock cultures were transferred twice in the appropriate medium and grown at 30 °C before use. Incubated strains were harvested during late exponential growth phase (36 h for *Prop. shermanii* and 11 h for *L. casei*) by centrifugation, rinsed twice in Ringer's solution, and suspended in cheese milk at the appropriate level for each experimental cheese.

Cheese Manufacture. Cheddar cheese was made on three occasions in small-scale cheesemaking equipment, using 20 L vats of pasteurized milk (72 °C × 15 s). Pasteurized milk was heated to 31 °C and inoculated with 2% (v/v) of lactic starter *(Lactococcus lactis* ssp. *cremoris* 223; Chr. Hansen's Laboratories, Little Island, Co. Cork, Ireland). After the addition of the starter, *Lactobacillus* or *Propionibacterium* strains were added as adjuncts. A total of four cheeses and a control cheese (starter culture only) were manufactured in each of the three trials. For cheese 1, *Lactobacillus casei* ssp. *casei* IFPL 731 was added to cheesemilk at 10^4 cfu/mL (0.2 mL of stationary-phase culture per 20 L of milk) together with the starter culture; for cheeses 2−4, *Prop. shermanii* NCDO 853 was added at levels of 10^5, 10^6 and, 10^7 cfu/mL (2, 20, and 200 mL of a stationary-phase culture per 20 L of milk), respectively, with the starter culture. Calf rennet was added to the cheesemilk (Chr. Hansen's Laboratories) at a level of 0.3 mL/L. The setting temperature was 30 °C, and the coagulum was cut ~30 min after renneting. The curds were cooked at 38 °C and cheddared until the pH reached 5.3−5.4. Milled curds were salted at a level of 2.5% (w/w), pressed overnight at 1 bar at 16 ± 1 °C, vacuum packed, and ripened at 7 °C for 6 months. All cheeses were analyzed in duplicate.

Bacteriological Analysis. Lactobacilli in the cheeses were enumerated on Rogosa Agar (Difco Laboratories, Detroit, MI incubated at 30 °C for 5 days. Numbers of propionic acid bacteria in cheese were estimated as described by Drinan and Cogan (1992) using sodium lactate agar with cloxacillin (4 μg/mL). The plates were incubated anaerobically for 7 days at 30 °C.

Organoleptic Assessment. During ripening, cheeses were graded blind by two graders from the Irish Department of Agriculture, Food, and Forestry for flavor (maximum score, 45 points) and texture (maximum score, 40 points). In practice, the graders use only 5 points on the scale: 35 (very poor) to 40 (very good) for flavor and 30 to 35 for texture.

Compositional Analysis. Samples of 1-day-old cheeses were analyzed for fat (Gerber method; IS, 1955), protein (macro-Kjeldahl; IDF, 1964), moisture (oven drying at 102 °C; IDF, 1982), and salt (Fox, 1963). The pH of each cheese was determined in a 1:1 cheese:water slurry using a standard pH meter (Radiometer, Copenhagen, Denmark).

Assessment of Proteolysis. Proteolysis was assessed in cheese samples at 1 day and 1, 3, and 6 months. Water-soluble extracts (WSE) of the cheeses were prepared according to the method of Kuchroo and Fox (1982). The nitrogen content of the WSEs (WSN) was determined in duplicate by the macro-Kjeldahl method (IDF, 1964). Aliquots of the WSEs were freeze-dried for analysis by reverse-phase, high-performance liquid chromatography (RP-HPLC) and polyacrylamide gel electrophoresis (PAGE).

The content of free amino acids in the WSEs was determined in duplicate by the cadmium−ninhydrin method of Folkertsma and Fox (1992). Results are presented as milligrams of Leu per gram of cheese by reference to a standard curve.

Urea−PAGE on cheese samples and WSEs was performed using a Protean II xi vertical slab−gel unit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.) according to the method of Andrews (1983). Gels were stained by the method of Blakesley and Boezi (1977).

WSEs were fractionated by solubility in 70% (v/v) ethanol. Absolute ethanol was added to aliquots of the WSEs to a final concentration of 70%. The mixture was held for 30 min at room temperature and then centrifuged at 3000*g* for 30 min at 20 °C. The supernatant, which contained the ethanol-soluble peptides, was filtered through Whatman No. 1 filter paper and the ethanol removed using a rotary evaporator at 30 °C under vacuum and freeze-dried. The ethanol-insoluble fraction was dispersed in distilled water and freeze-dried prior to analysis.

Peptide profiles of the WSE, 70% ethanol-soluble and -insoluble fractions were determined by RP-HPLC using a Waters 626 solvent delivery system with a Waters 600s controller, a Waters 717 plus autosampler, a Waters 486 UV spectrophotometric detector at 214 nm (Waters Corp., Milford, MA), and Nucleosil C_8 analytical (25 cm × 4.6 mm, 5 μm particle size, 300 Å pore size) and guard (4.6 mm × 1 cm) columns. The eluents used were 0.1% (v/v), trifluoro acetic acid in water (solvent A) and 0.1% (v/v) trifluoro acetic acid in acetonitrile (solvent B). Samples (10 mg/mL) were dissolved in sample buffer (10% solvent B in solvent A containing 4.5 mol/L urea) and centrifuged and the supernatant filtered through 0.45 μm cellulose acetate filters. Filtrate (50 μL) was applied to the column and eluted at a flow rate of 0.75 mL/min, using the following gradient: 100% solvent A for 5 min followed by a gradient from 0 to 50% solvent B over 55 min, elution with 50% solvent B for 6 min, a further gradient from 50 to 60% solvent B over 4 min, and finally 60% solvent B for 3 min.

Amino Acid Analysis. The concentration of free amino acids in the 12% trichloro acetic acid-soluble fraction of WSEs was determined using a Beckman model 6300 amino acid analyzer (Beckman Instruments Ltd., High Wycombe, U.K.) using a Beckman P-N 338052 Na cation exchange column (12 × 0.4 cm). A standard amino acid mixture was used to calibrate the column, and norleucine was added to all samples as an internal standard. Amino acids were postcolumn derivatized with ninhydrin and detected by absorbance at 440 nm for proline or 570 nm for all other amino acids.

Statistical Analysis. The results are the mean of six replicates (three samples, each analyzed in duplicate). The data were subjected to a Student test to determine significant differences ($P < 0.05$) between control cheese and experimental cheeses and between the experimental cheeses.

RESULTS

Microbial Analysis. Figure 1 shows the growth of lactobacilli and propionic acid bacteria during ripening in control and experimental cheeses. In all of the trials, the numbers of lactobacilli in the control cheeses were below 10^2 cfu/g at day 1 but increased during ripening and reached 10^7 cfu/g at the end of the 6 month ripening period. In the cheeses containing *L. casei* as adjunct,

Table 1. Grades[a] (Mean ± SD) of Experimental Cheeses[b]

	flavor			texture			overall quality[c]		
cheese[b]	4 weeks	12 weeks	24 weeks	4 weeks	12 weeks	24 weeks	4 weeks	12 weeks	24 weeks
control	38.3 ± 0.6	37.8 ± 0.8	38.1 ± 0.7	32.6 ± 0.7	32.3 ± 1.3	32.5 ± 1.0	71.0 ± 0	70.1 ± 1.5	70.6 ± 1.1
1	38.0 ± 0	38.3 ± 0.2	38.5 ± 0.5	33.0 ± 0	33.0 ± 0	33.0 ± 0	71.0 ± 0	71.3 ± 0.2	71.5 ± 0.4
2	38.5 ± 0.6	38.3 ± 0.5	38.5 ± 0.5	33.0 ± 0	33.0 ± 0	33.0 ± 0	71.5 ± 0.4	71.3 ± 0.4	71.5 ± 0.4
3	38.8 ± 0.3	38.3 ± 1.3	38.1 ± 0.7	33.0 ± 0	32.3 ± 0.7	32.8 ± 0.7	71.8 ± 0.2	70.6 ± 1.2	70.3 ± 0.8
4	38.0 ± 1.0	37.6 ± 0.6	37.5 ± 0	32.0 ± 2	32.0 ± 2.0	31.6 ± 0.3	70 ± 2.1	69.6 ± 1.8	69.1 ± 0.2

[a] Data from trials 1−3. [b] Maximum grades for flavor and body were 40 and 45, respectively. [c] Cheese 1 was inoculated with *L. casei* ssp. *casei*, cheese 2 with *Prop. shermanii* at 10^5 cfu/mL, cheese 3 with *Prop. shermanii* at 10^6 cfu/mL, and cheese 4 with *Prop. shermanii* at 10^7 cfu/mL. [d] Flavor + texture.

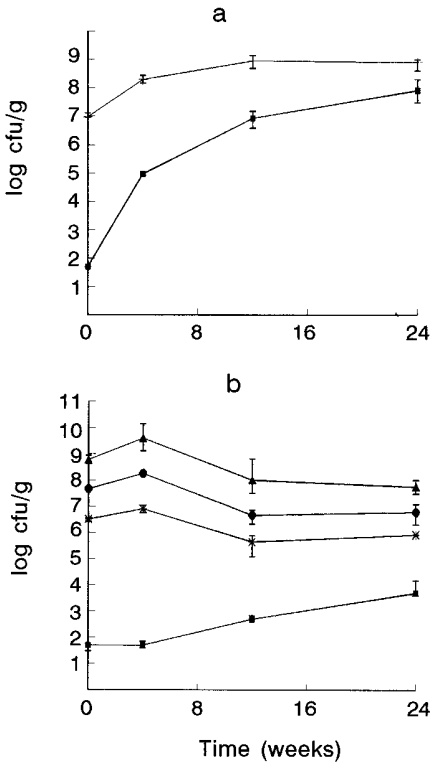

Figure 1. Counts of (a) lactobacilli and (b) propionibacteria during ripening of control cheese (■) and cheeses inoculated with *L. casei* ssp. *casei* (cheese 1) (+), *Prop. shermanii* at 10^5 cfu/mL (cheese 2) (*), *Prop. shermanii* at 10^6 cfu/mL (cheese 3) (●), and *Prop. shermanii* at 10^7 cfu/mL (cheese 4) (▲).

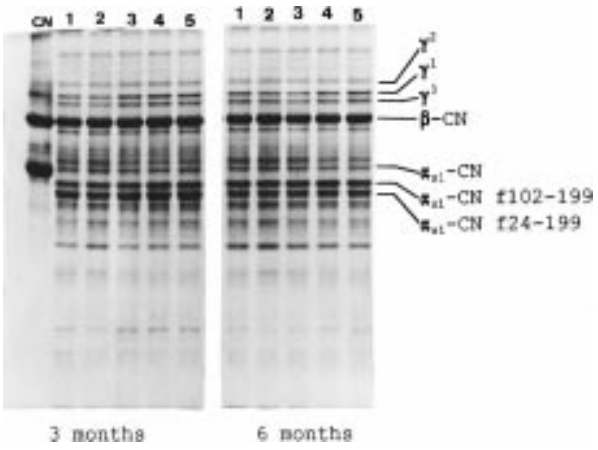

Figure 2. Urea−polyacrylamide gel electrophoretograms of Na caseinate (CN) and experimental cheese samples at 3 and 6 months of ripening: Lane 1, control cheese; lane 2, cheese 1; lane 3, cheese 2; lane 4, cheese 3; lane 5, cheese 4.

lactobacilli counts reached ~10^9 cfu/g after 8 weeks and remained at this level until the end of the ripening period (Figure 1a).

Counts of propionic acid bacteria in the control cheese increased throughout ripening but were always below 10^4 cfu/g in all trials. The numbers of propionic acid bacteria entrapped in the curd at day 1 in cheeses 2−4 were approximately 4×10^6, 5×10^7, and 6×10^8 cfu/g, respectively. *Propionibacterium* cells increased slighly at 4 weeks in the three trials but decreased by 1 or 2 log cycles thereafter (Figure 1b).

Sensory Assessment. Mean grade scores and standard deviations for cheeses after 4, 12, and 24 weeks of ripening are shown in Table 1. In general the experimental cheeses received good scores for flavor and texture. In each trial, cheeses 1−3 received the highest flavor scores and cheese 4 received lower scores for flavor and texture than the other cheeses. The flavor of cheese 4 was considered to be slightly sweet and nutty. Significant differences for overall quality ($P < 0.05$) were found at 4 weeks of ripening between the control cheese and cheese 3 and between cheese 1 and cheese 3 and at 36 weeks of ripening between cheese 4 and the other cheeses. No significant differences were found at 12 weeks of ripening due to variations in the values obtained in each trial.

The addition of *Prop. shermanii* cells did not cause problems with regard to openness or the development of eyes; although 1-month-old cheeses inoculated with *Propionibacterium* showed some cracks, these disappeared thereafter.

Chemical Composition. Experimental cheeses had an average moisture content of 39.1 ± 0.8%, an average fat content of 28.7 ± 0.9%, an average protein of 25.9 ± 0.7%, an average salt content of 1.5 ± 0.2%, an average pH of 5.2 ± 0, and an average salt in moisture content of 3.8 ± 0.5%. Since the moisture content was slightly high and the salt content was low, salt-in-moisture levels were also low. The rate of acid formation, which is a critical factor in the manufacture of cheese, was not affected by the addition of *L. casei* or *Prop. shermanii*.

Assessment of Proteolysis. Urea−PAGE electrophoretograms of cheese samples and WSEs during ripening (1, 3, and 6 months) showed no major qualitative differences in the banding pattern between the different cheeses (Figures 2 and 3). The formation of WSN (expressed as the percentage of total N) during ripening was also similar in all cheeses (results not shown); it increased from about 4% at day 1 to 23% at the end of the 6 months ripening period for all cheeses.

Figure 4 shows the total concentration of free amino acids (measured by reaction with cadmium−ninhydrin) in the WSEs of the control and experimental cheeses. In each trial, the cheeses with the highest level of propionic acid bacteria, cheeses 3 and 4, had the highest content of free amino acids. A significant increase ($P < 0.05$) in the final concentration of the free amino acids

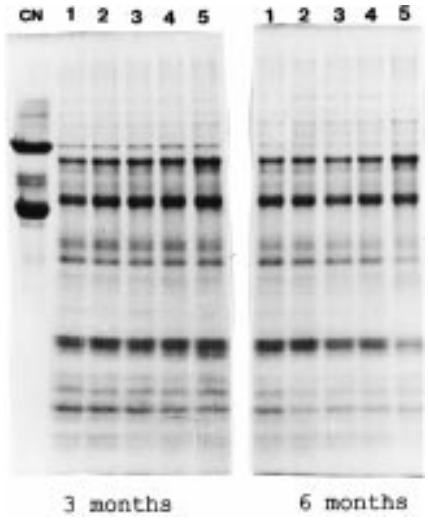

Figure 3. Urea−polyacrylamide gel electrophoretograms of Na caseinate (CN) and water-soluble extract of experimental cheese samples at 3 and 6 months of ripening: lane 1, control cheese; lane 2, cheese 1; lane 3, cheese 2; lane 4, cheese 3; lane 5, cheese 4.

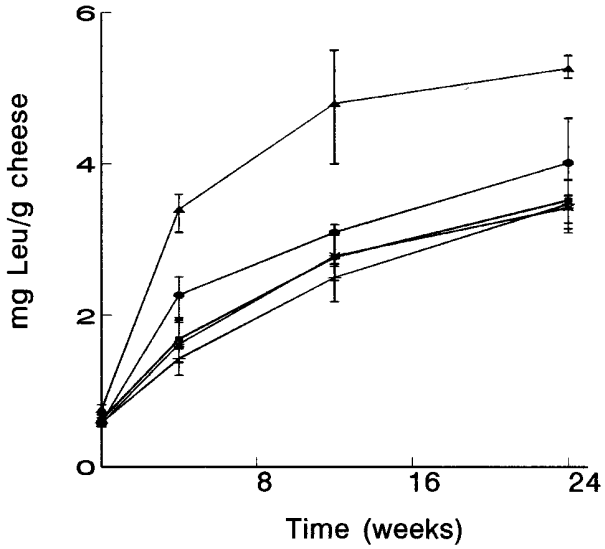

Figure 4. Development of total free amino acids during ripening in control cheese (■) and cheese 1 (+), cheese 2 (*), cheese 3 (●) and cheese 4 (▲).

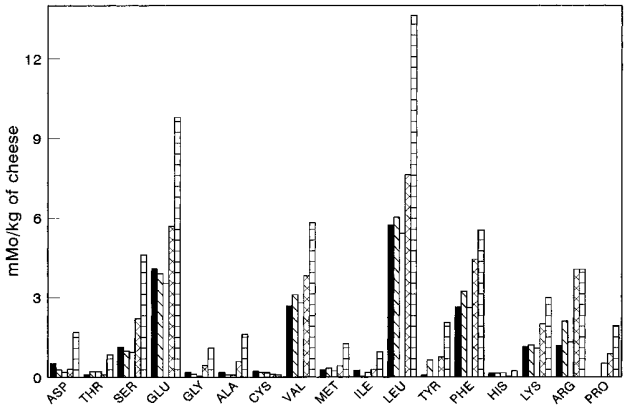

Figure 5. Concentration of individual amino acids in 3-month-old control cheese (solid bar) and cheeses 1 (slashed bar), 2 (open bar), 3 (cross-hatched bar), and 4 (horizontally striped bar). Data are from one trial.

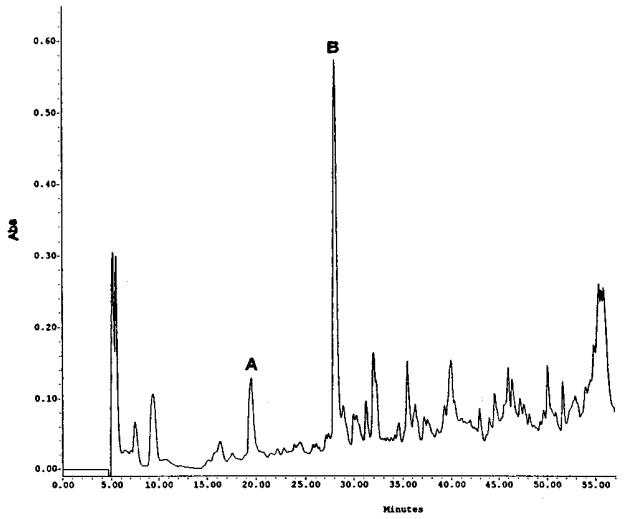

Figure 6. RP-HPLC chromatograms of the water-soluble extract from 3-month-old control cheese.

was observed in these cheeses at 8, 12, and 24 weeks of ripening. Control, cheese 1, and cheese 2 had similar levels of free amino acids, and their values were not significantly different ($P < 0.05$).

The concentration of individual amino acids in the WSEs of 3-month-old cheeses confirms this finding (Figure 5). The addition of *L. casei* (cheese 1) or *Prop. shermanii* at a low level (cheese 2) did not increase the concentration of free amino acids. The principal free amino acids were Leu and Glu, followed by Val and Phe and Arg and Lys. In each trial, proline was present at a high concentration in cheeses with added *Prop. shermanii*.

Peptide profiles of the WSE from the experimental cheese showed no qualitative differences, but some quantitative differences were observed, especially in cheeses inoculated with *Propionibacterium*. Hydrophobic material eluting between 30 and 58 min in the control cheese (Figure 6) decreased in cheeses containing high numbers of *Prop. shermanii* (i.e. cheeses 3 and 4). Peak A, eluting at 20 min, was larger in cheeses inoculated with *Propionicbacterium* than in the control and cheese 1. In contrast, peak B, eluting at 28.5 min, was reduced in cheeses 3 and 4.

Figure 7 shows the peptide profiles of the ethanol-soluble fraction of the control cheese. Only quantitative differences were observed between the chromatograms. Cheese 1 showed a decrease in the materials eluting between 51.5 and 57 min, which are quite hydrophobic. Again, peak areas for hydrohobic peptides, with retention times between 37 and 58 min, were smaller in cheeses 2−4, whereas hydrophilic peptides, eluting between 0 and 37 min, gave slightly larger peak areas in these cheeses compared to the control. Peak B decreased in cheeses 3 and 4 and increased in cheeses 1 and 2.

Chromatograms of the ethanol-insoluble fraction also showed some quantitative differences (Figure 8). Peaks D and E, eluting at 32 and 36 min, respectively, were smaller in cheeses 1, 3, and 4 than in the control and cheese 2, whereas peaks A and C were larger in cheeses 3 and 4. Similar to previous results for the WSE and ethanol-soluble fraction, peptides eluting in the hydrophobic region of the chromatogram, between 49 and 58 min, decreased in cheeses 3 and 4.

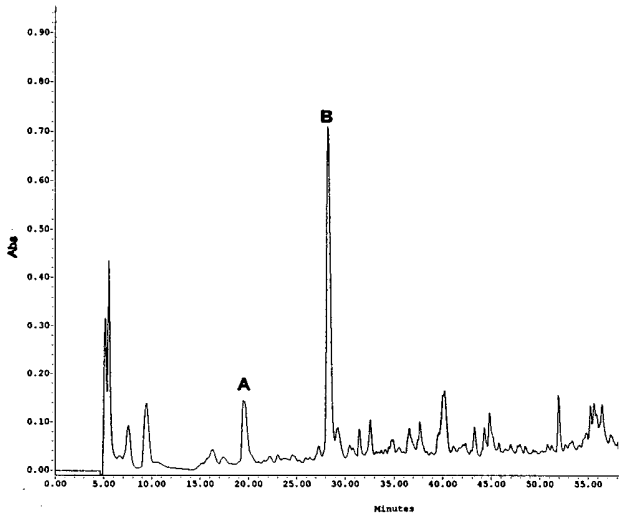

Figure 7. RP-HPLC chromatograms of the ethanol-soluble fraction from 3-month-old control cheese.

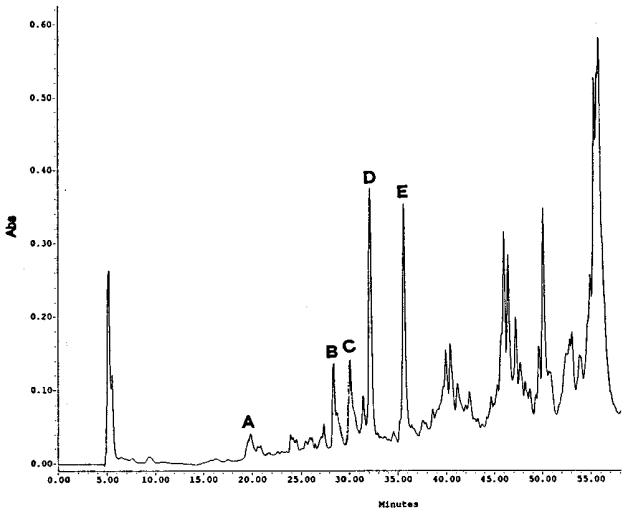

Figure 8. RP-HPLC chromatograms of the ethanol-insoluble fraction from 3-month-old control cheese.

DISCUSSION

In general, the experimental cheeses received similar or slightly higher scores for flavor and body/texture than the control cheese. Cheeses inoculated with *Prop. shermanii* had a distinct slightly sweet and nutty flavor, which resembled Swiss-type cheeses. At the highest level of adjunct (10^7 cfu/mL), this flavor was more accute and therefore these cheeses were downgraded. However, the graders considered that this flavor could be attractive to some consumers.

The pattern and extent of proteolysis in the experimental and control cheeses were found to be very similar, as indicated by urea−PAGE and WSN. Rennet, indigenous milk proteinases and starter proteinases are responsible for the proteolysis at this level (Visser, 1977). Although proteinases have been found in both microorganisms used as adjuncts (Requena et al., 1993; Dupuis et al., 1995), their contribution may be low or was masked by the coagulant and starter proteinases.

Significative differences were found between the experimental cheeses at the amino acid level. The large increase in total and individual amino acids observed in cheeses inoculated with *Prop. shermanii* at a high level reflect the high peptidase activity in these cheeses. No significative differences were found at the amino acid level between the control and experimental cheese inoculated with *L. casei*. These results are largerly in agreement with the finding of other authors who used *L. casei* as an adjunct (Lynch et al., 1996; Lane and Fox, 1996). The strain of *L. casei* ssp. *casei* used in this study possesses a broad range of peptidases comparable to starter *Lactococcus* (Parra et al., 1996). Several aminopeptidases, a prolidase, and a dipeptidase have been purified from this particular strain (Fernández-Esplá and Martín-Hernández, 1997; Fernández-Esplá et al., 1997a,b; Fernandez de Palencia et al., 1997a,b). Since a high number of lactobacilli was observed at day 1 of ripening, this particular strain may dominate the natural nonstarter microflora of these cheeses (cheese 1). Therefore, it may be possible that the limiting factor in the starter/*L. casei* cheeses was the release of enzymes at an appropiate level. The number of lactobacilli in cheese 1 showed no decline throughout the 6 month ripening period, while the number of propionic acid bacteria in cheeses 3 and 4 decreased approximately 100-fold between 1 and 6 months. The increase in the amino acid level could be due either to the metabolism of viable cells or to the action of enzymes released after cell death or autolysis, but the latter is considered to be more effective (Law and Sharpe, 1977; El-Soda et al., 1981). It should be noted that, in this study, some growth of wild NSLAB occurred in control cheeses and consequently they may contribute to proteolysis.

Although amino acid formation in Cheddar cheese has been found to correlate well with flavor development, the role of amino acids in flavor is not fully understood. It is generally accepted that amino acids per se are not responsible for Cheddar flavor, but they provide the essential background, acting as precursors for the formation of cheese flavor compounds by means of enzymatic and nonenzymatic reactions (Engels and Visser, 1994). Results of this study showed that the inclusion of *Prop. shermanii* as adjunct caused changes in the relative proportions of each amino acid and, at a high level, increased the content of free amino acids. Proline, which has been reported to impart the sweet flavor to Swiss-type cheeses, was present at a high level only in cheeses inoculated with *Propionibacterium*. Langsrud et al. (1977, 1978) showed that proline-specific peptidases, released by autolysis when propionibacteria reach their maximum number, are responsible for the production of proline in Swiss-type cheeses.

Although no qualitative differences were found in the peptide profile of the experimental cheeses, minor quantitative differences were evident. In general, hydrophobic peptides were present at lower levels in cheeses inoculated with *Prop. shermanii* at a high or medium level, indicating high peptidase activity in these cheeses.

In an attempt to accelerate the ripening of Cheddar cheese, Lloyd et al. (1980) added a yoghurt culture YB (*Streptococcus thermophilus, L. heveticus*, and *L. jugurti*) in combination with the normal starter and reported that the experimantal cheeses had a sweet Swiss-type flavor. The content of proline in the inoculated cheeses was increased to the level found in Swiss cheese.

Proline-containing peptides have been reported to exhibit bitterness (Ishibashi et al., 1988); this flavor defect may occur during acceleration of cheese ripening.

Results of this study reveal a possible role of *Prop. shermanii* as a debittering agent and also as a source for producing mature-flavored products. This role has been neglected compared with the capacity of *Propionibacterium* to produce CO_2 and volatile flavor compounds, such as acetic and propionic acids.

ACKNOWLEDGMENT

We thank Baukje Folkertsma and Therese Uniacke-Lowe for expert technical assistance, Lucia Parra for helpful discussion of the HPLC data, and Tanoj Singh for the electrophoresis and valuable suggestions.

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Received for review June 23, 1997. Revised manuscript received December 22, 1997. Accepted December 23, 1997. This research was funded by grant aids under the Food Sub-Program of the Operational Program for Industrial Development which is administered by the Irish Department of Agriculture, Food, and Forestry (supported by National and EU funds) and by the ALI94-0735 Project from the Spanish Interministerial Commission for Science and Technology. M.D.F.-E. was recipient of a FLORA exchange program grant.

JF970535K