Lait (1997) 77, 441-459

Original article

Contribution of starter lactococci and non-starter lactobacilli to proteolysis in Cheddar cheese with a controlled microflora

CM Lynch [1], PLH McSweeney [1], PF Fox [1], TM Cogan [2], FD Drinan [2]

[1] *Department of Food Chemistry, University College, Cork;*
[2] *National Dairy Products Research Centre, Moorepark, Fermoy, Co Cork, Ireland*

(Received 4 October 1996; accepted 18 February 1997)

Summary — Cheddar cheeses were manufactured under controlled microbiological conditions to study the relative contribution of starter and non-starter bacteria to cheese ripening. In each of two trials, four cheeses were manufactured with or without a starter culture (starter-free cheeses were chemically acidified using glucono-δ-lactone). Adjunct cultures of mesophilic lactobacilli were added to one chemically-acidified and one starter cheese. Assessment of proteolysis showed a major contribution by the starter in comparison to the non-starter bacteria to the formation of free amino acids and, to a lesser extent, water-soluble nitrogen. Reversed phase-HPLC of ethanol-soluble and -insoluble fractions of the water-soluble extracts detected some peptides (produced by lactobacilli) which were present in profiles of the starter-free cheese containing the adjunct but were not present in its corresponding control.

Cheddar cheese / starter bacteria / non-starter bacteria / proteolysis

Résumé — Contribution des lactocoques du levain et des lactobacilles non levain à la protéolyse d'un fromage de cheddar à microflore contrôlée. Des fromages de cheddar ont été fabriqués dans des conditions microbiologiques contrôlées, pour étudier la contribution relative des bactéries levain ou non levain à l'affinage du fromage. Dans chacun des deux essais, quatre fromages ont été fabriqués, avec ou sans levain (les fromages sans levain ont été acidifiés chimiquement à l'aide de glucono-delta-lactone). Des lactobacilles mésophiles ont été ajoutés à l'un des fromages acidifiés chimiquement et à l'un des fromages avec levain. L'estimation de la protéolyse a montré une contribution majeure du levain, comparée à celle des bactéries non levain à la formation des acides aminés libres, et dans une moindre mesure à celle de l'azote soluble dans l'eau. La chromatographie RP-HPLC des fractions soluble et insoluble dans l'éthanol des extraits solubles dans l'eau a mis en évidence quelques peptides (produits par les lactobacilles) présents dans les profils du fromage sans levain avec ajout de lactobacilles, et qui n'étaient pas présents dans le contrôle correspondant.

fromage / cheddar / levain / bactérie non levain / protéolyse

INTRODUCTION

During cheese manufacture, the number of starter bacteria increases rapidly from about 10^7 colony forming units (cfu)/mL of cheesemilk at the beginning of cheesemaking to > 10^9 cfu/g of curd at pressing. After salting, starter numbers decrease relatively rapidly during the early stages of maturation, due to the bactericidal effect of salt, low pH and depletion of a fermentable sugar (Chapman and Sharpe, 1981). However, non-starter lactic acid bacteria (NSLAB) are capable of growth in Cheddar cheese (typically < 39% moisture, 4–6% salt-in-moisture, pH 4.9–5.3 and 5–13°C ripening temperature). NSLAB are adventitious bacteria that gain entry to Cheddar cheese primarily during manufacture from the air, cheesemilk (via post-pasteurisation contamination) and cheesemaking equipment (Naylor and Sharpe, 1958; Franklin and Sharpe, 1963). NSLAB multiply during ripening, from typically about 50 cfu/g of curd express to 10^6–10^8 cfu/g in the mature cheese. In Irish Cheddar, the NSLAB are mainly species of mesophilic lactobacilli, eg, *Lb casei* ssp *casei*, *Lb casei* ssp *pseudoplantarum*, *Lb plantarum* and *Lb curvatus* (Jordan and Cogan, 1993).

The primary function of the starter bacteria during cheese manufacture is the production of lactic acid at an appropriate rate, but they also make an important contribution to proteolysis during ripening through the action of lactococcal caseino- and peptidolytic enzymes (Smid et al, 1991). Techniques devised to assess the importance of the starter in cheese ripening were first developed by Mabbitt et al (1955) and involve simulating lactic acid production during manufacture, usually using gluconoδ-lactone (GDL), which hydrolyses to gluconic acid, thereby reducing the pH. This technique has been used, among others, by Reiter et al (1969), O'Keeffe et al (1976) and Visser (1977). However, none of these authors considered the possible contribution of adventitious or adjunct lactobacilli.

Elimination of the non-starter flora requires the use of aseptic cheesemaking techniques. McSweeney et al (1994b) used an aseptic vat technique to study the influence of a mixed mesophilic *Lactobacillus* adjunct in starter-acidified Cheddar. Experimental cheeses containing the adjunct had improved flavour intensity and acceptability and higher levels of free amino acids than control cheeses containing 3 to 5 log cycles fewer NSLAB. Similar results were reported by Lynch et al (1996), who used the same aseptic vat technique to study the influence of different species of mesophilic lactobacilli on starter-acidified Cheddar cheeses during ripening.

Lane and Fox (1996) studied the contribution of starter and added lactobacilli to proteolysis in Cheddar cheese manufactured under non-aseptic conditions by biological (starter) or chemical (GDL) acidification. These authors found few differences in primary proteolysis between any of the cheeses but demonstrated the major contribution of starter enzymes (and the relatively small contribution of NSLAB) to the release of small peptides and amino acids during ripening. However, the effect of starter and added lactobacilli on flavour development in the cheeses was not investigated in this study. The objective of the present work was to further assess the relative contribution of starter and non-starter bacteria to proteolysis and flavour development in Cheddar manufactured under strictly controlled microbiological conditions so as to avoid excessive growth of adventitious NSLAB, which occurred in the study of Lane and Fox (1996).

MATERIALS AND METHODS

Microbial strains and cultures

A culture of *Lactococcus lactis* ssp *cremoris* UC 317, obtained from the culture collection

of the Department of Microbiology, University College, Cork, grown overnight at 21°C in sterile (110°C × 10 min) reconstituted low-heat skim milk powder (100 g/L), was used as starter. Strains of lactobacilli (*Lactobacillus casei* ssp *casei* DPC 2777, *Lb casei* ssp *casei* DPC 2786, *Lb casei* ssp *pseudoplantarum* DPC 2742, *Lb casei* ssp *pseudoplantarum* DPC 2745, *Lb plantarum* DPC 2748, *Lb curvatus* DPC 2767) were obtained from the culture collection of the National Dairy Products Research Centre, Moorepark, and had been isolated from a strongly-flavoured raw-milk Cheddar cheese (McSweeney et al, 1993). Each strain was grown individually in MRS broth (deMan et al, 1960), modified by reducing the glucose concentration to 10 g/L and omitting acetate and Tween 80, except for *Lb curvatus* which was grown in unmodified MRS broth since it grew more satisfactorily in the unmodified medium. The adjunct culture was prepared by mixing equal volumes of the six stationary-phase cultures. The species and proportions of lactobacilli were chosen to approximate the NSLAB microflora in a mature raw-milk Cheddar cheese (McSweeney et al, 1993).

Manufacture of controlled microflora cheese

Cheeses were made on two occasions in 25 L vats; all cheesemaking equipment was steam-sterilised at 137°C for 5 min prior to use. Milk for cheesemaking was pasteurised at 78°C for 15 s and stored in sterile containers. The higher than normal pasteurisation temperature was used to ensure the destruction of indigenous NSLAB. The cheese vats were placed in thermostatically controlled water baths situated in a laminar air-flow unit, modified to accommodate them. Four cheeses were manufactured on each occasion from 20 L batches of milk set at 31°C, two of which were acidified by *Lc lactis* ssp *cremoris* UC317 (inoculum, 20 mL /L) and the other two by a combination of direct addition of concentrated lactic acid to the cheese milk and sterile powdered GDL (Pfizer Chemical Corp, Ringaskiddy, Co Cork, Ireland) to the curd before salting. This chemical-acidification technique causes a decrease in curd pH at a very similar rate to that caused by a starter culture.

The *Lactobacillus* adjunct culture was added to one chemically-acidified and one starter-acidified cheese (~ 0.4 mL per 20 L cheese milk) immediately after the addition of starter. Rennet (7.5 mL filter-sterilised Maxiren, Gist-brocades, Delft, the Netherlands) was added to each vat and the coagulum cut after 40–50 min. The curds and whey were stirred at 31°C for 10–15 min and then heated to 39°C over 30 min. The curd/whey mixture was held at 39°C until the whey pH in each vat reached 6.2, at which point the whey was drained off. The starter-containing curd was cut into blocks which were turned every 15 min to promote acid production. GDL was added to the starter-free curd to reduce its pH and the curd was then turned every 15 min as for the starter-containing curd. The addition of 40 g GDL/kg of curd was necessary to reduce the pH of the starter-free curd to ~ 5.3 at milling. In some cases, further addition of GDL to the starter-free curd was necessary to reduce its pH to ~ 5.3 at milling; 6.7 g GDL/kg of curd was required to reduce pH by ~ 0.1 pH unit. When the pH of the curd in each vat was 5.3–5.4, the curd was milled and salted (~ 2.5 g NaCl/100 g curd). The starter-acidified cheeses were manufactured essentially according to the protocol of Kosikowski (1977). The steps involved in the manufacture of chemically-acidified Cheddar cheese and the kinetics of curd acidification are described in O'Keeffe et al (1975). The aseptic precautions observed during cheese manufacture were as described by McSweeney et al (1994b). The cheeses were pressed overnight at 0.15 MPa at ~ 20°C, vacuum packaged and ripened at 7°C.

Bacteriological analysis

Lactobacilli in cheese milk and cheeses were enumerated on *Lactobacillus* selection agar (LBS, Becton Dickinson, Cowley, UK), incubated at 30°C for 5 days. Coliforms in cheesemilk and cheeses were enumerated on Violet Red Bile Agar (VRBA, Oxoid, Basingstoke, UK), incubated at 30°C for 24 h. Total bacterial counts (TBC) in pasteurised cheese milk were determined on tryptone-glucose-yeast extract agar (Oxoid), incubated at 30°C for 2 days.

Sensory analysis

Cheeses were graded when 18 and 26 weeks old by an 8-member trained taste panel (including a commercial grader) at the National Dairy Products Research Centre, for flavour intensity and flavour acceptability on a 0–80 point scale (0–10 unacceptable; 10–30 poor; 30–50 acceptable; 50–70 good; 70–80 excellent). Samples were held for 1 h at room temperature before being presented to the panel members in semi-enclosed booths designed for sensory analysis. Samples were coded 1–4 as follows: 1, GDL; 2, GDL/ *Lactobacillus* adjunct; 3, starter; and 4, starter/*Lactobacillus* adjunct. The four samples from each trial were presented at separate sessions.

Compositional analysis

All cheeses were analysed when 1 month old 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 a 1:1 cheese:water slurry was determined using a standard pH meter (Radiometer, Copenhagen, Denmark).

Assessment of proteolysis

Water-soluble extracts (WSE) of the cheeses were prepared by the method of Kuchroo and Fox (1982). The nitrogen content of the WSEs was determined in duplicate by the macro-Kjeldahl method (IDF, 1964). Total free amino acids in the cheeses were determined in triplicate by the Cd-ninhydrin method (Folkertsma and Fox, 1992). WSEs were fractionated by ethanol (700 mL/L) into ethanol-soluble (smaller, hydrophilic peptides) and -insoluble (larger, hydrophobic peptides) fractions, as follows: absolute ethanol was added to aliquots of the WSEs to a final ethanol concentration of 700 mL/L. The mixture was held for 30 min at room temperature and then centrifuged at 3000 *g* for 30 min at 20°C. The supernatant (containing the ethanol-soluble peptides) was filtered through Whatman no 1 filter paper and the ethanol removed using a rotary evaporator (Model no RE 100, Bibby Sterelin Ltd, Stone, UK) at 30°C under vacuum. The pellet (containing the ethanol-insoluble peptides) was dispersed in distilled water and freeze dried prior to analysis by reversed phase (RP)-HPLC.

Peptide profiles of the ethanol-soluble and -insoluble fractions of the WSEs were determined by RP-HPLC using a Waters 626 solvent delivery system with a Waters 600s controller and a Waters 717 plus autosampler (Waters Corp, Milford, USA). Nucleosil C_8 (5 μm particles, 30 μm pore size) guard (4.6 × 10 mm) and analytical (4.6 × 250 mm) columns (Macherey-Nagel GmbH, Duren, Germany), were used. Column eluates were monitored at 214 nm using a Waters 486 detector interfaced with an IBM-compatible PC running on Millennium software. The chromatographic conditions were: solvent A: 1 mL/L, tri-fluoroacetic acid (TFA, sequential grade, Sigma, St Louis, USA) in deionized, HPLC-grade water (Milli-Q system, Waters Corp). Solvent B: 1 mL/L, TFA in CH_3CN (HPLC-grade, Labscan Ltd, Dublin, Ireland). Sam-

ples (4 mg/mL) were dissolved in solvent A and filtered through 0.45 μm cellulose acetate filters (Sartorius GmbH, Gottingen, Germany); 40 μL of filtrate were applied to the column and eluted at a flow-rate of 0.75 mL/min with 100% A for 5 min. A gradient of 0 to 50% B (0.91% B per min) was then commenced, followed by elution with 50% B for 6 min. A further gradient from 50 to 60% B (2.5% B per min) was then applied, followed by a final hold at 60% B for 3 min. The column was washed with 95% B for 5 min, followed by equilibration with 100% A for 5 min before the next injection.

Urea-PAGE was performed on cheese samples using a Protean IIxi vertical slab gel unit (Bio-Rad Laboratories Ltd, Watford, UK) and the stacking gel system described by Andrews (1983). The gels were stained directly by the method of Blakesley and Boezi (1977) with Coomassie brilliant blue G250.

RESULTS

Microbiological analysis

The microbiological status of the milk used for cheese manufacture in both trials is shown in table I. The uninoculated milk in both trials was free of coliforms, indigenous NSLAB and had low total bacterial counts. The milk in the vats inoculated with adjunct contained ~2.5 × 10^3 lactobacilli/mL. Growth of lactobacilli in the control and experimental cheeses in trials 1 and 2 is shown in figure 1. Adjunct lactobacilli grew rapidly in the experimental cheeses and were present at 10^6 to 10^7 cfu/g ex-press and ~10^8 cfu/g after 1 month. Thereafter, the numbers of lactobacilli remained high, with only a slight decrease, during the remainder of the ripening period. Starter-acidified cheeses remained free (< 10 cfu/g cheese) of adventitious NSLAB for ~1 month in both trials while the chemically-acidified control cheeses remained free for only 1 (trial 1) or 2 weeks (trial 2). Numbers of adventitious

Table I. Bacterial counts[1] in milk used for the manufacture of Cheddar cheese under controlled microbiological conditions.

Nombre de bactéries[1] dans le lait utilisé pour la fabrication de fromage de cheddar sous conditions microbiologiques contrôlées.

	Uninoculated milk	*Lactobacillus-inoculated milk*
Trial 1		
Coliforms	< 1	< 1
Lactobacilli	<1	2.6 × 10^3
TBC[2]	260	ND
Trial 2		
Coliforms	< 1	< 1
Lactobacilli	< 1	2.4 × 10^3
TBC	75	ND

[1] Colony forming units/mL. [2] Total bacterial count. ND: not determined.

[1] Unités formant colonie/mL. [2] Nombre total de bactéries. ND : non déterminé.

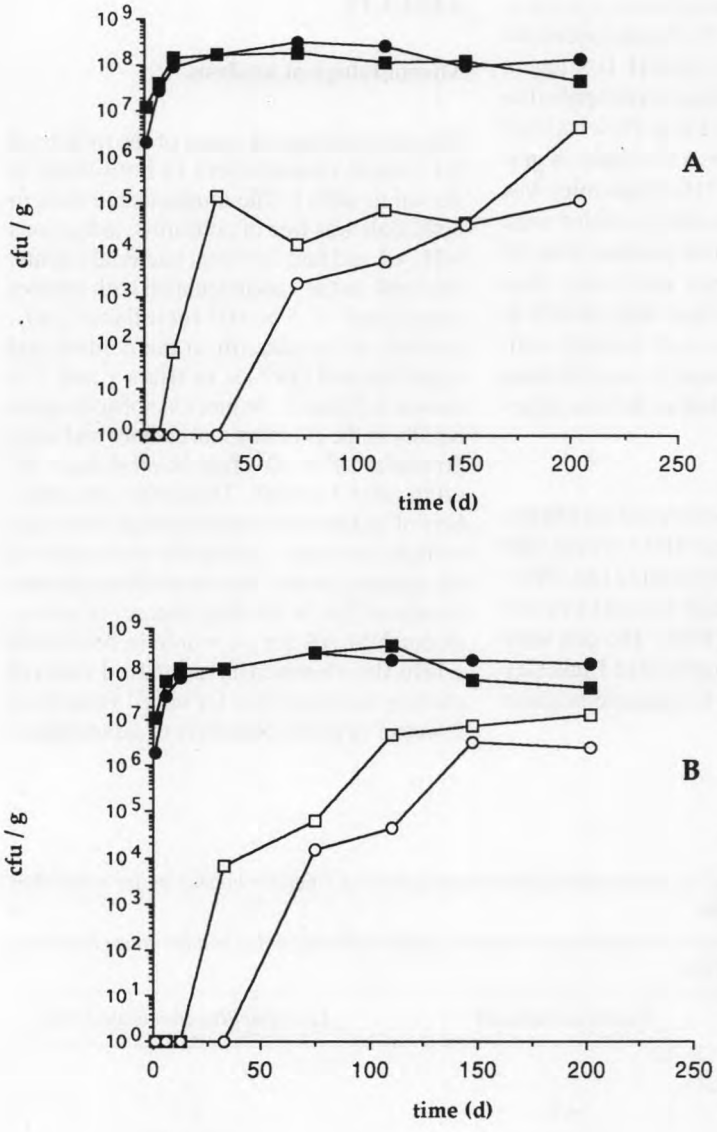

Fig 1. Growth of lactobacilli in Cheddar cheeses manufactured under controlled microbiological conditions, with or without adjunct lactobacilli, by chemical (❑, glucono-δ-lactone (GDL); ■, GDL/Lb adjunct) or biological (○, starter; ●, starter/Lb adjunct) acidification. **A.** Trial 1. **B.** Trial 2.

*Croissance des lactobacilles dans le fromage de cheddar fabriqué dans des conditions microbiologiques contrôlées, avec ou sans ajout de lactobacilles, par acidification chimique (❑ glucono-δ-lactone (GDL); ■ GDL/Lb) ou biologique (○ levain ; ● levain + Lb). **A.** Étude 1. **B.** Étude 2.*

NSLAB in the control cheeses remained $< \sim 5 \times 10^6$ cfu/g throughout ripening.

Compositional analysis

The chemical composition of cheeses from trials 1 and 2 is shown in table II. While all cheeses had compositions within the normal range expected for Cheddar cheese, trial 1 cheeses generally had slightly lower fat-in-dry-matter and higher salt-in-moisture levels than the cheeses in trial 2. In both trials, the pH of the chemically-acidified cheeses was higher than that of the starter cheeses. In

Table II. Mean composition[1] of Cheddar cheeses manufactured under controlled microbiological conditions.

Composition moyenne[1] des fromages de cheddar fabriqués sous conditions microbiologiques contrôlées.

	Fat (%)	*Protein (%)*	*Moisture (%)*	*FDM[2] (%)*	*NaCl (%)*	*S/M[3] (%)*	*pH*
Trial 1							
GDL[4]	29.5	28.0	37.3	47.1	1.65	4.4	5.55
GDL/Lb adjunct	29.0	27.2	36.5	45.7	1.60	4.4	5.22
Str[5]	29.0	29.7	36.7	45.8	1.46	4.0	5.24
Str/Lb adjunct	32.0	28.9	34.9	49.2	1.58	4.5	5.14
Trial 2							
GDL	30.8	25.9	36.4	48.4	1.44	4.0	5.38
GDL/Lb adjunct	32.3	25.9	34.9	49.6	1.41	4.0	5.16
Str	33.0	25.6	35.4	51.1	1.63	4.6	5.32
Str/Lb adjunct	32.5	24.7	36.7	52.2	1.43	4.0	5.03

[1] Mean of duplicate analyses. [2] Fat-in dry-matter. [3] Salt-in-moisture. [4] glucono-δ-lactone (chemically-acidified cheese). [5] starter-acidified cheese.

[1] Moyenne de deux analyses. [2] Matière grasse dans la matière sèche. [3] Sel-teneur en eau. [4] Glucono-δ-lactone (fromage acidifié chimiquement). [5] Fromage acidifié par levain.

addition, the pH of cheeses containing a *Lactobacillus* adjunct was lower than their corresponding controls.

Sensory analysis

Table III shows the mean of the grades (and standard deviations) received by the cheeses in trials 1 and 2 after 4.5 and 6.5 months of ripening. At both gradings, adjunct lactobacilli considerably intensified the flavour of the chemically-acidified cheeses in comparison to the control GDL cheeses but the flavour was considered unacceptable by many members of the panel and therefore the GDL/Lb adjunct cheeses were downgraded for flavour acceptability. The starter-acidified cheeses (with or without adjunct lactobacilli) received considerably higher grades for flavour intensity and flavour acceptability than the GDL control or GDL/Lb adjunct cheeses at both gradings. On both occasions, the starter/Lb adjunct cheese received slightly higher scores for flavour intensity but slightly lower scores for flavour acceptability than the starter control cheese.

Assessment of proteolysis

Since the results for both sets of cheeses were similar, the results for only trial 1 are shown.

Urea-PAGE electrophoretograms of cheese samples taken at different stages during ripening are shown in figure 2. At day 0 and 1 month, no qualitative differences and only small quantitative differences were observed between the cheeses. At 3 and 6 months, greater breakdown of β-CN was observed in the GDL control than in the

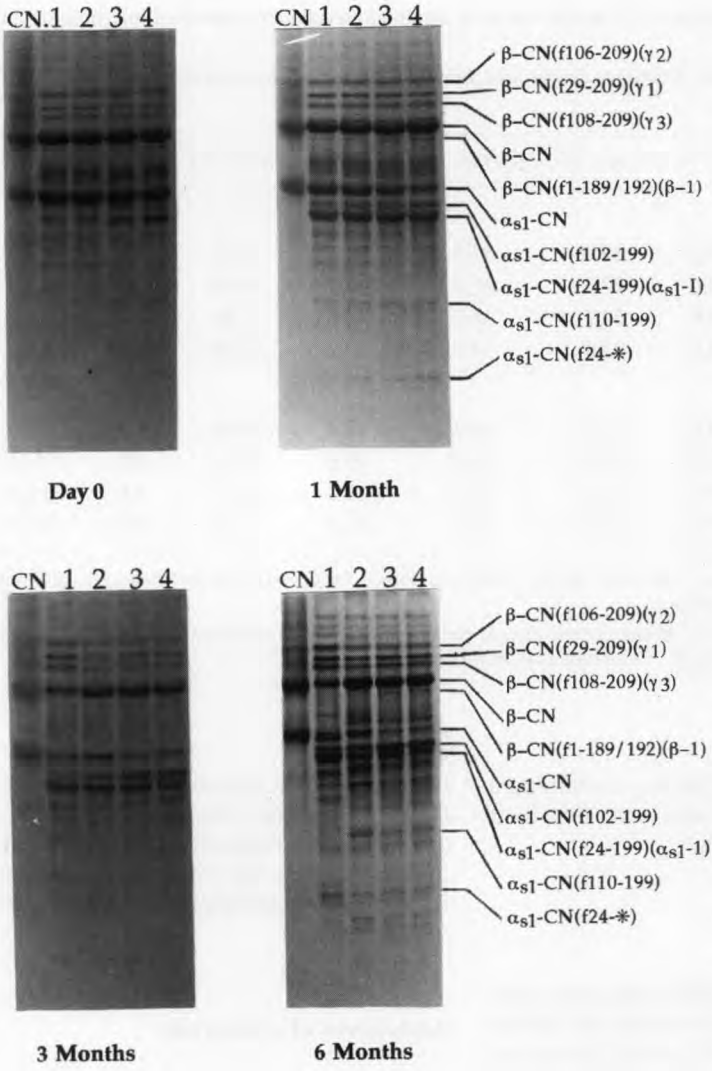

Fig 2. Urea-polyacrylamide gel electrophoretograms of bovine sodium caseinate (CN) and whole Cheddar cheese samples from trial 1 at different stages of ripening. Cheeses manufactured under controlled microbiological conditions using glucono-δ-lactone (GDL) (lane 1), GDL/Lb adjunct (lane 2), starter (lane 3) or starter/Lb adjunct (lane 4). * Undetermined C-terminus.

*Électrophorégramme urée-PAGE de caséinate de sodium (CN) et d'échantillons de fromage de cheddar de l'essai 1 à différents stades d'affinage. Fromages fabriqués dans des conditions microbiologiques contrôlées à l'aide de glucono-δ-lactone (GDL) (1), GDL + Lb (2), levain (3) et levain + Lb (4). * C-terminal non déterminé.*

other cheeses. Greater breakdown of α_{s1}-CN was observed in the starter-acidified cheeses (lanes 3 and 4, probably due to their lower pH) especially at 3 and 6 months. Qualitative and quantitative differences in peptides with higher electrophoretic mobility than α_{s1}-I CN (possibly originating from α_{s1}-CN) were observed between the chemically-acidified and starter-acidified cheeses, particularly at 6 months.

The formation of water-soluble nitrogen (WSN), expressed as a percentage of total N (WSN as % TN), in the cheeses is shown in figure 3. The starter-acidified cheeses generally had higher levels of WSN than chemically-acidified cheeses throughout ripening while the starter/Lb adjunct cheese had slightly higher levels of WSN than the starter control cheese throughout ripening. Somewhat unexpectedly, the WSN in the

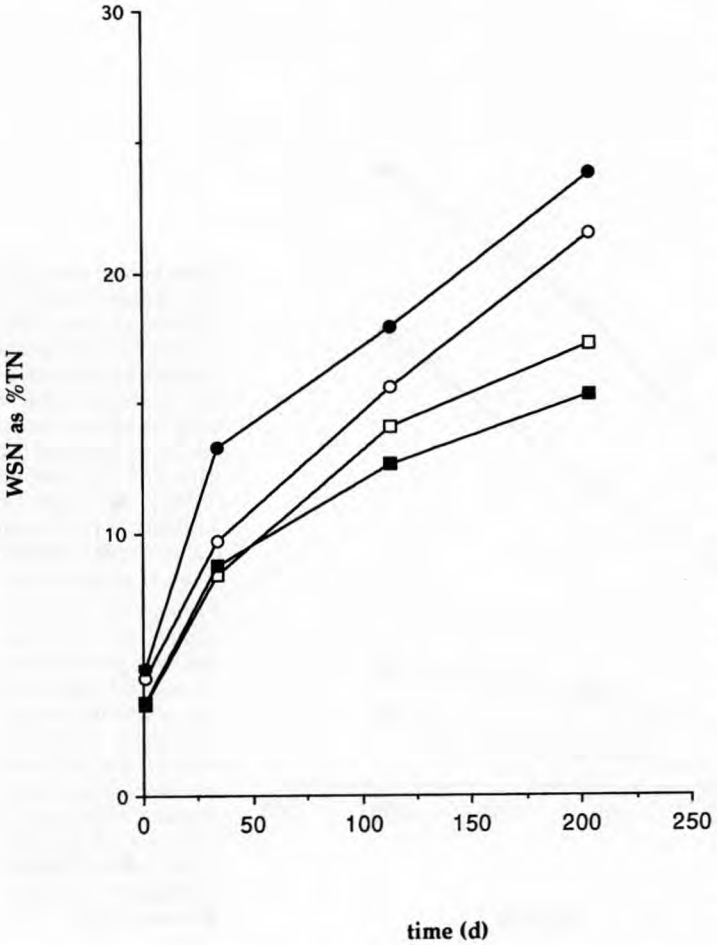

Fig 3. Formation of water-soluble nitrogen, expressed as a percentage of total nitrogen (WSN as % of TN) in Cheddar cheeses (trial 1) manufactured under controlled microbiological conditions, with or without adjunct lactobacilli, by chemical (❑, glucono-δ-lactone [GDL]; ■, GDL/Lb adjunct) or biological (○, starter; ●, starter/Lb adjunct) acidification.

Formation d'azote soluble dans l'eau, exprimé en pourcentage de l'azote total dans les fromages de cheddar (essai 1) fabriqués dans des conditions microbiologiques contrôlées, avec ou sans ajout de lactobactilles, par acidification chimique (❑ glucono-δ-lactone [GDL], ■ GDL/Lb) ou biologique (○ levain ; ● levain + Lb).

GDL control cheese was higher throughout ripening than in the GDL/Lb. adjunct cheese. The formation of total free amino acids (FAA) in the cheeses is shown in figure 4. The release of FAA in the GDL control cheese was very low, while the starter control cheese showed a progressive increase in the concentration of FAA throughout ripening. Both the GDL/Lb adjunct and the starter/Lb adjunct cheeses had higher levels of FAA than their corresponding control cheeses throughout ripening.

Peptide profiles of the ethanol-insoluble fractions of the WSEs from the cheeses are shown in figure 5 (3 months) and figure 6 (6 months). There were no quantitative or qualitative differences between the starter control and starter/Lb adjunct cheeses at 3 or 6 months. Some qualitative and quantitative differences were apparent between the GDL control and GDL/Lb adjunct cheeses at both times, particularly with respect to those peptides eluting between 45 and 55 min. However, these differences were smaller than those observed between the

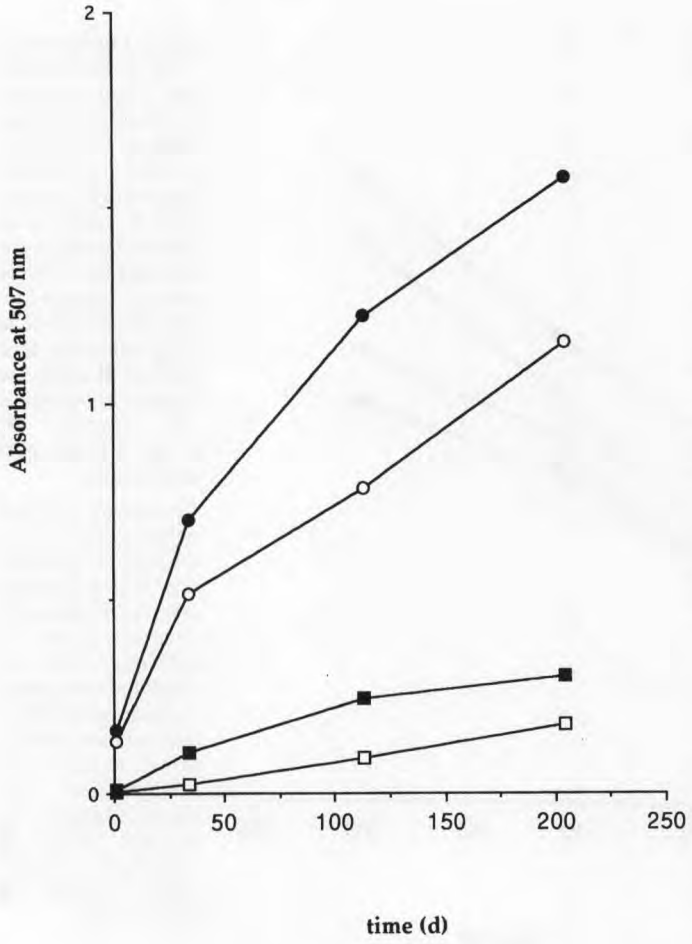

Fig 4. Formation of total free amino acids in Cheddar cheeses (trial 1) manufactured under controlled microbiological conditions, with or without adjunct lactobacilli, by chemical (❑, glucono-δ-lactone [GDL]; ■, GDL/Lb adjunct) or biological (○, starter; ●, starter/Lb adjunct) acidification.

Formation d'acides aminés libres totaux dans les fromages de cheddar fabriqués dans des conditions microbiologiques contrôlées, avec ou sans ajout de lactobacilles, par acidification chimique (❑ glucono-δ-lactone [GDL], ■ GDL/Lb ou biologique : ○ levain ; ● levain + Lb).

two GDL and the two starter-acidified cheeses.

RP-HPLC profiles of the ethanol-soluble fractions of the WSEs from the cheeses are shown in figure 7 (3 months) and figure 8 (6 months). Again, there were only small differences between the starter control and starter/Lb adjunct cheeses at both 3 or 6 months. Comparison between the profiles of the two GDL and the two starter-acidified cheeses showed considerable qualitative and quantitative differences between them. No differences were apparent between the chromatograms of the 1-month-old GDL control and GDL/Lb adjunct cheeses (not shown) but substantial differences were apparent during the later stages of ripening (especially at 6 months). Peptides eluting at 23, 31, 32, 37.5, 38.5 and 40 min (as well as quantitative differences in other peptides) were present in the profiles of the GDL/Lb adjunct cheese at 6 months but absent from the profile of the corresponding GDL control (fig 8). The peptides eluting at 23 and 31 min were also quite prominent in profiles

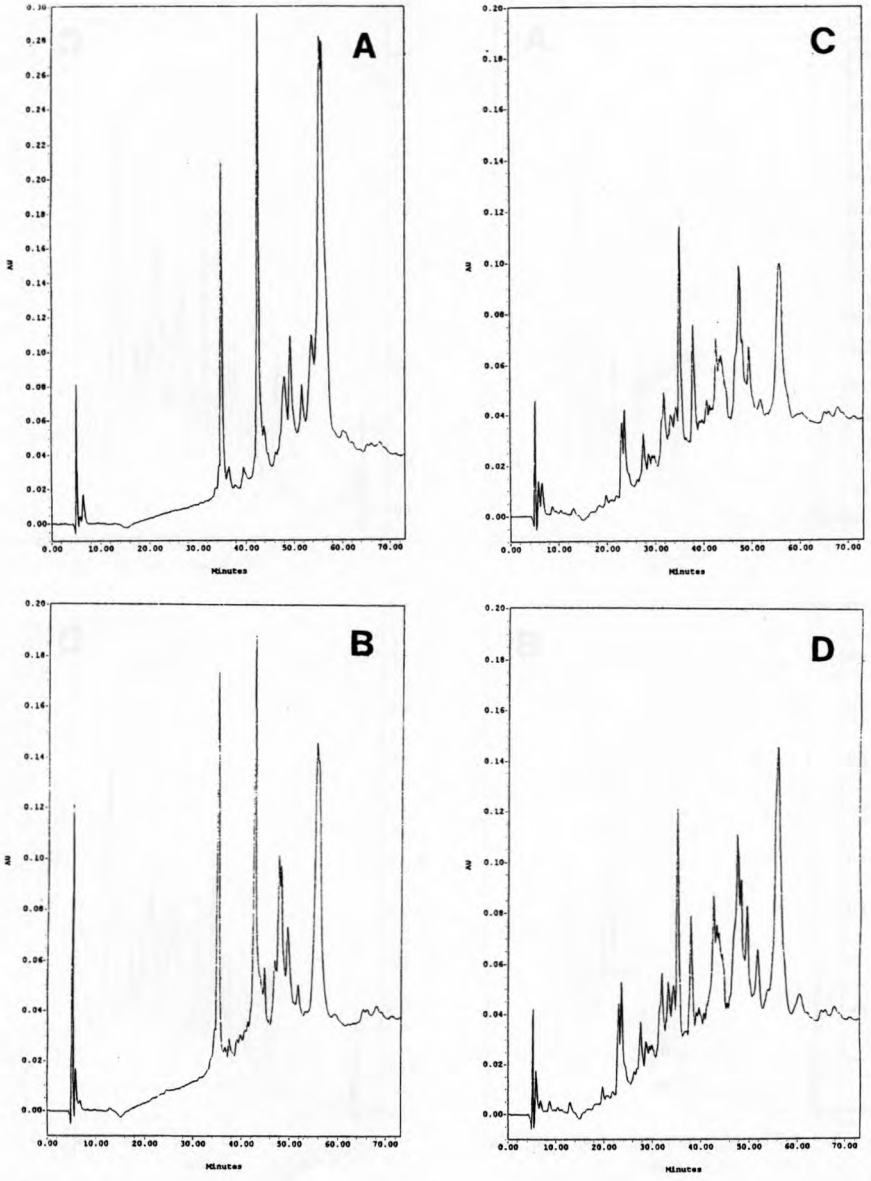

Fig 5. Reverse-phase HPLC chromatograms of the water-soluble/700 mL/L ethanol-insoluble fraction from 3-month-old Cheddar cheeses (trial 1) manufactured under controlled microbiological conditions, with or without adjunct lactobacilli by chemical (**A**, glucono-δ-lactone [GDL]; **B,** GDL/Lb adjunct) or biological (**C**, starter; **D**, starter/Lb adjunct) acidification.

*Chromatogramme RP-HPLC de la fraction soluble dans l'eau et insoluble dans l'éthanol (700 mL/L) des fromages de cheddar de 3 mois (essai 1) fabriqués dans des conditions microbiologiques contrôlées, avec ou sans ajout de lactobacilles, par acidification chimique (**A** : glucono-δ-lactone [GDL], **B** : GDL + Lb) ou biologique (**C** : levain, **D** : levain + Lb).*

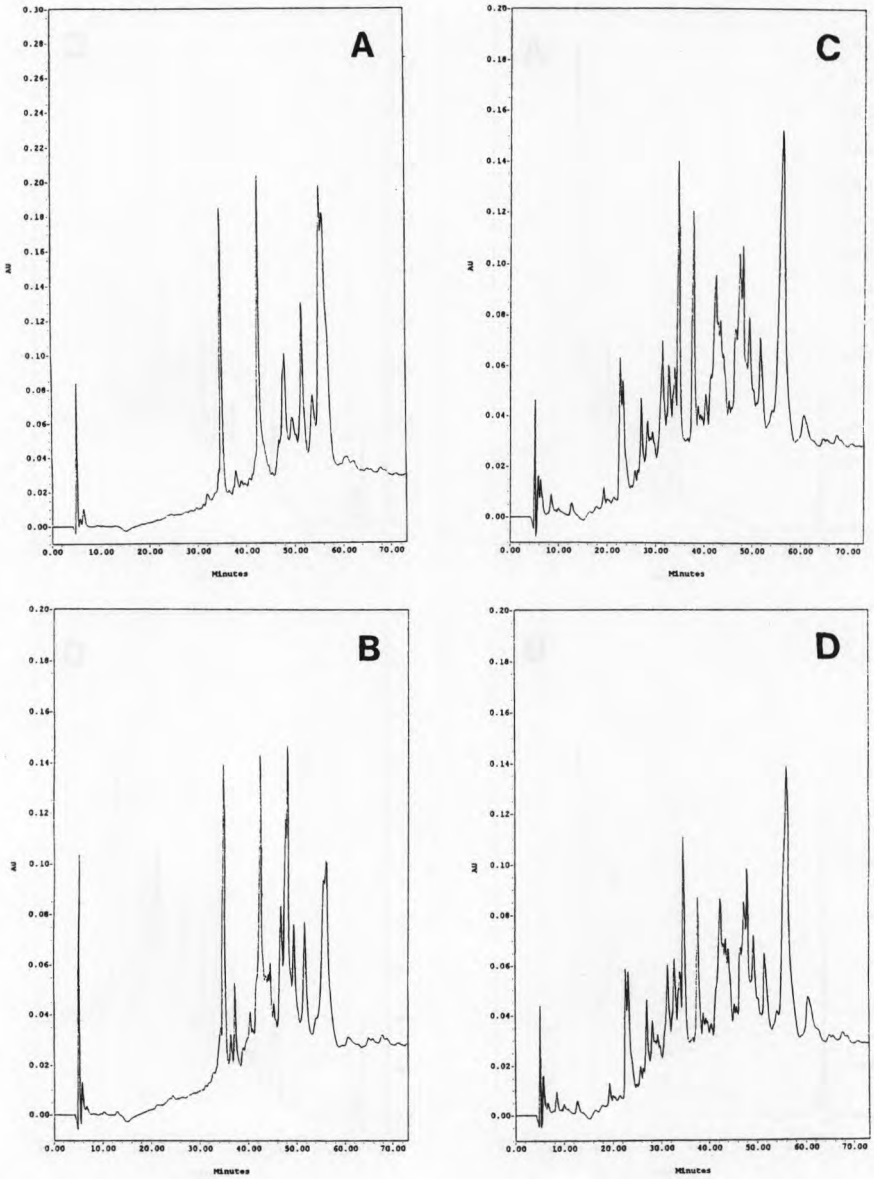

Fig 6. Reverse-phase HPLC chromatograms of the water-soluble/700 mL/L ethanol-insoluble fraction from 6-month-old Cheddar cheeses (trial 1) manufactured under controlled microbiological conditions, with or without adjunct lactobacilli by chemical (**A**, glucono-δ-lactone [GDL]; **B**, GDL/Lb adjunct) or biological (**C**, starter; **D**, starter/Lb adjunct) acidification.

*Chromatogramme RP-HPLC de la fraction soluble dans l'eau et insoluble dans l'éthanol (700 mL/L) des fromages de cheddar de 6 mois (essai 1) fabriqués dans des conditions microbiologiques contrôlées, avec ou sans ajout de lactobacilles, par acidification chimique (**A** : glucono-δ-lactone [GDL], **B** : GDL + Lb) ou biologique (**C** : levain, **D** : levain + Lb).*

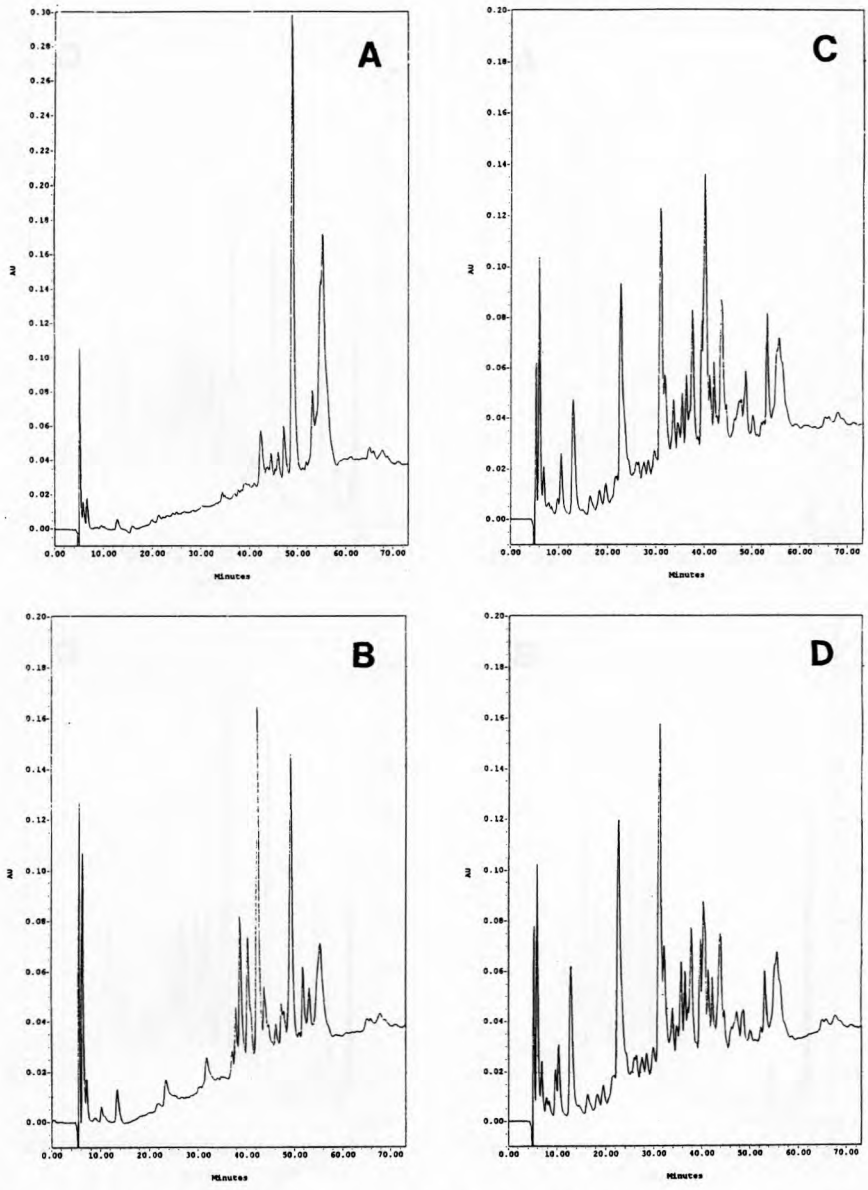

Fig 7. Reverse-phase HPLC chromatograms of the water-soluble/700 mL/L ethanol-soluble fraction from 3-month-old Cheddar cheeses (trial 1) manufactured under controlled microbiological conditions, with or without adjunct lactobacilli by chemical (**A**, glucono-δ-lactone [GDL]; **B**, GDL/Lb adjunct) or biological (**C**, starter; **D**, starter/Lb adjunct) acidification.

*Chromatogramme RP-HPLC de la fraction soluble dans l'eau et soluble dans l'éthanol (700 mL/L) des fromages de cheddar de 3 mois (essai 1) fabriqués dans des conditions microbiologiques contrôlées, avec ou sans ajout de lactobacilles, par acidification chimique (**A** : glucono-δ-lactone [GDL], **B** : GDL + Lb) ou biologique (**C** : levain, **D** : levain + Lb).*

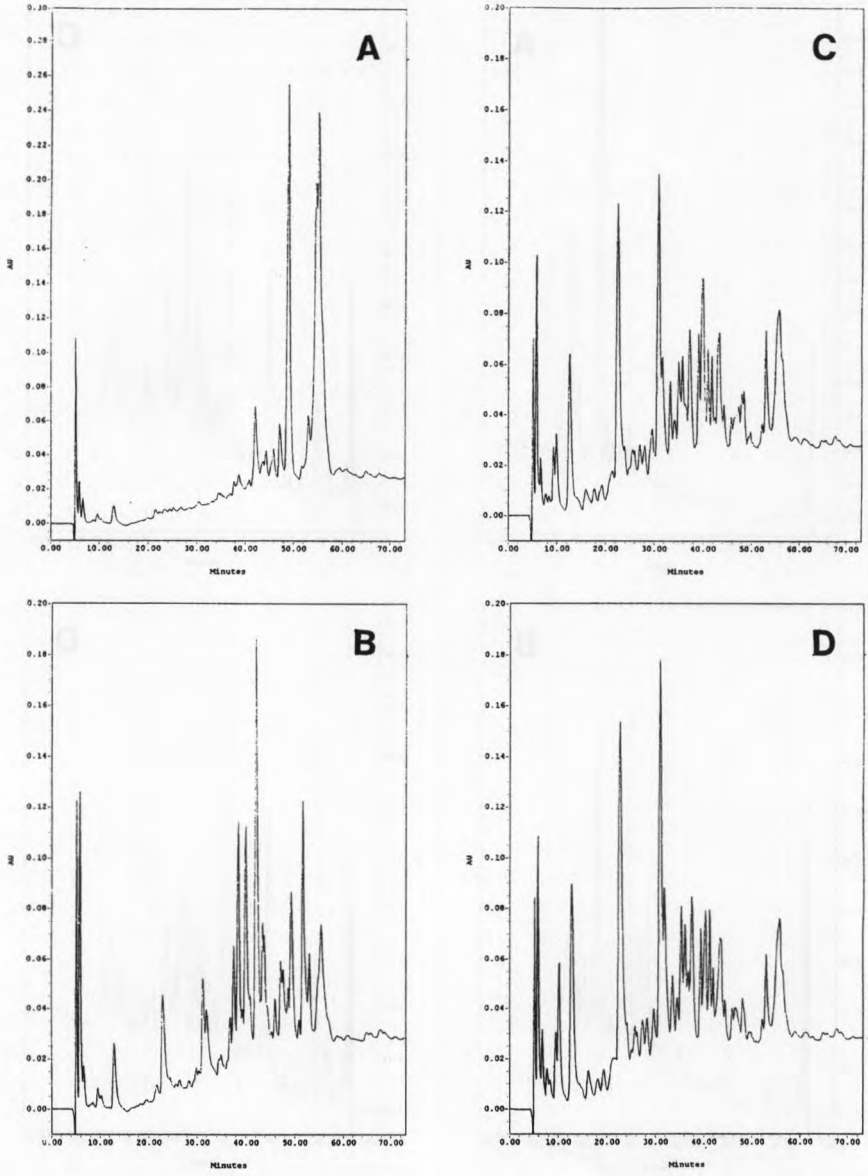

Fig 8. Reverse-phase HPLC chromatograms of the water-soluble/700 mL/L ethanol-soluble fraction from 6-month-old Cheddar cheeses (trial 1) manufactured under controlled microbiological conditions, with or without adjunct lactobacilli by chemical (**A**, glucono-δ-lactone [GDL]; **B**, GDL/Lb adjunct) or biological (**C**, starter; **D**, starter/Lb adjunct) acidification.

*Chromatogramme RP-HPLC de la fraction soluble dans l'eau et soluble dans l'éthanol (700 mL/L) des fromages de cheddar de 6 mois (essai 1) fabriqués dans des conditions microbiologiques contrôlées, avec ou sans ajout de lactobacilles, par acidification chimique (**A** : glucono-δ-lactone [GDL], **B** : GDL + Lb) ou biologique (**C** : levain, **D** : levain + Lb).*

of the ethanol-soluble fraction of the starter-acidified cheeses.

DISCUSSION

Microbiological analysis

Pasteurisation of the cheesemilk at 78°C for 15 s eliminated coliforms and indigenous NSLAB and reduced the total count to a very low level (table I). Milk of good microbiological quality is essential for cheese manufacture under controlled conditions, particularly with respect to indigenous NSLAB which, if present in the cheesemilk, grow rapidly during cheese ripening (McSweeney et al, 1993). The level of *Lactobacillus* inoculum in the experimental cheesemilk (~ 2.5×10^3 lactobacilli/mL) was chosen to approximate the numbers of indigenous *Lactobacillus* typically found in good quality raw milk (10^2–10^3 cfu/mL). Also, the strains of *Lactobacillus* used in the 'cocktail' were chosen to approximate the principal species found in mature Cheddar cheese manufactured from good quality raw milk (McSweeney et al, 1993).

The growth characteristics of the adjunct lactobacilli in the experimental cheeses (fig 1) may be considered typical and similar to those reported by other authors, eg, strains of *Lb casei* added to Cheddar cheesemilk at a level of 10^5/mL grew rapidly in the cheese to > 10^8 cfu/g after ~2 months and remained at this level to the end of a 12-month ripening period (Broome et al, 1990). Growth of lactobacilli in the experimental cheeses occurred more rapidly and higher numbers of lactobacilli were present for most of the ripening period than in typical commercial Cheddar cheese. The greater difficulty experienced in maintaining the chemically-acidified cheeses free of adventitious NSLAB in comparison to the starter-acidified cheeses was due to the more extensive handling of the curd required during manufacture and the greater quantities of residual lactose remaining in the starter-free curd. Growth of adventitious lactobacilli in the chemically-acidified controls was slightly more rapid than in the starter-free controls. This may have been due to the absence of starter-produced antimicrobial substances in the chemically-acidified controls. In addition, the higher pH of the chemically-acidified controls probably provided a slightly more favourable environment for the growth of lactobacilli than in the starter-acidified controls. However, in both trials, a 1–2 log cycle difference in numbers of lactobacilli was maintained throughout ripening between the control and experimental cheeses, demonstrating the effectiveness of the aseptic vat technique used. Lower numbers of lactobacilli were maintained in the GDL and starter control cheeses (particularly during the first 4 months of ripening) than in the controls in the study of Lane and Fox (1996).

Compositional analysis

The composition of all cheeses (table II) was within the normal range expected for Cheddar. The slightly higher pH of the chemically-acidified cheeses compared to the starter-acidified cheeses was due mainly to the difficulty in controlling the pH during chemical acidification owing to loss of GDL in the whey and the lack of precision in estimating the correct amount of GDL to add to the curd (which could not be weighed due to the risk of contamination). The slightly lower pH of the cheeses containing the *Lactobacillus* adjunct in comparison to their respective controls was probably due to metabolism of residual lactose by the adjunct lactobacilli with the production of lactic acid. Lynch et al (1996) also noted slightly lower pH values in cheeses containing mesophilic *Lactobacillus* adjuncts in comparison to controls.

Sensory analysis

The flavour of the GDL control cheese was considered to be bland in comparison to the other cheeses at both gradings (table III). It is a widely held view that proper, balanced Cheddar cheese flavour cannot be produced without starter bacteria (Urbach, 1995). The adjunct lactobacilli in the experimental cheeses appeared to cause some intensification in cheese flavour particularly in the GDL/Lb adjunct cheese; however, a high degree of variability was noted between the taste panel members. McSweeney et al (1994b) reported intensification of the flavour of Cheddar cheese containing adjunct lactobacilli and while cheeses containing adjuncts also scored better for flavour acceptability, their flavour was considered atypical. Therefore, it appears that a heterogeneous population of lactobacilli, present at high numbers (> 10^8 cfu/g) during ripening, intensified cheese flavour (especially in the absence of a starter) but the resulting flavour was considered atypical and was unacceptable to some, but attractive to other, members of a typical taste panel. Unfortunately, Lane and Fox (1996) did not assess the sensory qualities of the cheeses in their study.

Assessment of proteolysis

Urea-PAGE electrophoretograms (fig 2) of whole cheese samples at day 0 and 1 month showed essentially no qualitative differences between the cheeses. This was as expected since the level of proteolysis detectable by PAGE is mediated mainly by chymosin and plasmin (O'Keeffe et al, 1976). However, in comparison to the chemically-acidified cheeses, the starter-acidified cheeses did have slightly higher concentrations of a band

Table III. Average grades (± standard deviations) at 18 and 26 weeks of ripening of Cheddar cheeses manufactured under controlled microbiological conditions, with and without adjunct lactobacilli (0–10, unacceptable; 10–30, poor; 30–50, acceptable; 50–70, good; 70–80, excellent).

Classement moyen (± écart type) à 18 et 26 semaines d'affinage, de fromage de cheddar fabriqué dans des conditions microbiologiques contrôlées, avec ou sans ajout de lactobacilles (0–10, inacceptable ; 10–30, médiocre, 30–50, acceptable ; 50–70, bon ; 70–80, excellent).

	Flavour intensity		*Flavour acceptability*	
	Trial 1	*Trial 2*	*Trial 1*	*Trial 2*
18 weeks				
GDL[1]	22.5 ± 14.9	24.4 ± 9.4	16.9 ± 11.6	22.5 ± 11.7
GDL/Lb adjunct	44.4 ± 28.0	39.4 ± 20.4	14.4 ± 11.8	14.4 ± 16.8
Str[2]	42.5 ± 10.7	40.6 ± 11.5	38.8 ± 11.6	40.0 ± 10.4
Str/Lb adjunct	50.0 ± 9.6	48.1 ± 10.0	37.5 ± 10.4	33.8 ± 13.3
26 weeks				
GDL	23.8 ± 15.1	34.4 ± 13.0	21.3 ± 9.9	31.9 ± 10.3
GDL/Lb adjunct	53.8 ± 24.6	48.8 ± 27.9	19.4 ± 19.3	18.1 ± 16.5
Str	47.5 ± 16.5	49.6 ± 8.0	48.1 ± 14.1	44.4 ± 11.2
Str/Lb adjunct	50.6 ± 23.4	51.9 ± 18.7	44.4 ± 20.8	40.0 ± 20.9

[1] Glucono-δ-lactone (chemically-acidified cheese). [2] Starter-acidified cheese.

[1] Glucono-δ-lactone (fromage acidifié chimiquement). [2] Fromage acidifié par levain.

with slightly faster mobility than β-CN (probably β-CN f1-192 (β-I), produced by chymosin) and slightly higher concentrations of α_{s1}-CN f24–199 and α_{s1}-CN f102–199 (identified according to McSweeney et al, 1994a; Mooney and Fox, unpublished), suggesting slightly higher chymosin activity at the lower pH of these cheeses (Fox et al, 1994).

At 3 and 6 months, the greater breakdown of β-CN noted in the GDL control cheese (probably due to its higher pH) was accompanied by higher levels of the γ-caseins, which are produced by plasmin from β-CN (Grufferty and Fox, 1988). The dependence of the proteolysis of β-CN on pH has been demonstrated by Noomen (1978), who observed increased degradation of β-CN in Meshanger-type cheese with an elevated curd pH and a constant 4% salt-in-moisture. Creamer (1975) noted greater degradation of β-CN in a 14-week-old Gouda cheese in comparison to a Cheddar of the same age. This was attributed primarily to the higher pH of the Gouda in comparison to the Cheddar (5.38 and 4.95 respectively) and the resultant higher plasmin activity in the Gouda sample. The qualitative and quantitative differences in peptides (originating mainly from α_{s1}-CN) observed at 6 months between the chemically-acidified and starter-acidified cheese, as well as those observed between the GDL control and the GDL/Lb adjunct cheeses, probably arose from different residual rennet activities (ie, greater residual chymosin activity at lower pH). Similar trends in primary proteolysis were reported by Lane and Fox (1996).

The higher levels of WSN as % of TN (fig 3) found during ripening in the starter-acidified cheeses in comparison to the chemically-acidified cheeses are in agreement with the results of Visser (1977), who concluded that although rennet is the principal contributor to the formation of WSN, cheeses manufactured aseptically without a starter developed lower levels of WSN than those acidified with a starter. The higher levels of WSN in the GDL control cheese in comparison to the GDL/Lb adjunct cheese were probably due to the higher plasmin activity in the GDL cheese (due to its higher pH) which led to greater formation of proteose peptones (N-terminal fragments of β-CN produced by plasmin) which are soluble at pH 4.6 and therefore accumulated to a greater extent in the GDL control cheese (which had a higher pH and greater β-CN breakdown than the GDL/Lb adjunct cheese, as indicated by urea-PAGE). The GDL control (pH 5.52) of Lane and Fox (1996) had a slightly lower content of WSN than that of the GDL/Lb adjunct (pH 5.41) cheese throughout ripening. These authors suggested that the slightly higher level of WSN in the GDL/Lb adjunct cheese reflected proteinase/peptidase activity by the adjunct lactobacilli.

The limited formation of FAA (fig 4) in the GDL control cheese in comparison to the starter control cheese is agreement with the results of O'Keeffe et al (1976) and highlights the importance of the starter and the limited role of chymosin and plasmin in the production of FAA. The *Lactobacillus* adjunct promoted an increase in FAA in both the chemically-acidified and starter-acidified experimental cheeses. NSLAB have been shown to influence the production of FAA (Puchades et al, 1989; Broome et al, 1990; McSweeney et al, 1994b). The increased levels of FAA in the experimental cheeses in this study was probably due to peptidase activity by the adjunct lactobacilli. Similar trends in the formation of FAA were reported by Lane and Fox (1996).

Comparison of the peptide profiles of the ethanol-insoluble fractions of the WSEs from the starter control and starter/Lb. adjunct cheeses at 3 (fig 5) or 6 (fig 6) months indicated that, in the presence of starter, the adjunct lactobacilli were of little importance in the breakdown and forma-

tion of these peptides. The greater differences in peptide profiles observed between the GDL control and the starter control cheeses than between the GDL control and GDL/Lb adjunct cheeses points to the much greater role played by starter enzymes in the breakdown/formation of ethanol-insoluble, water-soluble peptides. Comparison of the peptide profiles of the ethanol-soluble fractions of the WSEs from the cheeses at 3 (fig 7) or 6 (fig 8) months again highlighted the greater importance of the starter in the breakdown/formation of these peptides; however, some important qualitative differences were observed between the GDL and GDL/Lb adjunct cheeses at both times. In particular, the peptides eluting at 23 and 31 (which were quite prominent in the profiles of the starter-acidified cheeses) were probably α_{s1}-CN f1-9 and α_{s1}-CN f1-13 (Fox et al, 1994), produced by the action of the lactococcal cell-envelope proteinase on α_{s1}-CN f1-23 (produced by chymosin). Therefore, it is possible that these peptides (and others) were produced in the GDL/NSLAB cheese by the action of proteinases and peptidases from NSLAB on α_{s1}-CN f1-23. Although Lane and Fox (1996) used ultrafiltration (UF) to fractionate WSEs, the results obtained were generally similar to those of the present study, ie, few differences were observed between the starter and starter/NSLAB cheeses but large quantitative and qualitative differences between the UF permeates and retentates of the GDL control cheese and those of the starter control and GDL/NSLAB cheeses were observed.

The contribution of starter bacteria to Cheddar cheese ripening appears to be much greater than that of NSLAB. Starter bacteria are essential for the development of proper Cheddar flavour while NSLAB appear to intensify cheese flavour (especially in the absence of a starter), but do not necessarily improve it. Both starter and NSLAB contribute very little to primary proteolysis in Cheddar cheese, ie, as detected by PAGE. Starter bacteria make a greater contribution to secondary proteolysis than NSLAB (the contribution of which appears to be mainly the formation of FAA). An important finding of this study is that while adjunct lactobacilli contributed relatively little to proteolysis in starter-containing cheeses, they produced small peptides in starter-free cheeses, probably including the breakdown products of α_{s1}-CN f1-23 in the water-soluble, ethanol-soluble fraction. The results presented here are largely in agreement with those of Lane and Fox (1996).

ACKNOWLEDGMENTS

The assistance of Ms C Lane in cheese manufacture, the technical assistance of Mr E O'Connor and the financial support of FORBAIRT are gratefully acknowledged. This research project was part funded from EU structural funds under the food sub-programme of the industry programme.

REFERENCES

Andrews AT (1983) Proteinases in normal bovine milk and their action on the caseins. *J Dairy Res* 50, 45–55

Blakesley RW, Boezi JA (1977) A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Anal Biochem* 82, 580–581

Broome MC, Krause DA, Hickey MW (1990) The use of non-starter lactobacilli in Cheddar cheese manufacture. *Aust J Dairy Technol* 45, 67–73

Chapman HR, Sharpe ME (1981) Microbiology of cheese. In: *Dairy microbiology*, Vol 2 (Robinson RK, ed) Applied Science Publishers, London, 157–243

Creamer LK (1975) β-Casein degradation in Gouda and Cheddar cheese. *Dairy Sci* 58, 287–292

deMan JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. *J Appl Bacteriol* 23, 130–135

Folkertsma B, Fox PF (1992) Use of the Cd-ninhydrin reagent to assess proteolysis in cheese during ripening. *J Dairy Res* 59, 217–224

Fox PF (1963) Potentiometric determination of salt in cheese. *J Dairy Sci* 46, 744–745

Fox PF, Singh TK, McSweeney PLH (1994) Proteolysis in cheese during ripening. In: *Biochemistry of milk products* (Andrews AT, Varley J, eds) Royal Society of Chemistry, Cambridge, 1–31

Franklin JG, Sharpe ME (1963) The incidence of bacteria in cheesemilk and Cheddar cheese and their association with flavour. *J Dairy Res* 30, 87–99

Grufferty MB, Fox PF (1988) Milk alkaline proteinase. *J Dairy Res* 55, 609–630

IDF (1964) *Determination of the protein content of processed cheese products, Standard 25.* International Dairy Federation, Brussels

IDF (1982) *Cheese and processed cheese. Determination of the total solids content, reference method 4A.* International Dairy Federation, Brussels

IS (1955) *Irish Standards 69. Determination of the percentage of fat in cheese.* Institute for Industrial Research and Standards, Dublin

Jordan KN, Cogan TM (1993) Identification and growth of non–starter lactic acid bacteria in Irish Cheddar cheese. *Irish J Agric Food Res* 32, 47–55

Kosikowski F (1977) Cheddar cheese and related types. *In: Cheese and fermented milk foods.* Edwards Bros Inc, Ann Arbor, MI, 228–260

Kuchroo CN, Fox PF (1982) Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft* 37, 331–335

Lane CN, Fox PF (1996) Contribution of starter and added lactobacilli to proteolysis in Cheddar cheese during ripening. *Int Dairy J* 6, 715–728

Lynch CM, McSweeney PLH, Fox PF, Cogan TM, Drinan FD (1996) Manufacture of Cheddar cheese with and without adjunct lactobacilli under controlled microbiological conditions. *Int Dairy J* 6, 851–867

Mabbitt LA, Chapman HR, Berridge NJ (1955) Experiments in cheesemaking without starter. *J Dairy Res* 22, 365–373

McSweeney PLH, Fox PF, Lucey JA, Jordan KN, Cogan TM (1993) Contribution of the indigenous microflora to the maturation of Cheddar cheese. *Int Dairy J* 3, 613–634

McSweeney PLH, Pochet S, Fox PF, Healy A (1994a) Partial identification of peptides from the water-insoluble fraction of Cheddar cheese. *J Dairy Res* 61, 587–590

McSweeney PLH, Walsh EM, Fox PF, Cogan TM, Drinan FD, Castelo-Gonzalo M (1994b) A procedure for the manufacture of Cheddar cheese under controlled bacteriological conditions and the effect of adjunct lactobacilli on cheese quality. *Irish J Agric Food Res* 33, 183–192

Naylor J, Sharpe ME (1958) Lactobacilli in Cheddar cheese. III. The source of lactobacilli in cheese. *J Dairy Res* 25, 431–438

Noomen A (1978) Activity of proteolytic enzymes in simulated soft cheeses (Meshanger-type). 1. Activity of milk protease. *Neth Milk Dairy J* 32, 26–48

O'Keeffe RB, Fox PF, Daly C (1975) Proteolysis in Cheddar cheese: influence of the rate of acid production during manufacture. *J Dairy Res* 42, 111–122

O'Keeffe RB, Fox PF, Daly C (1976) Contribution of rennet and starter proteases to proteolysis in Cheddar cheese. *J Dairy Res* 43, 97–107

Puchades R, Lemieux L, Simard RD (1989) Evolution of free amino acids during the ripening of Cheddar cheese containing added lactobacilli strains. *J Food Sci* 54, 885–888

Reiter B, Sorokin Y, Pickering A, Hall AJ (1969) Hydrolysis of fat and protein in small cheeses made under aseptic conditions. *J Dairy Res* 36, 65–76

Smid EJ, Poolman B, Konnings WN (1991) Casein utilisation by lactococci. *Appl Environ Microbiol* 57, 2447–2452

Urbach G (1995) Contribution of lactic acid bacteria to flavour compound formation in dairy products. *Int Dairy J* 5, 877–903

Visser FMW (1977) Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 3. Protein breakdown: analysis of the soluble nitrogen and amino nitrogen fractions. *Neth Milk Dairy J* 31, 210–239