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Original article

Influence of starter and adjunct lactobacilli culture on ripening of miniature washed-curd cheeses

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Abstract – Ten strains of *Lactobacillus* were tested as adjunct cultures in combination with two different *Lactococcus lactis* starters in miniature washed-curd cheeses manufactured under controlled bacteriological conditions. Growth of lactobacilli seemed to depend on the strain used, but was not influenced by the starter strain (either *L. lactis* subsp. *lactis* IL416 or *L. lactis* subsp. *cremoris* AM2). Lactococcal counts in miniature cheeses with AM2 starter and added lactobacilli were higher than in control cheeses without lactobacilli. Consistently good survival (~100% after four weeks) was observed for IL416, regardless of the presence of adjunct culture). In contrast, AM2 starter cell viability decreased slowly over the same time period. Gross composition and protein analyses were performed on the miniature cheeses. Our results indicate that production of soluble nitrogenous compounds was influenced by the lactobacilli adjunct, and depended on the starter strain. We conclude that the use of different combinations of starter and adjunct cultures can result in marked differences in bacterial populations and product properties. Such studies may be used to choose the combination of strains necessary to obtain a product with particular properties.

Lactobacilli adjunct culture / starter / proteolysis / cheese

Résumé – **Influence du levain et d'une culture secondaire de lactobacilles sur l'affinage des fromages miniatures type pâte pressée.** Dix souches de lactobacilles de la collection CNRZ ont été testées en tant que culture secondaire, en combinaison avec deux souches différentes de levain *Lactococcus lactis*, dans des fromages miniatures type pâte pressée fabriqués en conditions bactériologiques contrôlées. La croissance des lactobacilles semble dépendre de la souche utilisée, mais elle n'est pas influencée par la souche de levain (*L. lactis* subsp. *lactis* IL416 ou *L. lactis* subsp. *cremoris* AM2). La numération des lactocoques dans les fromages miniatures fabriqués avec la souche AM2 et addition de lactobacilles est plus élevée que dans les fromages témoins sans ajout de lactobacilles. Comme attendue, une bonne survie a été observée pour IL416 (~100 % après quatre semaines), et ce indépendamment de la présence d'une culture secondaire. Au contraire, la viabilité de AM2 a décru au cours de la même période. La composition globale et des analyses biochimiques ont été réalisées sur les fromages miniatures. Nos résultats indiquent que l'addition de lactobacilles influence la production de composés azotés solubles en liaison avec la souche de

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levain. Nous concluons qu'en utilisant différentes combinaisons de lactocoques et de cultures secondaires, on peut obtenir des différences marquées dans les populations bactériennes et les propriétés des produits. De telles études pourraient être utilisées pour choisir les combinaisons de souches nécessaires pour obtenir un produit avec des propriétés spécifiques.

Culture secondaire de lactobacilles / levain / protéolyse / fromage type pâte pressée

1. INTRODUCTION

Cheese ripening is a complex process involving proteolysis, lipolysis and metabolism of residual lactose, citrate and lactate [11]. These transformations are produced by enzymes of micro-organisms as well as residual chymosin and indigenous milk proteases (and lipases in raw-milk cheeses).

Starter lactic acid bacteria (LAB) ferment lactose to give lactic acid during cheese-making. They also play a role in cheese flavour development during ripening [10]. Starter bacteria are added at high concentrations to milk, and counts attain 10^8–10^9 CFU·g^{-1} after cheese manufacture; however, the bacteria die and lyse during ripening, at rates that vary from strain to strain [3]. The intracellular peptidases are then released into the curd, where they can hydrolyse peptides generated from primary proteolysis to free amino acids (FAA) [7]. Some amino acids are sapid compounds themselves, but FAA in cheese are mainly important as precursors of flavour, since LAB can metabolise them to ammonia, carbonyl and sulfur compounds, etc., all of which strongly influence cheese flavour [29]. Lactococci of the starter also participate in the metabolism of residual lactose, citrate and lactate to produce different volatile compounds that take part in cheese aroma [28]; they also show weak lipolytic activity [4].

The contribution to cheese ripening of the other important group of LAB in cheese, i.e. non-starter lactic acid bacteria (NSLAB), remains uncertain. In cheeses made with pasteurised milk, the adventitious NSLAB contaminate cheese after milk pasteurisation or survive the heat treatment [42]. The main source of contamination is probably the resident flora in the dairy plant and/or the raw milk itself [31]. In Cheddar cheese, adventitious NSLAB are able to grow from very low numbers to 10^7–10^8 CFU·g^{-1} during the first weeks of ripening, and dominate the cheese microflora after starter cell viability starts to decrease [11]. NSLAB are mainly mesophilic lactobacilli in cheeses made with pasteurised milk, but pediococci and enterococci have also been found [6, 29, 32].

One strategy used to study the influence of NSLAB in ripening involves addition of *Lactobacillus* strains isolated from raw milk or cheese as a secondary or adjunct culture in cheese-making experiments. The majority of studies have shown that FAA content is increased and flavour intensity enhanced in experimental cheeses, even though acceptability by consumers is not always improved [2, 21, 23–25, 34]. Experimental Cheddar cheese containing a complex adjunct culture of mesophilic lactobacilli developed a stronger flavour than the control [30] and in another system, experimental cheese had a higher score for flavour intensity than the control [41]. Other authors have suggested that lactobacilli cultures can control the secondary microflora of cheese, and as such, prevent the growth of adventitious strains of NSLAB that may potentially cause defects [27]. However, these results are controversial: it was also reported that lactobacilli are not needed to obtain a good quality Gouda cheese [17] and the influence of lactobacilli in Cheddar cheese flavour was even suggested to be detrimental [43]. The influence of non-starter lactobacilli is suggested to be dependent on the species and strains composing this flora, and their final

counts [33]. Furthermore, the starter strain and environmental factors (mainly temperature) seem to play a major role in secondary flora development [20].

Experimentation on cheese models for NSLAB study has proven to be complex. Even under the strict bacteriological conditions needed for cheese-making, control cheeses are susceptible to contamination by adventitious NSLAB, or the NSLAB may have survived pasteurisation [2, 17, 41]. Phage attack, even at levels that do not compromise acidification, can also affect properties of the product by changing proteolysis patterns [3].

The objective of this work was to screen the expression (growth and biochemical activities) of ten adjunct cultures of mesophilic lactobacilli, during the ripening process of miniature washed-curd cheeses manufactured with two different *Lactococcus* starter strains in controlled microbiological conditions.

2. MATERIALS AND METHODS

2.1. Cultures

2.1.1. Starter cultures

Two types of starter cultures were used in the cheese-making: *L. lactis* subsp. *lactis* IL416, and *L. lactis* subsp. *cremoris* AM2.

Strain IL416 was selected for its high resistance to phage infection [5], and AM2 for its autolysis due to activated prophage [22]. Both strains were obtained from the CNRZ collection.

Each strain was inoculated in sterilised (110 °C, 10 min), reconstituted (10% w/v), low-heat skim milk powder (Food Service, Le Pecq, France). Several ten-fold successive culture dilutions were prepared and incubated overnight at 25 °C. The first dilution (in increasing order) in which milk did not clot was used as starter, to optimise the uniformity of starter activity and pH between cheese-making experiments.

2.1.2. Adjunct cultures

Five strains of *Lb. plantarum* (identified as 1228, 1245, 1310, 1311 and 1572), one *Lb. pentosus* (1218) and four *Lb. casei* (1219, 1227, 1244 and 1308) were tested as adjunct cultures. All the strains were obtained from the CNRZ collection (this laboratory), and had been isolated from cheese [38].

Strains were cultured individually in MRS broth [9] at 30 °C overnight, then transferred (2%) and incubated for 8 h at 30 °C. Several ten-fold dilutions were prepared and incubated at 30 °C overnight. The dilutions with an optical density (O.D.) = 0.8 (approximately 10^8 to 5×10^8 CFU·mL^{-1} for the tested strains) were used in the experiments.

2.2. Cheese manufacture

Two experimental cheeses (containing lactobacilli adjunct culture) and two control cheeses (without lactobacilli) of approximately 40 g each were produced simultaneously per cheese-making day. The choice of starter strains was alternated from one cheese-making day to another. Each lactobacilli adjunct was tested with both starters.

The cheeses were made in controlled bacteriological conditions, according to a previously described protocol adapted from Saint-Paulin type cheese technology [13, 36]. Whole pasteurised homogenised pooled milk was purchased in the local market every two cheese-making days, and analysed for pH, fat, dry matter, casein and non-protein nitrogen content to check for uniformity of gross composition.

Miniature cheeses were ripened for 28 d at 12 °C and 80% relative humidity. They were sampled for physical, chemical and microbiological analysis at 1, 14 and 28 d of ripening.

2.3. Analyses

2.3.1. Gross composition of cheeses

The pH values were determined throughout the cheese-making and ripening process with a pH meter Orion 920A (Orion, Boston, USA) equipped with a penetration electrode (Mettler Toledo, Paris, France). Measurements were taken on 2 g of milk or curd samples that were removed under sterile conditions.

Gross composition was determined on one-d-old cheeses. Dry matter was analysed in duplicate using the IDF method (IDF standard 4:1958) and NaCl content was determined in triplicate with a potentiometric chloride analyser (Corning Limited, Halstead, Essex, England).

2.3.2. Bacteriological analyses and phage detection

The population of starter lactococci present in the cheese products after 1, 14 and 28 d of ripening was determined by plating sample dilutions on M17 agar and after 48 h of incubation at 30 °C [39]. To determine the population of NSLAB, samples were also plated on Lactobacilli Selective Agar (LBS, Baltimore Biological Laboratories, Rockville, USA) and incubated for 72 h under anaerobic conditions [26]. The absence of lytic phages during cheese-making was verified as described [16].

2.3.3. Assessment of proteolysis

Cheese samples were treated to obtain crude citrate extract, insoluble and soluble fractions at pH 4.4 and the soluble fraction in phosphotungstic acid (PTA), according to Gripon et al. [12]. Nitrogen content of the fractions was determined in duplicate by the macro-Kjeldahl method according to the IDF Standard 20B [15].

The insoluble fraction at pH 4.4 was analysed by reverse phase liquid chromatography (RP-HPLC) and the relation between the peak area for αs1 casein and the peptide αs1–I, resulting from chymosin activity, calculated as $\%\frac{\alpha s1 - I}{\alpha s1 - I + \alpha s1}$ was used as an index of primary proteolysis. A 25 mg casein sample was dissolved in 1.5 mL of a Bis-Tris buffer also containing urea and β-mercaptoethanol and analysed according to Visser et al. [44]. The HPLC equipment consisted of two pumps (models 501 and 510), an automatic injector Wisp 712, an interface module, and a model 484 Tunable Absorbance Detector (purchased from Waters: Millipore S.A., St. Quentin-en-Yvelines, France). A 250 mm × 3.9 mm Nucleosil C18, 5 µm–300 Å analytical column was used (Macherey Nagel, Gmbh & Co, Düren, Germany) with a C18 Bondapak cartridge as guard-column (Waters, St. Quentin-en-Yvelines, France). The chromatographic conditions were as described by Hynes et al. [14].

Ethanol 70% soluble extract of cheeses was also analysed by RP-HPLC using the equipment described above. The extract was prepared according to Lynch et al. [24]. Water-soluble extracts of the cheeses were obtained by blending 5 g of cheese and 15 mL of milliQ water with an Ultraturrax homogeniser (IKA-Werke, Gmbh & Co, Düren, Germany) at maximum speed for 2 min then warmed up to 40 °C and maintained for 1 h. The suspension was centrifuged at 3000 *g* and filtered through glass wool [18]. The filtered solution was adjusted to a final volume of 25 mL, from which aliquots of 1 mL were fractionated by adding absolute ethanol to a final ethanol concentration of 70% (v/v). For each sample, the mixture was maintained at room temperature for 1 h and micro-centrifuged at 12 000 *g* for 10 min. The supernatant was recovered and the ethanol was evaporated in a SpeedVac (Varian, Hicksville, USA). 120 µL of each Speed-Vac-concentrated liquid sample were injected into the HPLC chromatograph. Detection was performed at 214 nm and the column temperature was 40 °C.

Table I. Gross composition of control and experimental 1-d-old miniature cheeses. Dry matter and salt-in-moisture (S/M) means did not differ; pH was significantly different for the two starter strains ($P < 0.05$).

Starter strain	IL416	AM2
% Dry matter	45.14 ± 0.71	44.92 ± 0.76
% S/M	2.45 ± 0.21	2.39 ± 0.24
pH	5.18 ± 0.06	5.08 ± 0.05

The gradient, starting from 100% of solvent A (H_2O–trifluoroacetic acid (TFA), 1000:1.1 v/v) and 0% of solvent B (Acetonitrile-H_2O-TFA 600:400:1 v/v/v), was generated 10 min after injection. The proportion of solvent B was increased at 1% min^{-1} (80 min); 20% min^{-1} (1 min); 0% min^{-1} (4 min) and then returned to starting conditions, which took 1 min. These last setting conditions were maintained for 10 min.

Qualitative differences between peptide profiles of the ethanol 70% soluble extracts were evaluated by visually comparing chromatograms, and quantitative differences by comparing areas of individual peaks expressed as arbitrary area units.

3. RESULTS

3.1. Gross composition

The gross composition of the one-d-old miniature cheeses (Tab. I) successfully modelled washed-curd cheese varieties [35]. No significant differences in dry matter and salt-in-moisture (S/M) content were observed between starter strains. Average pH values were significantly lower for cheeses made with AM2 starter, than for those made with IL416 starter.

3.2. Microbiology

Bacterial cell counts of lactococci and lactobacilli were determined during ripening of cheeses prepared using the two tested starters with and without adjunct cultures (Figs. 1 and 2). The control cheeses were free of exogenous adventitious lactobacilli throughout the ripening period.

The bacterial counts of *L. lactis* IL416 remained constant during ripening (above 10^9 CFU·g^{-1} of cheese), indicating the good stability of the starter population. Lactobacilli did not dominate the microflora of the cheeses at any stage of ripening. For strains of *Lb. casei* 1244 and 1308, and *Lb. pentosus* 1218, cell counts increased from ~10^6 CFU·mL^{-1} in milk to ~10^7 CFU·g^{-1} in curd during cheese-making and then remained constant. *Lb. casei* 1227 increased to ~10^8 CFU·g^{-1} the first day and then remained constant. *Lb. plantarum* 1310, 1572 and 1245 populations all gained approximately 1 log during ripening. The other strains did not increase during cheese-making (Fig. 1). Lactobacilli cells were added at circa 10^6 CFU·mL^{-1}, but lactobacilli counts in 1-d-old cheeses were ~10^7 CFU·g^{-1}. This increase could be a consequence of cell concentration but also some growing that compensated for the loss of lactobacilli during whey removal and washing.

In contrast to IL416, the *L. lactis* AM2 population decreased ~hundred-fold during cheese ripening (to 10^7 CFU·g^{-1}), possibly due to lysis by an activated prophage. Nevertheless, in several experimental cheeses (containing lactobacilli), the starter population remained higher than in control cheeses (without lactobacilli). This effect was verified for *Lb. casei* 1227, 1244, 1308, *Lb. plantarum* 1572 and 1245 and *Lb. pentosus* 1218. It was suggested that lactococcal counts on M17 agar could be over-estimated in cheeses containing added lactobacilli [20]. This possibility was ruled out in our study because we observed that the tested *Lactobacillus* strains grew very slowly on M17 agar plates, and were not detected after 48 h of incubation under aerobic conditions. In addition, in experimental cheeses prepared with starter AM2 and *Lactobacillus* strains 1218 and 1572, lactobacilli counts were

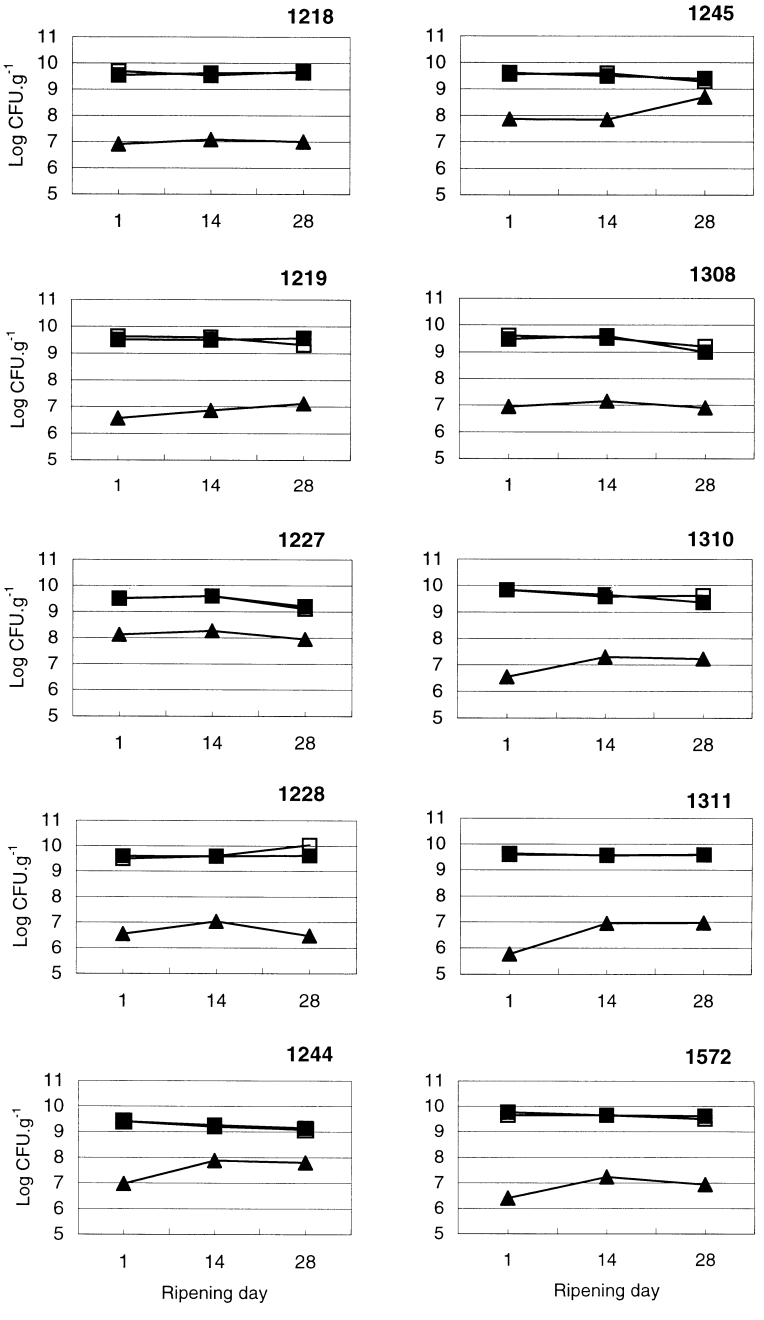

Figure 1. Growth curves of strain bacteria in miniature cheeses made with starter *L. lactis* IL416. □ Lactococci in control cheese; ■ Lactococci in experimental cheese; ▲ Lactobacilli in experimental cheese. Number in bold characters indicates *Lactobacillus* strain: 1218 *Lb. pentosus*; 1228, 1245, 1310, 1311 and 1572 *Lb. plantarum*; 1219, 1227, 1244 and 1308 *Lb. casei*.

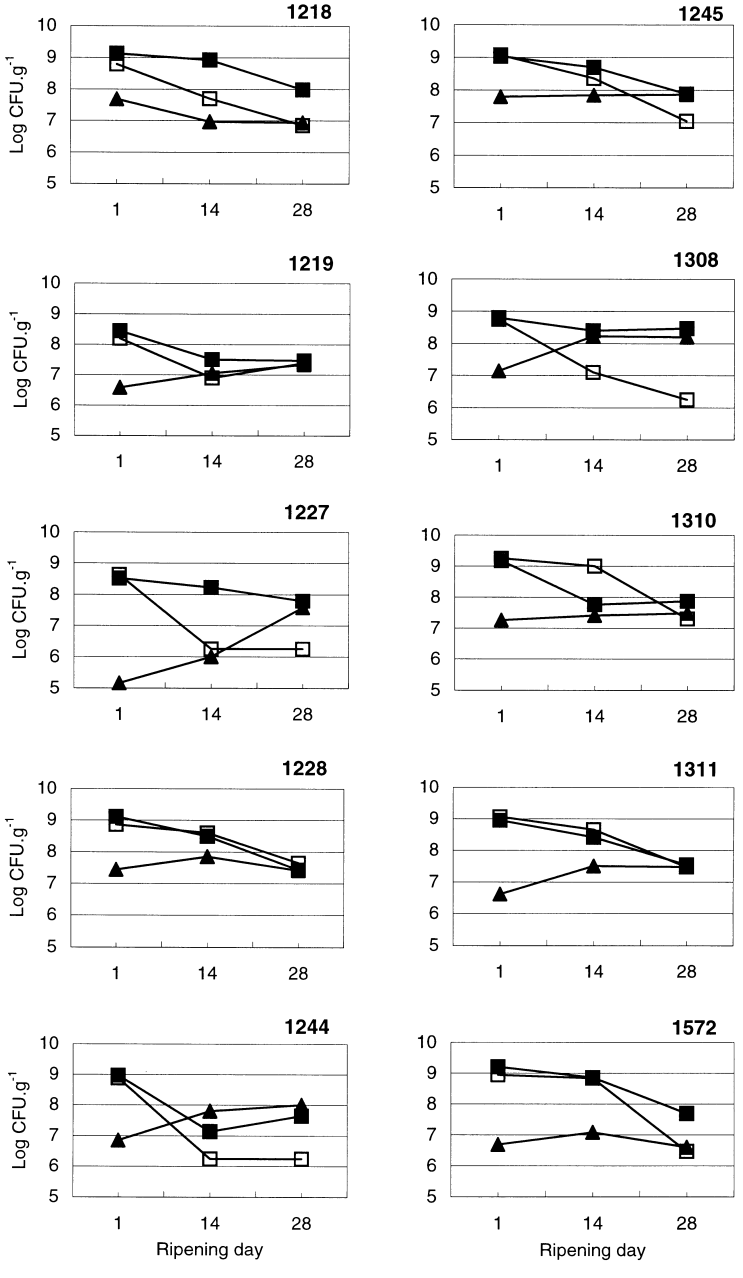

Figure 2. Growth curves of strain bacteria in miniature cheeses made with starter *L. lactis* AM2. □ Lactococci in control cheese; ■ Lactococci in experimental cheese; ▲ Lactobacilli in experimental cheese. Number in bold characters indicates *Lactobacillus* strain: 1218 *Lb. pentosus;* 1228, 1245, 1310, 1311 and 1572 *Lb. plantarum*; 1219, 1227, 1244 and 1308 *Lb. casei.*

Table II. Index of primary proteolysis of control and experimental miniature cheeses at 28 d of ripening; means did not differ significantly ($P < 0.05$).

Starter strain	IL416		AM2	
Lactobacillus adjunct	Without (control)	With (experimental)	Without (control)	With (experimental)
$\% \frac{\alpha s1 - I}{\alpha s1 - I + \alpha s1}$	37 ± 4	41 ± 1	43 ± 3	40 ± 5

ten-fold lower than those of lactococci at 14 and 28 d of ripening, and were therefore negligible. That was also true for cheeses prepared with starter AM2 and *Lactobacillus* strains 1245, 1227, 1228 and 1311 at 14 d of ripening (Fig. 2).

Lb. casei 1227 grew differently in miniature cheeses made with AM2 starter than in those made with IL416; in this context, CFU·mL^{-1} are at ~10^5 in one-d-old cheese, and reach ~10^8 CFU·g^{-1} at the end of the ripening period. *Lb. casei* 1308 was the only *Lactobacillus* adjunct strain that attained a more elevated population with AM2 than with IL416 starter; furthermore, AM2 strain survival was also improved in the cheese product.

3.3. Proteolysis

Chromatographic profiles of the insoluble cheese fractions at pH 4.4 were essentially the same for all cheeses (data not shown) and the relation $\% \frac{\alpha s1 - I}{\alpha s1 - I + \alpha s1}$ did not significantly differ ($P < 0.05$) (Tab. II).

We determined nitrogen content (expressed as % of total nitrogen) for control and experimental cheeses for both starters IL416 and AM2 at 28 d of ripening. The results are presented in Figure 3 for the soluble nitrogen content at pH 4.4 (SN) and for the phosphotungstic acid-soluble nitrogen content (PTA-N). For starter IL416, more than half the experimental cheeses showed higher SN levels than control cheeses (without lactobacilli), while SN values were very close to those of the control for other cheeses. SN levels of cheeses made with starter AM2 with and without lactobacilli were very close and did not show the same tendency. For both starter strains, *Lb. plantarum* 1310 was the adjunct resulting in the highest increment in SN levels. The PTA-N values for control cheeses made with AM2 starter varied more than those made with IL416. The production of PTA-N was enhanced for *Lb. plantarum* 1310 and 1572 with starter IL416. The effect of lactobacilli adjunct in cheeses manufactured with the AM2 strain was less evident. In some cases control cheeses showed a slightly higher level of PTA-N than experimental ones.

The chromatograms of the 70% ethanol soluble extracts revealed mainly quantitative differences between miniature control and experimental cheeses for the majority of the tested *Lactobacillus* strains. These results suggest that the starter strains play a more important role than the lactobacilli adjuncts in the production of peptides soluble in 70% ethanol (see chromatograms for IL416 and AM2 strains control miniature cheeses, Figs. 4a and 4b). Peptide profiles of control cheeses manufactured with the AM2 strain also showed some quantitative differences (Figs. 4b and 4c), probably because of the changes in AM2 starter viability. The differences agreed with the variability of PTA-N values for control cheeses manufactured with the AM2 strain. Thus, each experimental cheese had only to be compared with the control cheese manufactured the same day. Miniature cheese manufactured with both starters and *Lb. plantarum* 1572 adjunct showed qualitative differences compared with control cheese (without lactobacilli). Miniature cheese manufactured with AM2

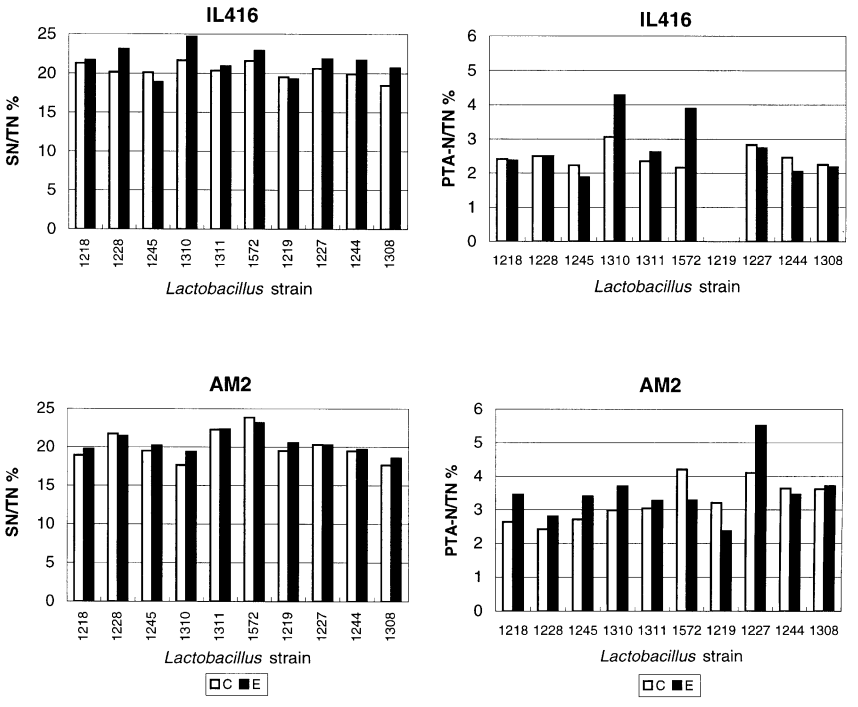

Figure 3. Soluble nitrogen at pH 4.4 (SN) and soluble nitrogen in phosphotungstic acid (PTA-N) expressed as % of the total nitrogen (TN), for control (C) and experimental (E) miniature cheeses at 28 d of ripening, made either with starter *L. lactis* IL416 or starter *L. lactis* AM2. Numbers under x-axe indicate *Lactobacillus* strain: 1218 *Lb. pentosus*; 1228, 1245, 1310, 1311 and 1572 *Lb. plantarum*; 1219, 1227, 1244 and 1308 *Lb. casei*.

starter and adjunct culture of the *Lb. plantarum* 1310 strain also differed from control cheese (Fig. 4c).

4. DISCUSSION

In the present study, we provide evidence that miniature cheeses as developed here provide a reproducible and simple model for washed-curd cheeses. We also show that the dynamics of starter and lactobacilli adjunct culture populations can vary according to the combination of strains used in cheese-making. The presence of the adjunct culture can also result in altered proteolytic end products, according to the starter and the adjunct strains.

We observed significant differences in starter strain viability according to the strain used; starter IL416 viable cell counts remained constant during ripening, while the AM2 strain population decreased by ~hundred-fold in control cheeses, as was expected due to autolysis by an activated prophage. In most experimental cheeses (containing lactobacilli), the AM2 starter population remained higher than in control cheeses (without lactobacilli). This effect was verified for six *Lactobacillus* strains and to our knowledge, has not been reported to date. The opposite effect for a starter of *L. lactis* subsp. *cremoris* and a *Lb. casei* adjunct was previously reported [2]. The results suggest that lactobacilli culture may have a positive effect on survival of AM2 starter.

Even though differences were found for *Lb. casei* 1227 and 1308, presumed lysis of

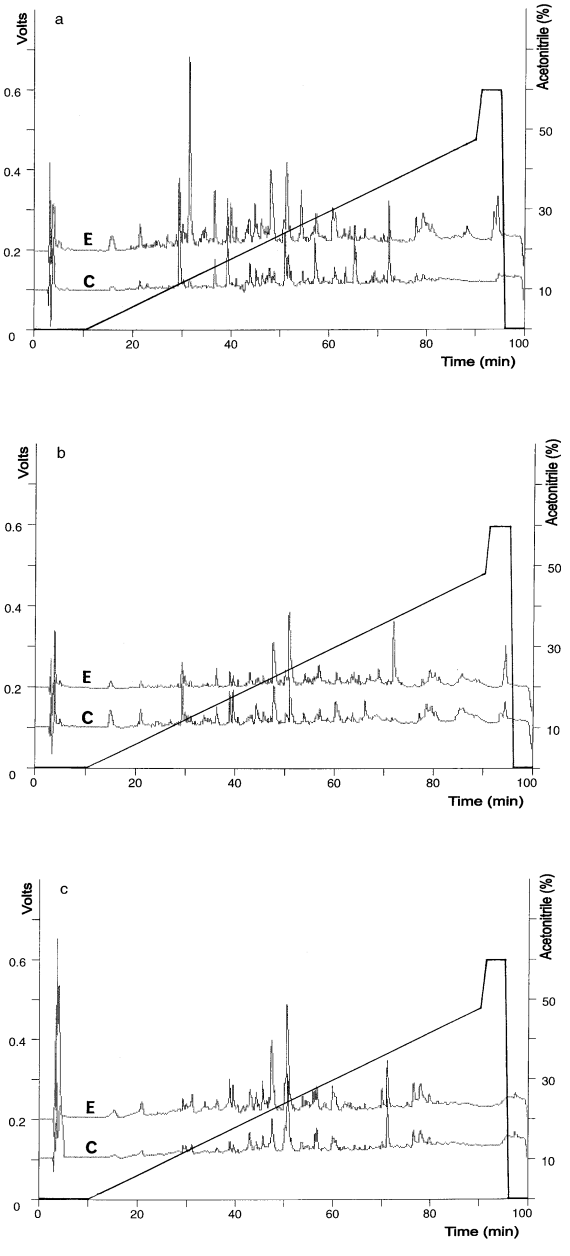

Figure 4. RP-HPLC chromatograms of 70% (v/v) ethanol soluble extract for miniature cheeses at 28 d of ripening: (**a**) *L. lactis* subsp. *lactis* IL416 starter, with (E, experimental) and without (C, control) adjunct culture of *L. plantarum* 1572; (**b**) *L. lactis* subsp. *cremoris* AM2 starter with and without adjunct culture of *L. plantarum* 1572; (**c**) *L. lactis* subsp. *cremoris* AM2 starter with and without adjunct culture of *L. plantarum* 1310.

the AM2 strain did not improve growth of the other *Lactobacillus* strains (Fig. 2). Thomas [40] demonstrated that lactobacilli are able to grow on lactococcal cell homogenate, so it is generally supposed that lytic starters improve lactobacilli development in cheese. The required concentration is about 1 mg of cells (dry matter)·mL^{-1}, which is equivalent to ~10^9 cells·g^{-1} of cheese, i.e. the complete starter population, conditions we get with a hundred-fold drop in viability. In those studies, carbon, and not nitrogen, appears to be the limiting factor in cheese for the growth of NSLAB. In our cheeses, lactobacilli were added to cheese-making milk where they can utilise lactose during the first stages of cheese-making, thus possibly abolishing the need for added nutrients provided by lysis of the starter. Lane et al. [20] reported similar results for four types of starter, among them the AM2 strain.

Primary proteolysis was similar for all control and experimental cheeses, showing that lactobacilli did not participate in breakdown of intact caseins in situ. Lane and Fox [19] found differences in primary proteolysis between cheeses prepared with and without a lactobacilli adjunct, and using glucono-delta-lactone to acidify the medium instead of LAB starter culture. In our study, as in others where milk was acidified by a LAB starter, such a difference was not detected [19, 23, 25, 37]. The similarity of $\% \frac{\alpha s1 - I}{\alpha s1 - I + \alpha s1}$ values for experimental and control cheeses also confirmed the reproducibility of miniature cheeses, as it depends on chymosin activity and therefore on water activity, pH and cheese composition [8]. We conclude that the tested *Lactobacillus* strains do not possess proteases able to hydrolyse entire caseins in the present experimental conditions.

Secondary proteolysis products were different for control and experimental cheeses. Several authors have reported that lactobacilli adjuncts increased the total amount of free amino acids (FAA) in Cheddar cheese [17, 24, 25, 34]. In our model, the level of PTA-N, which is an index of total FAA [1], was greatest for experimental cheeses manufactured with starter IL416 and adjunct cultures of *Lb. plantarum* 1310 and 1572. Differences in secondary proteolysis for these cheeses were also mirrored in peptide profiles obtained by ethanol soluble extract RP-HPLC. The level of PTA-N was higher in several control cheeses manufactured with the AM2 starter than in the corresponding experimental cheeses (containing lactobacilli). That may be due to greater viability of AM2 starter in experimental cheeses and a consequent reduction in amounts of released starter peptidases. On the contrary, lactobacilli addition may improve PTA-N production. In this case, both contrary effects may be involved, so that some experimental cheeses – notably those with *Lb. casei* 1227 – had greater PTA-N levels than control.

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