Impact of broken cells of lactococci or propionibacteria on the ripening of Saint-Paulin UF-cheeses: extent of proteolysis and GC-MS profiles

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Impact of broken cells of lactococci or propionibacteria on the ripening of Saint-Paulin UF-cheeses: extent of proteolysis and GC-MS profiles

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Abstract — UF-cheeses have been successfully developed in many countries. However, proteolysis and the general extent of ripening were shown to be far slower compared to traditional varieties. The absence of starter lysis has been cited as a plausible explanation. In this work, propionibacteria and lactococci cells were disrupted using a new pilot homogenizer. Crude broken suspension (CBS), or cell-free extract (CFE), obtained after centrifugation, were added to UF-St Paulin retentate (concentration factor 6) made with a usual lactic starter. RO water was added to the control. Proteolysis was estimated by NCN, NPN and free amino acids. Neutral volatile compounds were determined by GC-MS. A low extent of ripening was noted in the control and the absence of starter lysis was effectively proved using immunodetection of lactococci cytoplasmic proteins. The addition of lactococci CBS or CFE increased free amino acid content (1.5 to 3 times) whereas propionibacteria CBS or CFE exhibited no significant increase, even when cheeses were ripened at 20 °C instead of 12 °C. By contrast, addition of propionibacteria CBS generated a significant increase in several volatile compounds like alcohols and ketones, whereas CFE did not, showing that the presence of live cells was required to form these compounds. CBS or CFE of lactococci did not significantly change the volatile compound profile. In conclusion, it was possible to influence the ripening of UF-cheese by the addition of crude broken bacterial cell suspensions. Other strains and species should now be investigated.

ultrafiltration / UF-cheese / GC-MS profile / broken cells / ripening / proteolysis / *Lactococcus* / propionibacteria

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Résumé — Impact d'extraits cellulaires de lactocoques et de propionibactéries sur l'affinage de fromages UF : étendue de la protéolyse et profils CG-MS. Les fromages UF se sont développés avec succès dans de nombreux pays. Cependant, sans action technologique complémentaire, la protéolyse et par conséquent l'affinage sont nettement plus lents que dans les produits obtenus par les procédés traditionnels. L'absence de lyse du levain pourrait constituer une des causes possibles. Dans ce travail, des cellules de *P. freudenreichii* et de *Lactococcus* sp. ont été « cassées » à l'aide d'un nouvel homogénéisateur pilote. Les suspensions cassées brutes (CBS) ou les extraits intracellulaires (CFE) obtenus après centrifugation, ont été ajoutés à des rétentats UF de type St Paulin (concentration ×6) acidifiés à l'aide d'un levain lactique mésophile commercial. De l'eau osmosée était ajoutée au témoin. La protéolyse a été estimée par la mesure des teneurs en NCN, NPN et en acides aminés libres ; les composés volatils neutres par CG-MS. Dans le témoin, cette protéolyse était très limitée et l'absence de lyse du levain commercial (*L. lactis*) a été effectivement démontrée. L'addition de CBS ou de CFE de *Lactococcus* augmentait la teneur en acides aminés libres (1,5 à 3 fois). Cet accroissement n'était pas observé lors de l'addition de CBS ou de CFE de *P. freudenreichii,* même lorsque la température d'affinage était élevée à 20 °C au lieu de 12 °C. Par contre, l'addition de CBS de *P. freudenreichii* entraînait une augmentation des teneurs en produits volatils neutres : alcools et cétones. L'absence d'effet similaire avec le CFE montrait la nécessité de la présence de cellules entières. L'addition de CBS ou CFE de *Lactococcus* ne changeait pas significativement les teneurs en composés volatils neutres. En conclusion, l'addition d'homogénéisats de suspensions cellulaires peut modifier l'affinage des fromages UF. L'addition d'autres souches et d'autres espèces doit maintenant être explorée.

ultrafiltration / fromage UF / CG-MS profil / homogénéisats cellulaires / protéolyse / affinage / *Lactococcus* / propionibacteria

1. INTRODUCTION

Introduced 32 years ago by Maubois, Mocquot and Vassal [28], the MMV process has achieved successful commercial development for many cheese varieties adapted to the habits and the culture of each region of the world : examples of this are fresh, soft and semi-hard cheeses such as Pavé d'Affinois, Saint-Paulin in France [11, 21], Feta in Denmark and in Iran [50], Minas Frescal in Brazil [49, 47], Quark in Germany [33]; Twarog in Poland [32]; Teleme in Greece [48]; Ricotta and Cream cheese in the USA [4, 29] as well as hard cheeses such as Cheddar or Swiss cheese through the LCR concept [13, 30]. Economic advantages explain the success of the MMV process. Indeed yields are significantly increased (up to 20%) because the whey proteins are incorporated into the drained curd, there are reduced rennet requirements, reduced fat losses in whey and a simplification of the cheesemaking process [11, 23, 34]. Moreover, the differential concentration of milk proteins and fat before coagulation leads to a smoother and more homogeneous texture of UF-cheeses compared to homologous traditional cheeses [23, 24]. Such an interesting property has led to new cheese varieties.

However, for matured UF-cheese varieties, made from five-fold or fully concentrated milk, proteolysis and the general extent of ripening were shown to be far slower compared to traditional cheeses [39, 20]. This disadvantage was often attributed to the presence of whey proteins in the UF-cheeses [20, 23, 2]. However, this hypothesis is still controversial [30]. Indeed, the main reason for this retarded maturation is likely to be due to the high buffering capacity of UF-cheeses which retards the rate of lactic starter autolysis [11] and consequently the hydrolysis of the casein network. The negative role of the high level of calcium salts in UF-cheeses was emphasized as early as 1974 by Brulé et al. [3], suggesting several suitable ways to adjust

the mineral content of UF-retentate : reduction of milk pH before or during ultrafiltration, increase of ionic strength by addition of NaCl, or both methods [30]. Whey proteins in MMV cheeses are mainly an inert filler, as suggested by Koning et al. [20], because they are resistant to hydrolysis by chymosin, lactic starter bacterial proteases and plasmin [14, 20, 35]. The high content of β-lactoglobulin in UF-cheeses could also inhibit protease activity [2, 18].

To accelerate the ripening rate in UF-cheeses, the same methods used to improve the ripening of traditional cheeses can be attempted : (i) elevation of storage temperature; (ii) addition of attenuated starters [19]; (iii) addition of cell-free extracts of starter bacteria [8]; (iv) increase of microbial population or non-starter bacteria; (v) addition of proteases and lipases or (vi) a combination of methods [9, 6, 7]. Some of these methods have been assessed. Hickey et al. [15] added a proteolytic strain, *Lactobacillus helveticus* YB1, with the normal starter. They reported that the level of free amino acids in UF-cheese was increased to that observed with strain *L. lactis* subsp. *cremoris* E8 alone in traditional Cheddar cheese. Goudédranche et al. [12] improved the texture and the flavor of UF-Saint-Paulin and Camembert cheeses by adding 0.5 or 1.0 $g \cdot kg^{-1}$ of lysozyme to liquid pre-cheese. They observed a slight increase in proteolysis during ripening and an improvement of the organoleptic qualities of the UF-cheese. Bastian et al. [1], by the addition of urokinase to UF-Havarti and Saint-Paulin cheeses in order to activate plasmin, observed an increase of NPN and NCN levels and a greater degradation of β-casein, which they correlated with a slightly improved flavor. Spangler et al. [39] accelerated the ripening of UF-Gouda cheese by adding a mixture of liposome- entrapped enzyme and freeze-shocked *Lactobacillus helveticus*. They observed a significant increase in TCA- and PTA-soluble nitrogen and more intense flavor without bitter flavor development.

To our knowledge, the addition of extra amounts of starter proteinases and peptidases, as suggested by Bech [2] in order to increase the hydrolysis of casein, has not yet been described. UF-retentates are interesting materials because all the added pool of enzymes is retained in the curd and in the subsequent cheese, which is a strong advantage compared to traditional cheesemaking where 90% of the added enzymes are lost in the whey.

The objective of this study was to investigate the impact on the ripening of UF-Saint-Paulin cheeses of adding crude broken suspensions (CBS, a mixture of whole cells, cell walls and cell-free extract) or cell-free extract alone (CFE) of *Lactococcus* sp. or *Propionibacterium freudenreichii*. Cells were broken by using a new pilot high pressure homogenizer, as recently described [37].

2. MATERIALS AND METHODS

2.1. Origin of the strains and growth conditions

A freeze-dried commercial mixture of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *diacetylactis* (MM 101 – EZAL) was obtained from Rhodia-Texel (Dangé St-Romain, France) and kept at 4 °C. *Propionibacterium freudenreichii* TL 24 (CNRZ 725) was obtained from the culture collection of the Laboratoire de Recherches de Technologie Laitière (INRA Rennes, France). It was stored at –18 °C on YEL [27] containing glycerol (15% v/v) and cultivated on the same medium at 30 °C.

2.2. Preparation of cell suspensions and cell disruption by a pilot homogenizer

Freeze-dried *Lactococcus* sp. MM 101/10u (10 g) was suspended in 100 mL of RO sterile water (3.5×10^{10} $cfu \cdot mL^{-1}$).

Propionibacterium freudenreichii TL 24 was first cultivated in YEL broth for 24 h at 30 °C (OD_{650}~1), cells were harvested by centrifugation (9.000 *g*, 10 min, 4 °C) and suspended in 240 mL RO sterile water to achieve a cell concentration of 2.5×10^{10} cfu·mL^{-1}. Suspensions (75 mL) of both *P. freudenreichii* TL 24 and lactococci cells were subjected to the disrupting effect of a pilot homogenizer Stansted/fluid power LTD (Sodexim, Muizon, France) at a pressure of 200 mPa, as described elsewhere [37]. Thirty millilitres of the treated suspension containing whole cells, broken cells and a pool of enzymes were collected and named "crude broken suspension" (CBS). The rest of the suspension was centrifuged (10.000 *g*, 15 min, 4 °C) to remove whole cells and large cell wall fragments. Thirty millilitres of the supernatant were collected and named "cell free extract" (CFE). The efficiency of cell disruption was verified by cell viability in specific medium culture and by cell dry weight, before and after homogenization, as described by Saboya et al. [37].

2.3. Ultrafiltration and manufacture of UF-cheese

Two sets of experiments were conducted with different UF devices as described below. In both cases, the milk was first standardized at 2.8% fat and pasteurized at 90 °C / 4 s (Actijoule pilot, Société Actini, Evian, France). The temperature during the UF process was 48–50 °C. Dilution of the retentate (addition of RO water 1.12 vol/vol) was done when the six-fold retentate achieved a concentration. The UF was continued until a retentate with a T.S. content of 40 g per 100 g was obtained. The retentate was then cooled to 30 °C and inoculated with a *Lactococcus* starter, MM101. Freeze-dried powder was diluted to achieve 10^7 cfu·mL^{-1} of retentate. NaCl was then added to 0.75% (w/w). The fermentation was conducted for 18 h at 30 °C (pH reached : 5.1–5.2), and a further 0.65% of NaCl was added. The retentate was divided into several parts and treated with CBS and CFE as described below.

For the experiments involving homogenized MM101-*Lactococcus* cells as additive (LAC trial), the milk was ultrafiltrated using CARBOSEP M1 pilot UF equipment (150 kg·mol^{-1} cut-off membrane and a surface of 6.8 m^2 – Société Rhodia-Orelis, Miribel, France) for 80 min. The retentate was divided into four lots of 6 kg and treated as follows :

a) UF-control cheese = retentate + 30 mL RO water;

b) UF / CBS Lact 5 mL = retentate + 5 mL lactococci CBS + 25 mL RO water;

c) UF / CBS Lact 30 mL = retentate + 30 mL lactococci CBS;

d) UF / CFE Lact 30 mL = retentate + 30 mL lactococci CFE.

For the experiment involving broken cells of *P. freudenreichii* as additive, a MEMBRALOX membrane UF pilot equipment was used (0.05 μm pore size membrane and a surface of 1.8 m^2 – Société T.I.A., Bollene, France). The UF process was conducted for 120 min. The retentate was divided into three 6 kg lots and treated as follows:

a) UF-control cheese = retentate + 30 mL RO sterile water;

b) UF / CBS Prop 30 mL = retentate + 30 mL *P. freudenreichii* CBS;

c) UF / CFE Prop 30 mL = retentate + 30 mL *P. freudenreichii* CFE.

Rennet extract (1.8 mL) diluted five-fold with RO water (SKW Biosystems) was added to each retentate which was then set in a mould of 26 × 36 × 10.5 cm. Two hours later, when the pre-cheeses became firm, they were cut into 12 blocks of 10 × 9 × 8.5 cm and incubated for 4 h at 30 °C; then, the cheeses were taken from the moulds and held at 12 °C for 5 weeks. After three days, the cheeses were packed with a

plastic film. Some of the cheeses treated with *P. freudenreichii* TL 24 CBS were ripened at 20 °C after 10 d at 12 °C.

2.4. Chemical composition of UF-cheeses

Samples of cheese were taken after 1, 7, 13, 20 and 27 d. At day one, pH, Total Solids (TS), fat, salt, lactose and total nitrogen (TN) were determined. The pH was measured with a standard CG837 pH meter with InLAB 427 electrodes (Mettler Toledo, Viroflay, France) by direct insertion into the cheeses. TS were estimated by drying at 102 (± 2) °C for 7 h, according to IDF [16]. Fat was determined using the acid butyrometric methods of Van Gulik [17]. Salt was determined using a "Chloruremetre Corning 926" (Humeau Laboratoires, La Chapelle-sur-Erdre, France). The residual lactose in the cheeses was measured by the phenol-sulfuric acid colorimetric assay of Dubois et al. [5]. Total nitrogen (TN) in cheese was determined by the Kjeldahl method and a convertion factor of 6.38.

Protein breakdown in the cheeses was measured over 5 weeks by the evolution of soluble nitrogen at pH 4.6 (non-casein nitrogen or NCN) or 12% TCA-soluble N (non-protein nitrogen or NPN), according to Rowland [36]. The results were expressed as a percentage of total nitrogen content in the cheese.

2.5. Bacteriological analysis

Cheese samples (10 g) were taken at different times of ripening and diluted in 90 g of trisodium citrate (20 g·L^{-1}) using a Waring Blendor (Prolabo, 94126 Fontenay-sous-Bois, France) at low speed for 20 s and then at high speed for 40 s. Decimal dilutions were prepared with the same solution. Viability of *Lactococcus* was determined by the spread plate technique on M17 agar plates [40] after incubation at 30 °C for 48 h. Viability of *Propionibacterium* was determined using a selective medium LGA [26]; the LGA plates were incubated anaerobically at 30 °C for 5–6 d. The results were expressed as logarithm (log) of colony forming units (cfu) per g cheese.

2.6. Aqueous cheese extracts

Soft aqueous cheese extracts were obtained as described by Valence et al. [46]. The cheese samples (4.5 g) were diluted in 18 mL RO water and homogenized manually in plastic bags with filters (Humeau, La Chapelle-sur-Erdre, France). The solution was harvested and centrifuged at 10.000 *g* and 4 °C for 15 min. The supernatant, i.e., the aqueous extract, was filtered through a 0.45 µm membrane filter (Sartorius, 91127 Palaiseau, France) and stored at –18 °C until used.

2.7. SDS-PAGE analysis

Proteins in the aqueous cheese extracts, as well as CBS and CFE, were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE), as described by Valence and Lortal [44]. Samples were mixed (v/v) in Laemmli buffer and loaded on the gel (14% separating gel). Migration was carried out at 25 °C at a constant voltage of 180 V for 1.5 h.

2.8. Starter autolysis

Autolysis of *Lactococcus* starters was monitored during ripening using antibodies against cell-free extracts of *L. cremoris* (*LC-cytosol*). For this purpose, cells of *L. cremoris* CNRZ 205 (type strain) were collected during the exponential growth phase (OD_{650} of 0.8) by centrifugation (7.000 *g*, 20 min, 15 °C) and washed twice in cold RO water (15% of the initial culture). Then the pellets were stored for 48 h at –20 °C before being resuspended in cold sterile water (6% of the initial volume of broth) and submitted to 138 mPa for 3 min in a precooled French press apparatus (two

runs). Suspensions were centrifuged (48.000 *g*, 30 min, 4 °C) to eliminate unbroken cells and cell wall particles. The supernatant was ultracentrifuged at 150.000 *g* for 3 h at 4 °C to eliminate ribosomes. The supernatant obtained was called LC-cytosol. Polyclonal antibodies were produced by two rabbits against entire LC-cytosol obtained as described above (100 µg of protein per injection, 6 weeks of immunization) (Eurogentec, Seraing, Belgique).

Aqueous extracts of cheese were subjected to SDS-PAGE, followed by Western blotting transfer (1 h 15, constant current 250 mA) onto a nitrocellulose sheet (0.45 µm pore size, Millipore, 78051 St-Quentin-en-Yvelines, France) [43]. Cytosolic proteins of *L. cremoris* were detected using rabbit serum anti-LC-cytosol (stored at –30 °C and diluted at 1:2500 in PBS-tween just before use). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L)-HRP conjugate (Biorad) was used as the secondary antiserum with DAB (3,3'diaminobenzidine tetrahydrochlorine) (Amresco, Solon, USA) as visualizer [45].

2.9. Free amino acids

The free amino acid content was determined after deproteinization of the sample by sulfosalicylic acid (Merk-Eurolab, Grosseron S.A., Saint Herblain, France) according to Mondino et al. [31]. Aqueous cheese extracts (1 mL) were treated with 50 mg of sulfosalicylic acid, shaken for 15 s and incubated for 1 h at 4 °C. The mixtures were centrifuged at 5.000 *g* for 15 min at 4 °C. Supernatants were filtered through a 0.45 µm membrane (Sartorius, 91127 Palaiseau, France) and the filtrate was diluted six-fold with a 0.2 mol·L^{-1} lithium citrate buffer, pH 2.2 before injection. Amino acid analysis by ion exchange chromatography was carried out using a Pharmacia LKB Alpha Plus Aminoacid Analyser (Amersham Pharmacia Biotech Europe GMBH, Orsay, France), as described by Spackman et al. [38].

2.10. Neutral volatile compounds (GC-MS profiles)

Cheese samples were thawed just before analysis and diluted (1:5) with sodium citrate (0.5 mol·L^{-1}, pH 8.8). Neutral volatile compounds were analyzed by head-space-GC-MS, as described by Thierry et al. [41]. Seven grams of cheese were weighted in a 35 mL-non-fritted sparger. Briefly, compounds were trapped on a Vocarb 3000 trap (Supelco, Bella Fonte, PA, USA) thermally desorbed at 250 °C, cryofocused at –100 °C before being injected and separated on a HP5 capillary column (Agilent Technologies 60 m × 0.32 mm 1 µm film thickness). They were detected by a HP7972A quadruple mass spectrometer (Agilent Technologies, Les Ulis, France) after ionization by electronic impact and identified either by comparison of spectra with those of Nist 75 K Mass Spectral Database and/or spectra and retention times with those of reference compounds.

3. RESULTS

3.1. Cell breaking by high pressure homogenization

A pressure of 200 mPa has been shown to be highly efficient for breaking Gram-positive cells, including lactococci and propionibacteria used in this study, without completely denaturing intracellular cheese-related enzymes [37]. In this work, the survival rate was similar to those obtained previously, 4.6% for lactococci and 28.8% for propionibacteria. Thus, in the lactococcal CBS, 1.6×10^9 cells·mL^{-1} were still viable (4.6% of the initial value), mixed with the intracellular content from 3.3×10^{10} broken cells·mL^{-1}. In the case of *P. freudenreichii* CNRZ 725, the CBS contained

7.2×10^9 live cells·mL^{-1} as well as the cellular content from 1.75×10^{10} broken cells·mL^{-1}.

CBS and CFE exhibited the same protein profiles when compared by SDS-PAGE just after homogenization, indicating that soluble proteins were not significantly lost during the centrifugation step (data not shown). Thus, CBS and CFE differed mainly by the presence of viable cells in CBS.

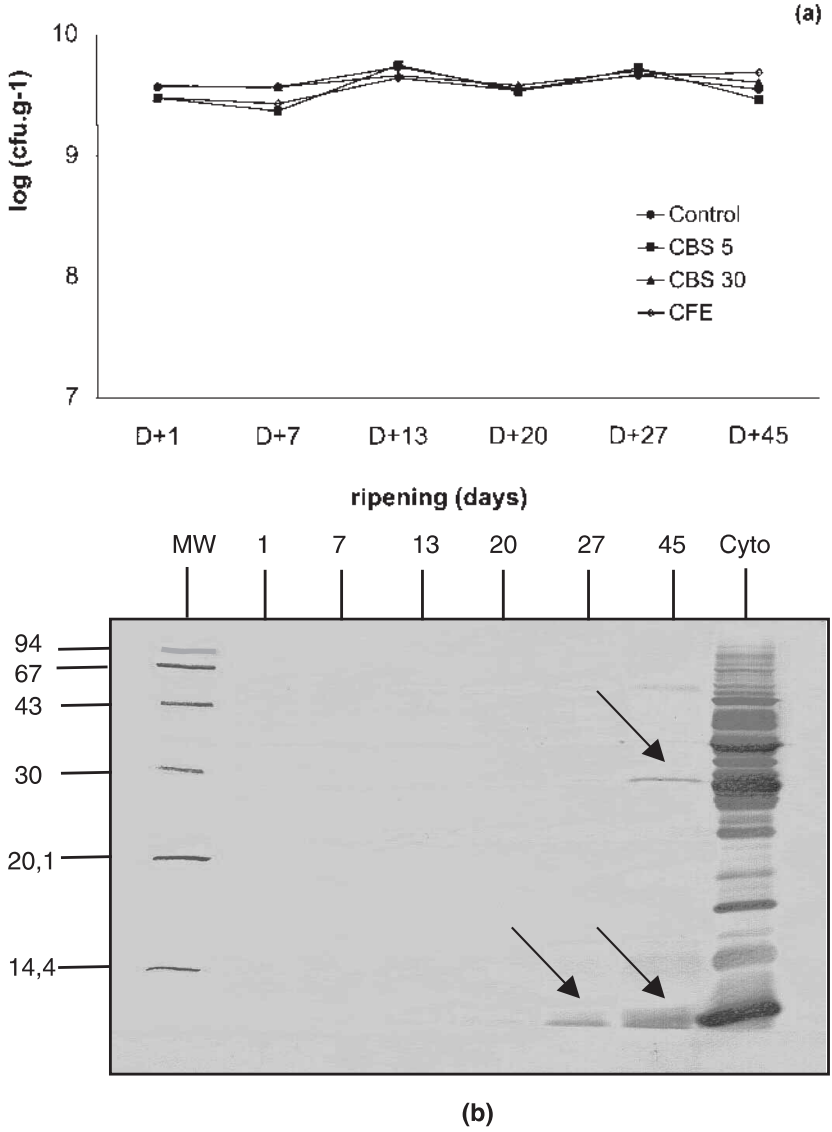

Figure 1. (**a**) Enumeration of *Lactococcus* sp. in UF-cheeses at 12 °C during ripening period (● Control; ■ CBS 5; ▲ CBS 30; ○ CFE). (**b**) Immunodetection of *Lactococcus* cytoplasmic proteins in the control; the age in days of the cheeses was indicated on each lane (MW = Molecular weight, kDa; Cyto = cytoplasmic proteins of *Lactococcus lactis*).

3.2. Chemical composition of one-day-old UF-cheeses

The composition of the control and experimental cheeses at day one were identical, i.e.: pH 5.23 (± 0.02), 40.4% (± 0.1) total solids, 16.4% (± 0.1) fat, 1.5% (± 0.04) salt and protein 18.9% (± 0.5). The residual lactose was less than 1 $g \cdot kg^{-1}$ at day one, due to the diafiltration treatment. The chemical composition was thus in agreement with previous data for UF-St-Paulin cheese [11].

3.3. Bacterial populations during cheese ripening

Populations of *Lactococcus* starter were identical at day one in all cheeses (control and experimental), i.e. around 3–4.0 × 10^9 $cfu \cdot g^{-1}$ (Figs. 1 and 2). No decrease in viability was noted over 6 weeks of ripening. The absence of lysis of *Lactococcus* starter was confirmed by immunoblotting of the aqueous extracts from one to 45 days-old using antibodies anti-LC cytosol. No intracellular proteins were detected either in the control or in the experimental cheeses until day 27; they then appeared weakly in the sample from 45-day-old cheese (Fig. 1b).

In the trials with propionibacteria, the initial number of *P. freudenreichii* was around 10^7 $cfu \cdot g^{-1}$ of cheese in the CBS-treated cheese and only 10^2 $cfu \cdot g^{-1}$ in CFE-treated cheese (Fig. 2). No change was noted when the cheeses were ripened at 12 °C but a slight growth of propionibacteria occurred when cheeses were incubated at 20 °C, + 1.2 log unit in the CBS-treated cheese. In the blank without additive, no propionibacteria were detected at day one, and less than 100 $cfu \cdot g^{-1}$ at the end of ripening.

In the trials with broken lactococci, it was not possible to selectively enumerate the additional viable lactococci (in the CBS supplemented cheese) because the additive was identical to the starter used for cheesemaking. However, a theoretical calculation can be done as all cells and enzymes added are retained in the UF-retentates : 30 mL of CBS (containing 1.6×10^9 $cfu \cdot mL^{-1}$ lactococci) was added to 6 kg of retentate, and thus theoretically 6×10^6 cells of live *Lactococcus* cells were added / g of retentate in the CBS assay, in parallel with the intracellular content of 1.6×10^8 $cells \cdot g^{-1}$. In the CFE supplemented cheese, the intracellular content corresponding to 1.6×10^8 $cells \cdot g^{-1}$ was added, but without a significant number of live cells.

3.4. Impact on the ripening of the addition of broken propionibacteria

When ripened at 12 °C, changes in the pH were the same in the experimental and control cheeses, increasing until day 20 from 5.2 to 5.35. When ripening was performed at 20 °C, the change was the same until day 20 but then pH continued to increase in the cheese treated with CBS (pH 5.45).

In terms of proteolysis, the effect was limited and mainly dependant on the temperature of ripening. When the cheeses were ripened at 20 °C, NCN/NT % was 12% higher than in the cheeses at 12 °C, but there was no further increase due to the addition of CBS or CFE (data not shown). A slight increase of NPN / NT % compared to the control was noted at day 27 when CBS or CFE was added, but only at 20 °C (Fig. 3). The sum of the free amino acids was 32.5 $\mu mol \cdot g^{-1}$ of cheese in the control, and about 32.7 $\mu mol \cdot g^{-1}$ and 34 $\mu mol \cdot g^{-1}$ for CBS and CFE treated cheeses, respectively. The profiles at day 27 were very similar to the control except for slight qualitative differences: in the presence of CBS, Asn, Asp and His content decreased significantly, whereas Glu, Pro, Ala and

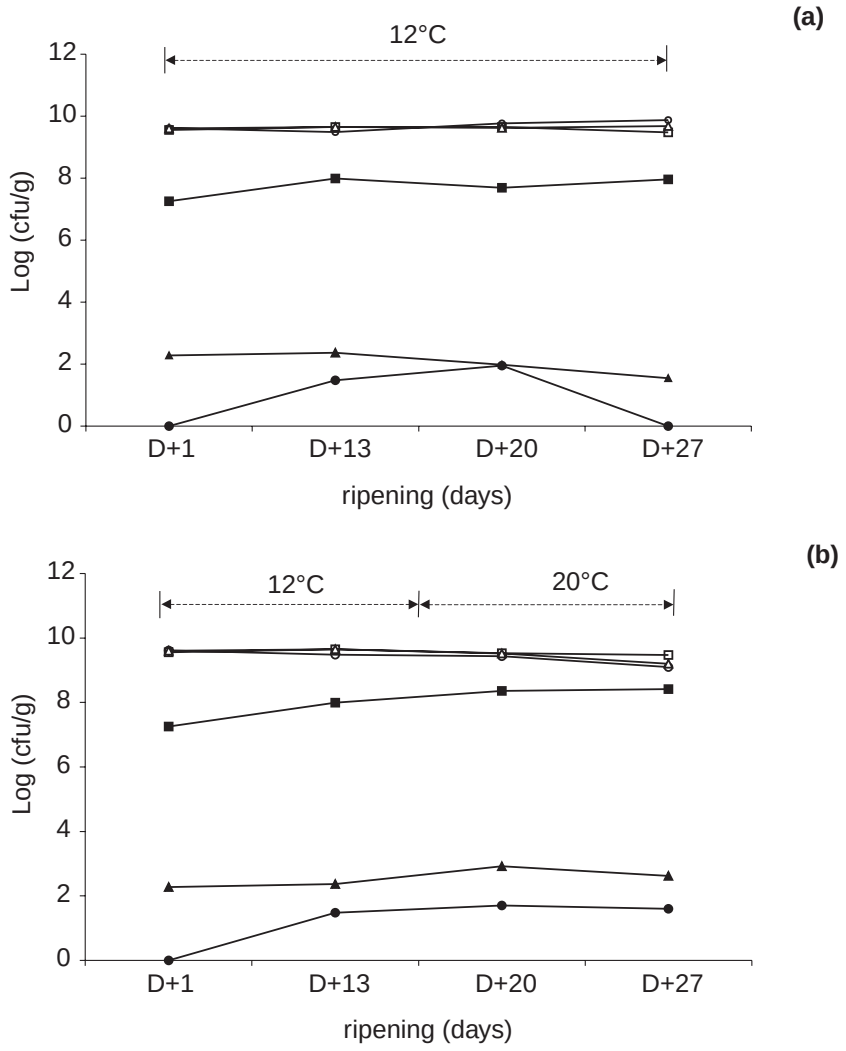

Figure 2. (**a**) Enumeration of *Lactococcus* sp. (○ Control; □ CBS; △ CFE) and *P. freudenreichii* (● Control; ■ CBS; ▲ CFE) in UF-cheeses treated with *P. freudenreichii* at 12 °C and (**b**) at 20 °C during ripening period.

Leu were present in larger amounts (+ 15 to + 40%) (Fig. 4a).

Regarding neutral volatile aroma compounds, GC-MS analysis of control cheeses revealed relatively few compounds (Tab. I), mainly alcohols, some ketones and some aldehydes. In the presence of CBS, the amount of some of them increased (two- or eleven-fold), like 1-propanol, 1-butanol, 1-pentanol, 3-methyl butanol, and 2-methyl butanol. Moreover, other compounds appeared, mainly esters and sulfur compounds like dimethyl disulfide (Tab. I). On the other hand, no significant effect of the CFE was observed in terms of neutral volatile aroma compounds, showing that live cells are required.

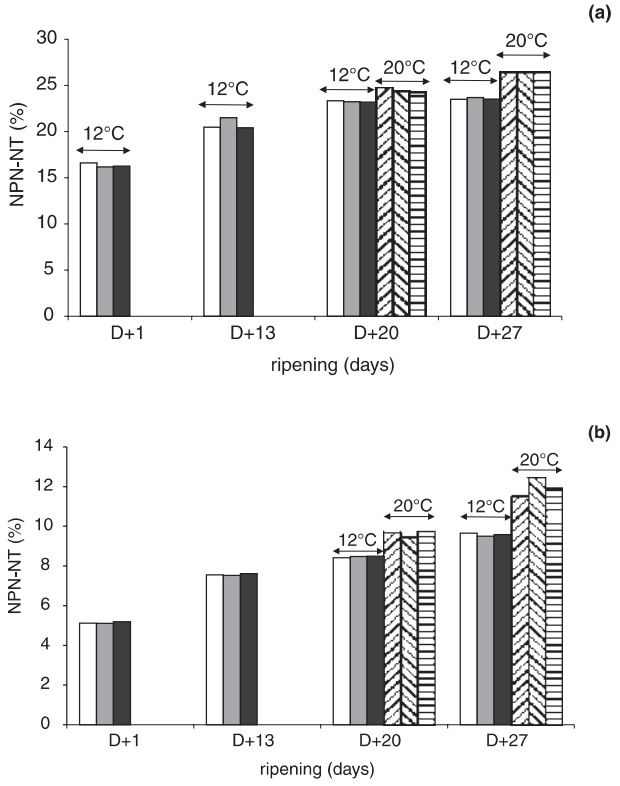

Figure 3. (**a**) NCN-NT (%). (**b**) NPN-NT (%) indexes in UF-cheese treated with *P. freudenreichii* and ripened at 12 ºC (□ Control; ■ CBS; ■ CFE) or 20 ºC (▨ Control; ▧ CBS; ▤ CFE).

3.5. Impact of the addition of broken lactococci on ripening

The same continuous increase of pH was observed until day 27 (5.2 to 5.5) for the blank and the cheeses treated with CBS or CFE. No significant effect was observed on primary proteolysis since no significant change in NCN/NT or NPN/NT indexes was noted. Also, SDS-PAGE analysis showed no differences in the profiles (data not shown). By contrast, an obvious impact on the free amino acids was observed (Fig. 4b). The sum of the free amino acids was 14.5 μmol·g^{-1} for the control at day 27, and, respectively, 20.5, 30.6 and 32.3 μmol·g^{-1} of cheese with CBS (5 mL), CBS (30 mL) and CFE. Qualitatively (Fig. 4b) the concentration of most amino acids increased 1.5 to 3 times when CFE was added. The result was similar with CBS (30 mL) but slight differences can be observ-ed in the profiles; the addition, in parallel with cytoplasmic content, of live *Lactococci*, had an influence on the release or catabolism of some amino acids. This result was surprising since the quantity added was relatively low (6 ×10^6 cells per g of cheese) compared to the high level of the starter itself (10^9·g^{-1}).

Neutral volatile compounds were estimated at day 27 and also at day 45. As in the aforementioned experiments, the control contained the same few aroma compounds. No increase and no new compounds were

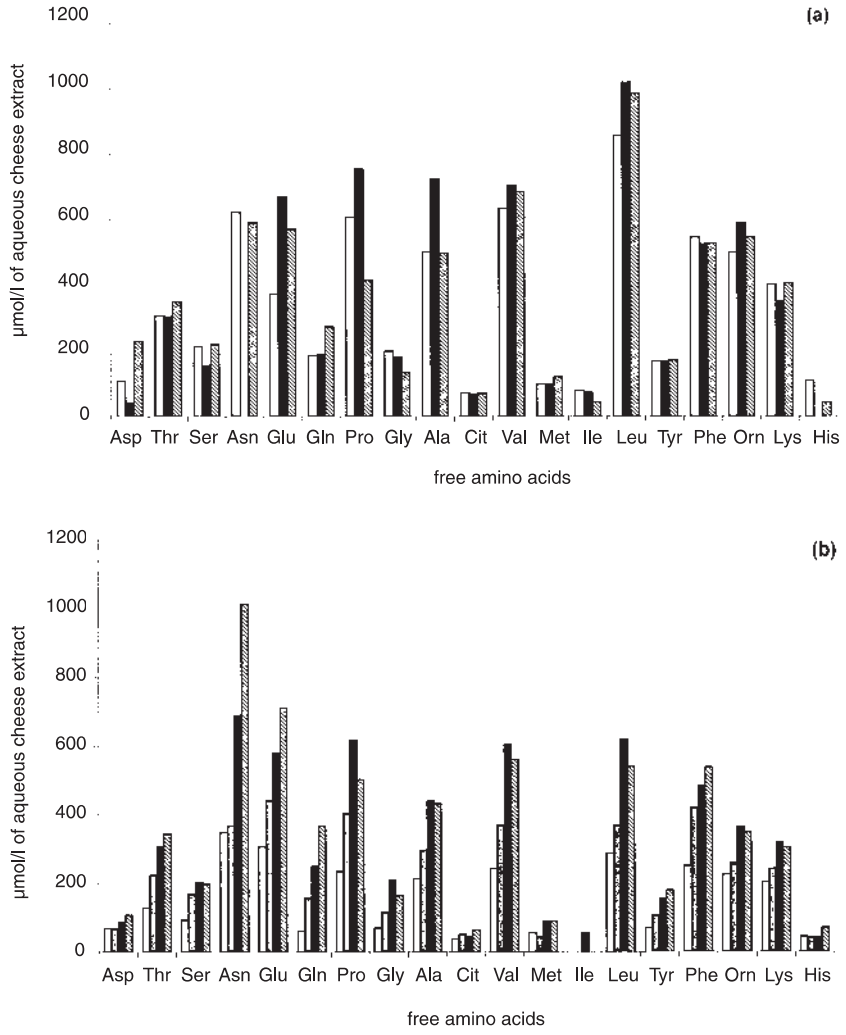

Figure 4. Free amino acid composition in the 27 day-old UF-cheese treated with *P. freudenreichii* (**a**) and ripened at 20 ºC (□ Control; ■ CBS; ▤ CFE) and in the UF-cheese treated with *Lactococcus* (**b**) at day 27 at 12 ºC (□ Control; ■ CBS 5; ■ CBS 30; ▤ CFE).

noted when CFE or CBS were added (data not shown).

4. DISCUSSION

Both enumeration of viable cells of added starters and immunoblotting data related to intracellular enzyme markers confirm that in cheeses made from milk concentrated by UF at milk pH, no lysis of *Lactococcus* starter was detected, as previously hypothesized by Goudédranche et al. [12] and Mistry and Maubois [30]. Such a change in a phenomenon essential for the normal ripening of cheese can be related to the environmental conditions of *Lactococcus* in this type of UF-cheeses.

The internal aqueous phase of UF-cheeses has a very high content in calcium salts [3] which limits the decrease in pH induced by lactic acid production by *Lactococci* and this could also inhibit lysis, as shown for *Lactobacilli* [25]. Relations between cellular lysis and the buffering capacity of the aqueous phase of cheese is a research area which should be studied more deeply.

Table I. Values of areas of neutral volatile compounds identified by GC MS in the UF-cheese treated with *P. freudenreichii* (CBS and CFE) at day 27 at 20 °C.

Volatile Compounds	Identification Method	Control	CBS	CFE
		$\times 10^6$	$\times 10^6$	$\times 10^6$
Alcohols				
1-propanol	MS	9	174	8
1-butanol	MS	97	242	62
3-methyl butanol	MS/R	1.4	3.4	ND
2-methyl butanol	MS/R	2.1	31.9	0.4
1-pentanol	MS	2.5	5.5	1.5
Aldehydes				
3-methyl butanal	MS/R	10.4	1.7	3.3
Ketones				
2,3-butanedione	MS/R	90	175	103
2,3-pentanedione	MS	5	4	1.2
2-pentanone	MS	7.2	9.9	6.7
2-hexanone	MS	0.5	2.3	0.5
2-heptanone	MS/R	11.2	17	10
2-nonanone	MS/R	1.7	2.2	1.2
Esters				
Ethyl acetate	MS	3.8	4.5	1.7
Ethyl propanoate	MS	ND	1.2	ND
Ethyl butanoate	MS/R	3.9	3.5	3.6
Propyl propanoate	MS	ND	0.9	ND
Propyl butanoate	MS	ND	1.3	ND
butyl butanoate	MS	0.6	0.8	0.6
Sulfur Compounds				
Dimethyl disulfide	MS/R	ND	2.85	ND

ND = not detected.
MS = identified by mass spectrum or comparison with data bank.
MS/R = identified by mass spectrum or comparison with compound reference.

Addition of broken cells obtained with the new high pressure homogenizer appears to be an interesting way to substitute lack of lysis of *Lactococcus* in UF-cheeses. Surprisingly, addition of CBS or CFE of *Lactococcus* did not significantly increase the NPN/NT ratio in the cheeses during ripening in spite of the significant increase in the concentration of free amino acids (1.5 to 3 times). Similar observations have been made by Hickey et al. [15] and by Spangler et al. [39] after the addition of *Lb. helveticus* or freeze-shocked *Lb. helveticus* cells, respectively. Stability of NPN content might be due either to complete destruction of the *Lactococcus* proteinase by homogenization; such a hypothesis appears highly unlikely considering the results observed by Saboya et al. [37] on cell enzymes submitted to the same technological treatment, or to an inhibition of this enzyme by a component concentrated by UF as proposed by Bech [2]. On the contrary, intracellular peptidases are still active and able to degrade peptides contained in UF-cheeses to free amino acids, as they do in traditional cheeses [22]. The lower increase in free amino acids observed after addition of CBS might be related to a partial consumption of the released amino acids by the remaining 10^6 cfu·g^{-1} live cells added to this suspension.

Addition of CBS or CFE from *Lactococcus* to UF-cheeses does not seem to produce more volatile compounds than in the control cheese. Such an observation confirms the low impact per se of *Lactococcus* intracellular enzymes on cheese flavor development [22].

Increase of ripening temperature from 12 to 20 °C, assayed in this study during the addition of *Propionibacteria* CBS or CFE, caused an increase in proteolysis of the casein network by 12% as measured by the NCN/NT ratio. This increase was probably due to the temperature activation of rennet and proteinase of live cells of the added starters [10]. Ripening at 20 °C also induced peptide breakdown, as shown by the 19% increase of the NPN/NT ratio in the reference cheeses. Addition of *Propionibacterium* CBS to UF-cheeses caused only a slight additional degradation of peptides (+ 8%) when ripened at 20 °C. No significant impact was seen with CFE. As was observed for experiments with *Lactococcus,* data for total free amino acids did not agree with peptidolysis results but a survey of individual amino acids demonstrates that disappearance of Asn, probably consumed by the remaining living cells of *Propionibacterium* in CBS, was the cause of this discrepancy.

Data obtained for volatile compounds showed no significant differences between the control and *Propionibacterium* CFE treated cheeses. On the other hand, addition of CBS induced a net increase of several compounds, including primary alcohols, esters, ketones and dimethyl disulfide, 2-methyl butanol and 3-methyl butanol, probably from the respective catabolism of Ile and Leu by *Propionibacterium* cells [42]. Propionic and acetic acids, which are the main products of *Propionibacterium* metabolism, were not determined in this study, but were very probably formed, resulting in the formation of ethyl and propyl esters of propanoic acid. Dimethyl disulfide can result from degradation of Met by *Propionibacterium* [42]. In addition, the presence of *Propionibacterium* was associated with a higher amount of some methyl ketones and of diacetyl (2,3-butanedione).

In conclusion, this work provides the first demonstration of the absence of *Lactococcus* starter lysis in UF-cheeses and confirmed the low extent of the ripening through the analysis of proteolysis and neutral volatile aroma compounds. On the other hand, it was shown that it is possible to modify these two ripening indices by adding broken cells obtained by high pressure homogenization, a technology which opens a new area of investigation to im-

prove UF-cheese ripening. Other strains and species with efficient peptidase activity and higher flavoring ability should now be tested.

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