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## **► To cite this version:**

Florence Valence, R. Richoux, Anne Thierry, A. Palva, Sylvie S. Lortal. Autolysis of *Lactobacillus helveticus* and *Propionibacterium freudenreichii* in Swiss cheeses: First evidence by using species-specific lysis markers. *Journal of Dairy Research*, 1998, 65 (4), pp.609-620. hal-02694510

**HAL Id: hal-02694510**

**<https://hal.inrae.fr/hal-02694510>**

Submitted on 1 Jun 2020

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## Autolysis of *Lactobacillus helveticus* and *Propionibacterium freudenreichii* in Swiss cheeses: first evidence by using species-specific lysis markers

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(Received 24 November 1997 and accepted for publication 25 February 1998)

**SUMMARY.** *Lactobacillus helveticus* and *Propionibacterium freudenreichii* are essential starters in Swiss cheesemaking and the release of their intracellular enzymes through autolysis could significantly influence ripening. To provide evidence of this lysis, cheese made from microfiltered thermized milk inoculated with *Lb. helveticus* ITGLH77, *Prop. freudenreichii* ITGP23 and a commercial *Streptococcus thermophilus* was assayed. Starter viability was determined and lysis was monitored during ripening by protein analysis with SDS-PAGE of aqueous cheese extracts and by immunoblot detection of intracellular proteins: dipeptidase (PepD) for *Lb. helveticus* and methylmalonyl coenzyme A mutase for *Prop. freudenreichii*. We verified that the species specificity of these lysis markers was towards the cytoplasm of all the species currently used in Swiss cheese. *Lb. helveticus* exhibited an almost complete loss of viability (99.9%) from the beginning of ripening in the cold room; concomitantly PepD appeared in the cheese extracts and was detected until the end of ripening. Damaged *Lb. helveticus* cells were also visualized by scanning electron microscopy. In addition, free PepD was also successfully detected in commercial Swiss-related cheeses. All these results clearly demonstrated the autolysis of *Lb. helveticus* in Swiss cheese. *Prop. freudenreichii* ITGP23 grew during warm room ripening and no loss of viability was detected after maximal growth ( $10^9$  cfu/g cheese). Free methylmalonyl-coenzyme A mutase was detected at the end of ripening during cold storage, when the cheese extracts were concentrated 20-fold, demonstrating that the autolysis of *Prop. freudenreichii* was tardy and limited.

The contribution of lactic acid bacteria to cheesemaking has long been considered to be due exclusively to the presence of live microorganisms producing lactic acid and proteolytic enzymes. Nevertheless, it seems increasingly obvious that lactic acid bacteria starters continue to influence ripening markedly even after their death by releasing their numerous intracellular peptidases and esterases through autolysis (Hansen, 1941; Law *et al.* 1974). Autolysis of lactococci has been clearly demonstrated in Cheddar (Wilkinson *et al.* 1994*a, b*; Crow *et al.* 1995*a*) and Saint-Paulin cheese (Chapot-Chartier *et al.* 1994). As expected, the extent of lysis was shown to have a

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direct effect on the extent of proteolysis (Crow *et al.* 1995*b*; O'Donovan *et al.* 1996; Kawabata *et al.* 1997). For these reasons, autolysis is now regarded as the rate-limiting step of proteolysis in hard and semi-hard cheeses.

Swiss-type cheeses are of obvious economic importance since ~ 850 000 tonnes of these varieties are produced every year all around the world (Syndicat Interprofessionnel du Gruyère Français, 1997). Their ripening requires several weeks and a decrease of viability of thermophilic lactic acid bacteria starters has been frequently described, suggesting that autolysis occurs (Demeter *et al.* 1953; Accolas *et al.* 1978; Demarigny *et al.* 1996; Thierry *et al.* 1998). Several species, including *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lb. delbrueckii* subsp. *lactis* and *Propionibacterium freudenreichii*, are used together in order to carry out the acidification step as well as the ripening process (Langsrud & Reinbold, 1973). Autolysis of these species has been studied in growth media and buffers, leading to a better knowledge of their autolysins (Sandholm & Sarimo, 1981; Lortal *et al.* 1997*a*), but very few assays have been carried out in cheese-like media, and most of these were conducted with *Lb. helveticus*. Ohmiya & Sato (1970) followed the autolysis of *Lb. helveticus* in an aseptic rennet curd and Bie & Sjöström (1975*a, b*) did the same in a casein liquid substrate and in semi-hard cheeses. Both groups monitored lysis by the release of DNA into the curd but encountered detection problems due to DNA degradation. Recently, damaged cells of *Lb. helveticus* and *Prop. freudenreichii* were visualized in Grana by scanning electron microscopy, indicating that both species underwent autolysis in this cheese (Bottazzi, 1993; Cappa *et al.* 1997).

The aim of this work was to investigate whether *Lb. helveticus* and *Prop. freudenreichii* undergo autolysis in Swiss cheese during processing and ripening. They were chosen from the different species inoculated because of their efficient cytoplasmic peptidase and esterase activity (Dupuis & Boyaval, 1993; Prost & Chamba, 1994; Sasaki *et al.* 1995). To study the autolysis, species-specific lysis markers had to be developed first because several species are present in Swiss cheese, growing and dying simultaneously. Cheeses were made on a small scale and the autolysis of both species was demonstrated using immunological detection of the lysis markers in aqueous cheese extracts.

#### MATERIALS AND METHODS

##### *Organisms and culture conditions*

*Lb. helveticus* ITGLH77 (LH77) and ITGLH1 (LH1) and *Prop. freudenreichii* ITGP23 (P23) are commercial starters from the Institut Technique du Gruyère collection. LH77 and P23 were selected on the basis of their high autolysis in buffers as described by Valence & Lortal (1995) for *Lb. helveticus* and by Lemée *et al.* (1994*b*) for *Prop. freudenreichii*. *Str. thermophilus* was a commercial starter (TA060) from Texel (F-86220 Dangé Saint-Romain, France). *Lb. delbrueckii* subsp. *lactis* CIP101028 was from the Institut Pasteur collection (F-75724 Paris Cédex 15, France) and *Lactococcus lactis* subsp. *lactis* CNRZ 144 was obtained from the Centre National de la Recherche Zootechnique (F-78352 Jouy-en-Josas, France).

Strains were stored at  $-80^{\circ}\text{C}$  in MRS broth (Difco, Detroit, MI 48232, USA) for *Lb. helveticus* and *Lb. lactis*, in sodium lactate broth (Malik *et al.* 1968) for *Prop. freudenreichii* and in M17 broth (Biokar 088, F-60000 Beauvais, France) for *Str. thermophilus* and *Lc. lactis*. The media were supplemented with 150 ml glycerol/l prior to freezing. For propagating the strains, the appropriate media without glycerol were used and growth was monitored by measuring  $A_{650}$ .

For cheese trials, cultures were revived from frozen stocks first by a transfer in reconstituted skim milk (100 g Lait G/l; Standa Industrie, F-14050 Caen, France) at 2 ml inoculum/l until lactic acid as measured by Dornic acidity reached 7.5 g/l, followed by a second transfer on Phagex commercial medium (Standa Industrie) at 100 ml inoculum/l until lactic acid reached 9.5 g/l for *Lb. helveticus*, or by two consecutive transfers in sodium lactate broth at 2 ml inoculum/l until  $A_{650}$  reached 1 for *Prop. freudenreichii*.

#### *Preparation of crude cytoplasm for determination of lysis marker specificity*

Cells were harvested during the exponential growth phase by centrifugation at 7000 *g* for 20 min and washed twice in cold distilled water. The pellet was resuspended in a volume of cold water 1/15th that of the initial volume of the culture, then subjected to treatment in a French Press apparatus at 4 °C and 138 MPa for 5 min (one run) for *Lb. helveticus* and *Lb. lactis* and at 138 MPa for 10 min (two runs) for *Prop. freudenreichii*, *Str. thermophilus* and *Lc. lactis*. Suspensions were centrifuged at 40000 *g* for 30 min to eliminate unbroken cells and cell walls. The supernatants represented crude cytoplasm, and were stored at -18 °C until used.

#### *Cheese manufacture*

Small scale experimental Swiss cheeses were made from 10.3 kg thermized and microfiltered milk by a standard procedure described previously (Buisson *et al.* 1987) with the following modifications. The starter was composed of *Str. thermophilus*, *Lb. helveticus* ITGLH77 and *Prop. freudenreichii* ITGP23; no *Lb. lactis* species were added. Cheeses were divided into four pieces (4 × 200 g) before waxing and were ripened at 11 °C (cold room) for 21 d, at 24 °C (warm room) for 48 d and at 4 °C (cold storage) for 15 d. From a single batch of milk three repetitions were carried out on three successive days. Three cheeses were produced per day, in order to provide 12 pieces of cheese for sampling. Samples were taken just after pressing (day 0), after brining (day 1) at the middle (day 16) and end (day 22) of the time in the cold room, at the middle (day 45) and end (day 69) of the time in the warm room, and after 1 week (day 76) and 3 weeks (day 92) cold storage.

#### *Chemical analysis of cheeses*

Total solids (International Dairy Federation, 1982), fat (Heiss, 1961) and NaCl (International Dairy Federation, 1988) contents were determined in the ripened cheese on day 69. During ripening (days 22, 33, 40, 47 and 69) lactate (kit from Boehringer, D-68298 Mannheim 31, Germany), propionate, acetate (Berdagué, 1986) and cheese volume (Richoux & Kerjean, 1995) were monitored.

#### *Aqueous cheese extracts*

Standard extracts were obtained essentially as described by Kuchroo & Fox (1982) with the following modifications. Each cheese sample (20 g) was homogenized with an Ultra-Turrax disperser (Bioblock, F-67403 Illkirch, France) for 2 min in 80 ml of water and the suspension was held at 38 °C for 20 min before centrifugation at 3000 *g* and 4 °C for 15 min. After removal of the upper solid fat layer the supernatant was filtered through Whatman no. 1 paper and then a 0.45 µm acetate filter (Sartorius, F-91127 Palaiseau, France). Aqueous cheese extracts were stored at -18 °C until the SDS-PAGE and immunoblotting assays were carried out.

For gentle extracts, each grated cheese sample (1.5 g) was ground manually in

6 ml distilled water in a filter stomacher bag (Humeau, F-44240 Treilli re, France) at 20  C for 5 min. The supernatant was filtered through a 0.45  m acetate filter (Sartorius). Gentle aqueous cheese extracts were stored at  18  C until used.

#### *Starter viability*

A 10 g cheese sample was homogenized with a Waring Blendor (Prolabo, F-94126 Fontenay-sous-Bois, France) once for 20 s at maximal speed, then twice for 10 s at minimal speed in 90 g trisodium citrate solution (20 g/l). The viability of starter lactobacilli in cheese was measured by plating serial dilutions of samples on MRS (pH 5.4) agar and incubating at 37  C for 48 h. *Prop. freudenreichii* cells were enumerated on the selective medium Pal Propiobac (Standa Industrie) after incubation at 30  C for 5 d, and *Str. thermophilus* cells on M17 agar plates (Biokar 088) after incubation at 43  C for 16 h. Non-starter lactic acid bacteria were enumerated on FH agar plates (Isolini *et al.* 1990) after incubation at 37  C for 3 d. Anaerobic growth conditions (Anaerocult A; Merck, F-94736 Nogent-sur-Marne, France) were used for all species studied and results were expressed as cfu/g cheese.

#### *Genomic DNA preparation and pulsed field gel electrophoresis*

In order to verify the identities of the strains enumerated on MRS (pH 5.4) agar and on Pal Propiobac, 20 colonies were randomly picked from 25–50 colonies on count plates on day 16 for *Lb. helveticus* and day 69 for *Prop. freudenreichii*, then subjected to genomic macrorestriction profile analysis according to the procedure of Lortal *et al.* (1997b) and Lem e *et al.* (1994b) for *Lb. helveticus* and *Prop. freudenreichii* respectively. Macrorestriction profiles obtained were compared with those of LH77 and P23 strains.

#### *Scanning electron microscopy*

Cheese samples were taken for electron microscopy at days 22 and 69. Two 1   1   3 mm pieces were cut from the internal portion of the cheese and fixed in glutaraldehyde (2.5 ml/l, pH 7.2) at 4  C for 48 h. Samples were washed twice in 25 mM-cacodylate buffer, pH 7.5, then fixed in 10 g osmium tetroxide/l at 4  C for 15 h followed by dehydration in alcohol at 100, 250, 500, 750 and 950 ml/l successively. The sample was critical point dried in CO<sub>2</sub>, mounted on an aluminium stub and coated with 15 nm gold. Microscopy was on a Philips XL20 scanning electron microscope at an acceleration voltage of 15 kV.

#### *SDS-PAGE analysis of total protein content of aqueous cheese extracts*

SDS-PAGE was carried out as described by Valence & Lortal (1995) at 20  C with an SDS-polyacrylamide (140 g/l) separating gel, pH 8.8 and a constant voltage of 180 V. Samples were mixed with an equal volume of Laemmli buffer (62.5 mM-Tris HCl, pH 6.8 containing 100 ml glycerol/l, 20 g SDS/l and 50 ml 2-mercaptoethanol/l) and boiled for 2 min (Laemmli, 1970); 20  l of each sample was loaded on the gel.

#### *Detection of the species-specific lysis marker using immunoblot assay*

A 53.4 kDa dipeptidase (PepD) was selected as specific lysis marker for *Lb. helveticus*. This peptidase has been shown to be intracellularly located and its DNA and amino acid sequences show no homology with any of the numerous lactic acid bacterial peptidases described so far (Vesanto *et al.* 1996). The sample used for immunization had been overexpressed in *Escherichia coli*, purified and immunized in an active form (octamer form). Immunization was with three injections (20  g antigen per injection) per rabbit, and immunization time was 6 weeks.

In the case of *Prop. freudenreichii*, the finding of a specific lysis marker was much easier, mainly because that species is phylogenetically very far from the lactobacilli and streptococci involved in Swiss cheesemaking. An intracellular enzyme required in the metabolism of propionic acid, i.e. the methylmalonyl coenzyme A mutase (MMCoA), was selected. This enzyme comprises two subunits,  $\alpha$  and  $\beta$ , of 62 and 70 kDa respectively (Marsh *et al.* 1989). MMCoA has been reported only in propionibacteria and in animal tissues (Kellermeyer *et al.* 1964). From 400  $\mu$ g of *Prop. shermanii* MMCoA from recombinant *Esch. coli*, polyclonal antibodies against MMCoA were produced by four successive intradermic injections of 50  $\mu$ g MMCoA which were carried out on days 0, 14, 28 and 56 in two rabbits (Eurogentec, B-4102 Seraing, Belgium).

Samples were subjected to SDS-PAGE and then transblotted on to a nitrocellulose sheet (0.45  $\mu$ m pore size: Millipore, F-78051 Saint-Quentin-en-Yveline, France) as described by Towbin *et al.* (1979). PepD and MMCoA were detected with rabbit sera anti-PepD and anti-MMCoA mutase respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, St Louis, MO 63178, US) was used as the secondary antiserum with 4-chloro-1-naphthol and H<sub>2</sub>O<sub>2</sub> substrates.

## RESULTS

### *Specific lysis markers for Lactobacillus helveticus and Propionibacterium freudenreichii*

Immunoblotting analysis of the cytoplasmic extracts of *Lb. helveticus* strains LH77 and LH1 with polyclonal PepD antiserum confirmed the presence of PepD protein of the expected size also in these strains (Fig. 1). Fig. 1 provides evidence that the polyclonal antibodies against PepD did not cross react with cytoplasmic extracts of *Str. thermophilus*, *Lc. lactis* and *Prop. freudenreichii*. For *Lb. lactis* a cross reacting protein was clearly detected but with a molecular mass slightly lower (51 kDa). A mixture of cytoplasmic extracts from *Lb. helveticus* and *Lb. lactis* (1:1) produced two close bands that could be distinguished (Fig. 1, lane 7), further confirming the specificity of PepD and its validity as a lysis marker. However, in the course of the cheese assays, the species *Lb. lactis* was omitted in order to avoid any doubt about the immunoblot detection of PepD.

In order to establish the sensitivity of PepD immunodetection, different quantities of total cytoplasmic proteins (from 1 to 34  $\mu$ g) were analysed by immunoblotting, and showed that a quantity of PepD as low as 2.1  $\mu$ g could be detected. For > 17  $\mu$ g protein, the intensity of the signal detected remained constant (results not shown).

A cytoplasmic extract of *Prop. freudenreichii* P23 subjected to immunoblot analysis with rabbit serum anti-MMCoA made it possible to detect the two  $\alpha$  and  $\beta$  subunits of the expected sizes (Fig. 2). A cytoplasmic extract of *Lb. helveticus* did not cross react with serum anti-MMCoA (results not shown). The sensitivity of the assay was determined and showed that 1.6  $\mu$ g per lane of the total cytoplasmic proteins of *Prop. freudenreichii* was sufficient to achieve the detection of the two subunits of the MMCoA; the signal detected for the  $\alpha$  subunit was more intense than that for the  $\beta$  subunit. For > 6.6  $\mu$ g protein, the intensity of the signal detected remained constant (Fig. 2).



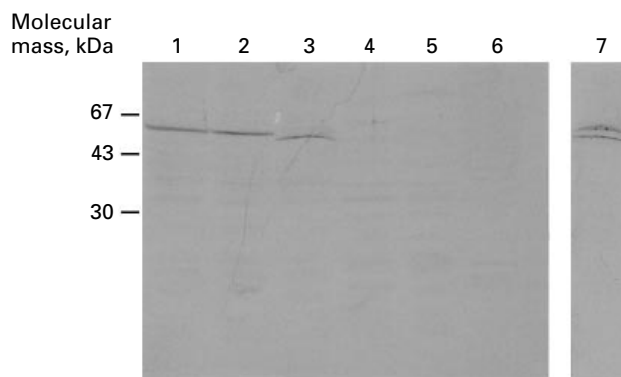


Fig. 1. Specificity of dipeptidase for use as a *Lactobacillus helveticus* lysis marker. Immunoblot of cytoplasm from various species used in Swiss cheesemaking using serum anti-dipeptidase: lane 1, *Lb. helveticus* LH77; lane 2, *Lb. helveticus* LH1; lane 3, *Lb. delbrueckii* subsp. *lactis*; lane 4, *Streptococcus thermophilus*; lane 5, *Lactococcus lactis* subsp. *lactis*; lane 6, *Propionibacterium freudenreichii*; lane 7, 1:1 mixture (v/v) of *Lb. helveticus* LH77 and *Lb. delbrueckii* subsp. *lactis*. Molecular masses of standard proteins are indicated on the left.

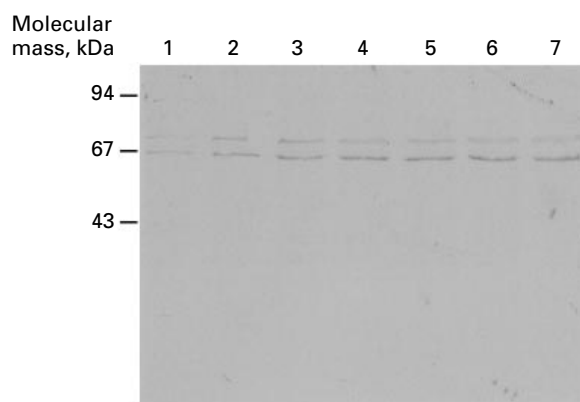


Fig. 2. Sensitivity of methylmalonyl coenzyme A mutase detection by immunoblotting estimated by loading increasing quantities of *Propionibacterium freudenreichii* P23 cytoplasmic proteins: lane 1, 1-6; lane 2, 3-2; lane 3, 6-6; lane 4, 9-6; lane 5, 13; lane 6, 19 and lane 7, 26  $\mu$ g.

### Chemical analysis of the cheeses

The chemical analyses were in agreement with previous values for Swiss cheeses prepared on a small scale (Buisson *et al.* 1987) and on an industrial scale (Steffen *et al.* 1993). During ripening, as expected, the depletion of lactate was concomitant with the production of acetate and propionate and was completed on day 47.

### Viability of starter and non-starter bacteria during ripening

Maximal numbers of *Lb. helveticus* cells were reached at the end of the pressing (day 0) followed by a decrease in numbers, moderately (0.3 log unit) during brining (day 1) and significantly (3 log units) during cold room ripening. At the end of ripening, *Lb. helveticus* was hardly detectable (< 100 cfu/g cheese; Fig. 3). At day 16 the macrorestriction profiles of 20 colonies, randomly sampled during the enumeration of starter, were shown to be identical to the reference profile of *Lb. helveticus* LH77. The viability of *Str. thermophilus* decreased in the same way as *Lb. helveticus*, reaching  $10^4$  cfu/g at the end of the ripening. The viable count of *Prop.*

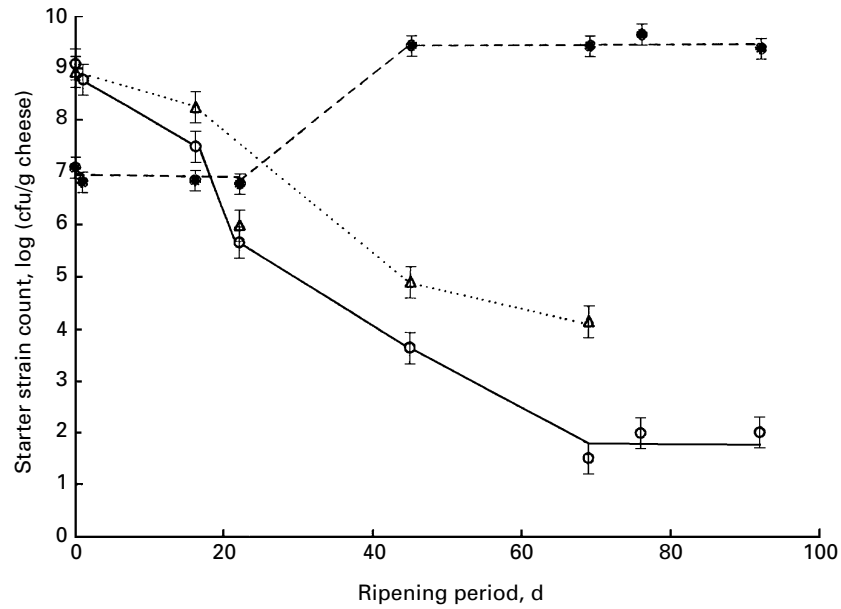


Fig. 3. Counts of starter strains in experimental cheeses during ripening: ○, *Lactobacillus helveticus* LH77; ●, *Propionibacterium freudenreichii*; △, *Streptococcus thermophilus*. Values are means from three trials with SD indicated by vertical bars.

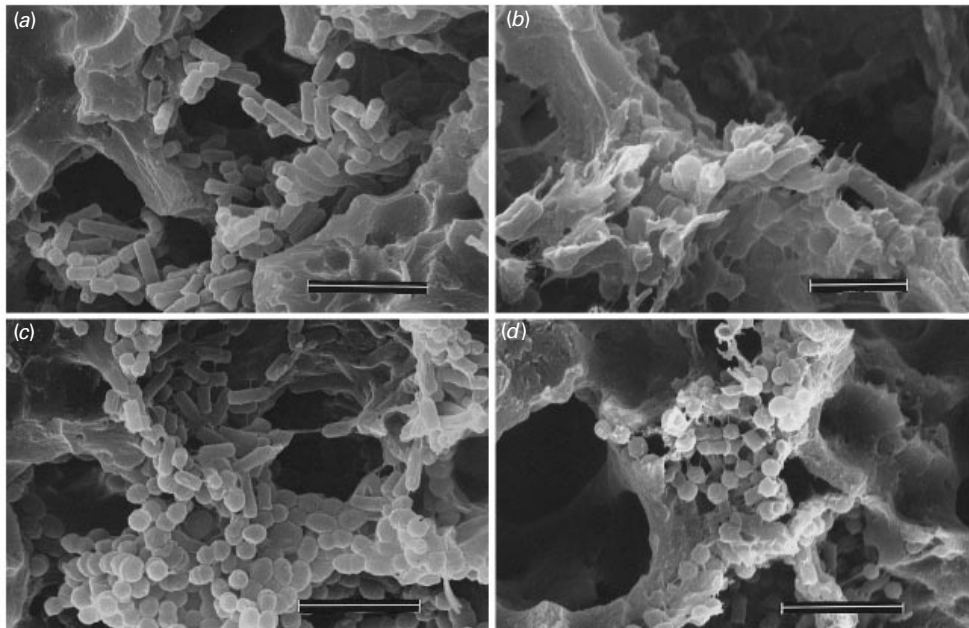


Fig. 4. Electron photomicrographs of starters in Swiss cheese during ripening. Samples were taken at (a, c) day 0, (b) day 22 and (d) day 69. (a) Undamaged rod-shaped cells of *Lactobacillus helveticus*, (b) damaged cells of *Lb. helveticus*, (c) mixed colony of intact *Lb. helveticus* and *Streptococcus thermophilus* cells and (d) cells of *Propionibacterium freudenreichii* exhibiting a coccoid morphology. Scale bars: (a, b, c) 5 µm, (d) 2 µm.



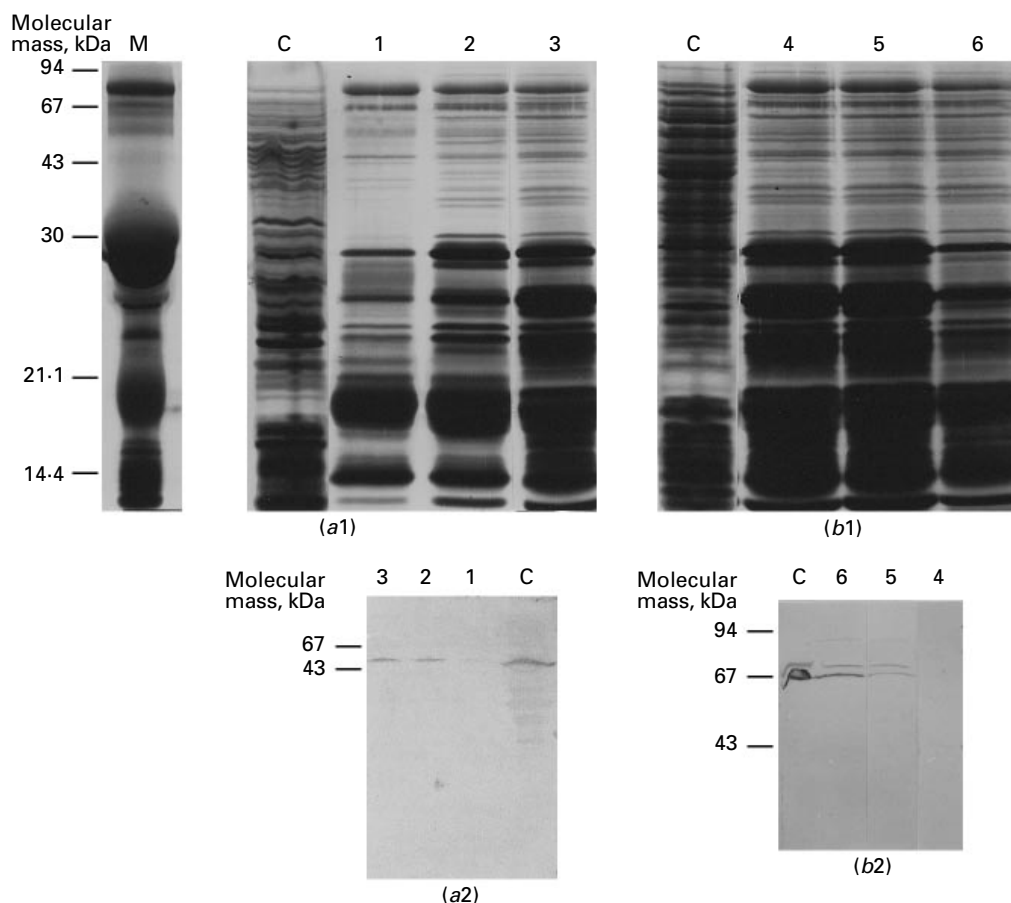


Fig. 5. Autolysis of starter strains in experimental Swiss cheeses. (a1) SDS-PAGE and (a2) immunoblotting with anti-dipeptidase serum showing the autolysis of *Lactobacillus helveticus* by the early appearance of cytoplasmic proteins including dipeptidase in the aqueous cheese extracts: M, milk; C, LH77 crude cytoplasm; aqueous cheese extracts on: lane 1, day 0; lane 2, day 16; lane 3, day 22. (b1) SDS-PAGE and (b2) immunoblotting with anti-methylmalonyl coenzyme A mutase serum showing the late autolysis of *Propionibacterium freudenreichii* by the appearance of free methylmalonyl coenzyme A mutase in the aqueous cheese extracts only after cold storage of the cheeses: C, P23 crude cytoplasm; aqueous cheese extracts on: lane 4, day 69; lane 5, day 76; lane 6, day 92. Molecular masses of standard proteins are indicated on the left.

*freudenreichii* P23 did not change during the cold room period but increased during warm room ripening to a maximum population of  $2.5 \times 10^9$  cfu/g cheese. After that, no decrease in counts of *Prop. freudenreichii* was observed (Fig. 3). The macrorestriction profiles of 20 randomly sampled colonies from Pal Propiobac plates on day 69 were shown to be fully identical to *Prop. freudenreichii* P23.

Since the cheeses were made from microfiltered milk, the non-starter lactic flora was not detectable at the beginning of the ripening and did not exceed  $10^6$  cfu/g at the end of the ripening.

#### *Appearance of bacteria in cheese*

Scanning electron microscopy revealed well-shaped lactobacilli at the beginning of the ripening process (Fig. 4a). It was notable that lactobacilli and streptococci were frequently mixed on the same colony (Fig. 4c). At day 22, damaged *Lb.*

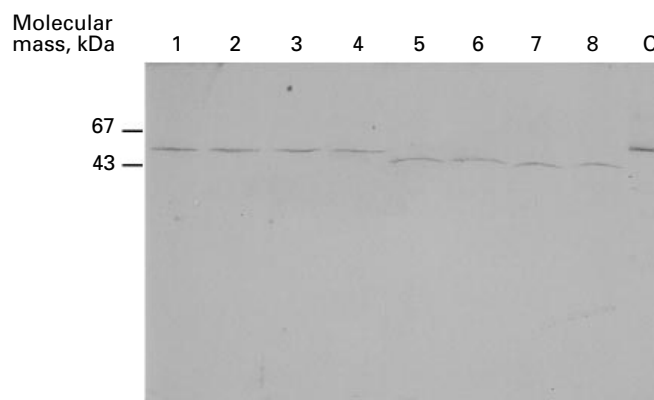


Fig. 6. Detection of dipeptidase lysis marker in commercial Swiss and related cheeses. Two extraction methods, standard (SM) and gentle (GM), were compared: lane 1, Parmesan GM; lane 2, Parmesan SM; lane 3, Comté GM; lane 4, Comté SM; lane 5, Beaufort GM; lane 6, Beaufort SM; lane 7, Emmental GM; lane 8, Emmental SM. C, LH77 crude cytoplasm. Molecular masses of standard proteins are indicated on the left.

*helveticus* LH77 cells were found (Fig. 4*b*), whereas the colonies of *Prop. freudenreichii* P23 appeared unchanged, i.e. coccoid and seemingly surrounded by polysaccharides throughout ripening (Fig. 4*d*).

#### *Detection of the lysis markers in the experimental cheeses*

Aqueous cheese extracts were first subjected to SDS-PAGE analysis and the protein profiles obtained were compared with protein profiles of *Lb. helveticus* and *Prop. freudenreichii* cytoplasm and milk (Fig. 5). Between days 16 and 22, for an equal volume loaded per lane, we were able to detect an increase in the number of bands in cheese extracts, particularly for proteins with molecular masses > 30 kDa. As the milk SDS-PAGE profile did not contain proteins in the range 30–60 kDa, proteins detected in this size frame from cheese extracts most probably came from bacterial cells (Fig. 5*a, b*).

Results of the immunoblotting of cheese extracts with polyclonal PepD antibodies are shown in Fig. 5*a*). PepD was detected in the cheese extracts as a faint signal on day 16 but in much higher quantities at the end of the cold room period (day 22). No variations in the intensity of PepD could be detected thereafter up to the end of ripening (results not shown). The standard aqueous extraction method involved an Ultra-Turrax step, which may have caused disruption of the weakened cells. However, the immunoblot profiles were exactly the same with the gentle aqueous extraction procedure developed in this study (results not shown). These results demonstrated that *Lb. helveticus* undergoes autolysis at the beginning of ripening.

The immunoblotting analysis of the cheese extracts with polyclonal MMCoA antibodies did not allow the detection of lysis marker at the first attempt. However, a weak but clear signal from MMCoA was observed in 20-fold concentrated standard and gentle extracts on day 76, indicating a weak autolysis of *Prop. freudenreichii* during cold storage after ripening (Fig. 5*b*).

#### *Detection of the lysis markers in commercial Emmental, Beaufort, Comté and Parmesan cheese*

Aqueous cheese extracts (standard and gentle extraction methods) of commercial Swiss and related cheeses obtained from a local supermarket were subjected to SDS-PAGE and immunoblotting analyses. Regardless of the extraction method used,

SDS-PAGE profiles in the different cheeses were very similar both to each other and to our experimental small scale cheeses (results not shown). For Parmesan and Comté cheeses, PepD was clearly detected, showing that *Lb. helveticus* underwent autolysis (Fig. 6). With Emmental and Beaufort cheeses, only weak signals of a molecular mass slightly lower than that of *Lb. helveticus* PepD were detected. The cross reacting band was identified as the 51 kDa protein detected in the crude cytoplasm of *Lb. lactis*, giving indications of autolysis of this species in these cheeses.

#### DISCUSSION

The autolysis of *Lb. helveticus* in Swiss cheese has been demonstrated for the first time in this study. The release of intracellular PepD occurred mainly during cold room ripening and was coincident with the decrease in lactobacilli counts and with the scanning electron microscopy observations of damaged cells. Whatever the method of extraction used (standard or gentle) the PepD appeared at the same time, indicating that bursting of weakened cells during the extraction procedure was unlikely. The presence of a very faint band of free PepD at the beginning of the cold room period, together with the 0.3 log reduction in counts during brining, could indicate that the autolysis of *Lb. helveticus* started just at the end of pressing. At this stage, *Lb. helveticus* encountered an effective nutrient starvation (depletion of galactose) at a pH (5.4) and a temperature (11 °C) allowing the expression of the autolysins (Lortal *et al.* 1991). The detection of PepD in aqueous extracts from commercial Comté and Parmesan supported the idea that autolysis of *Lb. helveticus* is a general phenomenon in Swiss and related cheeses. This is in agreement with frequent observations of a decrease in thermophilic starter counts during the ripening of Swiss and related cheeses (Accolas *et al.* 1978; Coppola *et al.* 1997; Demarigny *et al.* 1996; Thierry *et al.* 1998). Nevertheless, before this is taken to be general, it should be verified for a larger number of *Lb. helveticus* strains.

Secondary proteolysis (production of small peptides and free amino acids) is an essential step during ripening. *Lb. helveticus* possesses numerous intracellular peptidases and at least two of them (APII and APIV) have been claimed to be involved in processing bitter peptides (Prost & Chamba, 1994). This implies not only their release in the curd through autolysis but also their ability to retain significant activity after being released. This is plausible, since it was recently shown that peptidases from thermophilic lactic species can retain their activity for several weeks in Emmental juice (Gagnaire *et al.* 1998). Moreover, various lactococcal intracellular enzymes, including peptidases, are stable in the cheese curd (Wilkinson *et al.* 1994*b*). Thus, the ability of *Lb. helveticus* to release its cytoplasmic content should be taken into consideration in starter selection. The extent of autolysis in the cheese probably depends on the strain and on the technological conditions (heating, brining, initial lactose content, pH), but the strain dependence should be the most promising factor to study. Indeed, previous experiments in buffers, growth media and dairy substrates have underlined a significant variation in autolytic activity among *Lb. helveticus* strains (Ohmiya & Sato, 1975; Bie & Sjöström, 1975*a, b*; Lortal *et al.* 1997*a*).

In the case of *Prop. freudenreichii*, the main carbon source (lactate) was depleted from the middle of the warm room period. This represented nutrient starvation for 22 d at pH 5.5 and 24 °C, conditions that usually induce autolysis (Lemée *et al.* 1994*a*). Nevertheless, autolysis of *Prop. freudenreichii* occurred very late, during cold storage, and its extent was apparently limited, since a concentration step of the cheese extracts was necessary to detect the MMCoA. At the same time, no significant

reduction in counts was found, further supporting the idea of a very limited extent of autolysis for *Prop. freudenreichii*. The early autolysis of *Lb. helveticus* demonstrated in this study was much more likely to influence the ripening process and this is currently under investigation.

From a methodological point of view, the immunoblotting detection developed to monitor lysis has many advantages: it is adequately sensitive, it is possible to analyse several samples at the same time and it is not dependent on the enzymic activity of markers. Indeed the variations in activity were regarded as a subtle problem when enzymic lysis markers were used (Wilkinson *et al.* 1994b). Furthermore, taking into account their species specificity, the antisera anti-PepD and anti-MMCoA mutase could probably be used to detect the lysis of *Lb. helveticus* and *Prop. freudenreichii* in other ecosystems.

We are deeply indebted to Dr Leadlay (University of Cambridge, Department of Biochemistry, Tennis Court Road, Cambridge, UK) for providing us with pure methylmalonyl coenzyme A mutase, and to E. Vesanto for the rabbit sera anti-PepD. We sincerely thank M. Le Hénaff for advice on western-blotting analysis and S. Guezenc for skilled technical assistance.

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