Lactobacillus helveticus as a tool to change proteolysis and functionality in Swiss-type cheeses

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ABSTRACT

Lactobacillus helveticus exhibits a great biodiversity in terms of protease gene content, with 1 to 4 cell envelope proteinases. Among them, proteinases PrtH and PrtH2 were shown to have different cleavage specificity on pure α_{s1} -casein. The aim of this work was to investigate the proteolytic activity of 2 *L. helveticus* strains in cheese matrix: ITGLH77 (PrtH2 only) and ITGLH1 (at least 2 proteinases, PrtH and PrtH2). Cell viability, proteolysis, autolysis, and stretchability of experimental Emmental cheeses were measured during ripening. The peptides identified by mass spectrometry showed very different profiles in the 2 cheeses. Regardless of the casein origin, the number of different peptides containing more than 20 amino acids was greater in cheeses manufactured with strain ITGLH77. This accumulation of large peptides, including those from α_{s1} - and α_{s2} -caseins, was in agreement with the lower overall extent of proteolysis obtained in ITGLH77 cheeses, which can be attributed to the presence of one cell envelope proteinase of the lactobacilli strains or lesser release of intracellular peptidases into the cheese aqueous phase. In parallel, stretchability was measured throughout ripening time. Emmental strands observed by confocal laser scanning microscopy showed microstructure similar to that of mozzarella strands. Stretchability was correlated with a specific type of peptide (hydrophobic), as shown by principal component analysis, and with a lower degree of proteolysis.

Key words: *Lactobacillus helveticus*, cell envelope proteinase, stretchability, peptide identification

INTRODUCTION

Stretchability is a techno-functional characteristic of some cheeses, such as Emmental, Mozzarella, and Cheddar, and is one of the most desirable cooking properties for consumer products (e.g., pizza, pasta, fondue). Stretchability varies between cheeses depending on cheese-making processes, cheese composition, and ripening conditions used (Guinee, 2002). Stretching properties also depend on the presence of some species in the ecosystem, such as thermophilic lactobacilli (Hong et al., 1998; Guinee, 2002; Oommen et al., 2002; Richoux et al., 2009). It was demonstrated, for example, that *Lactobacillus helveticus* led to greater stretchability (3 times higher) than *Lactobacillus delbrueckii* (Richoux et al., 2009). Cell wall proteases of lactobacilli are thought to be important actors in generating stretching properties (Oberg et al., 1991; Oommen et al., 2002) via the release of hydrophobic medium-size peptides (Richoux et al., 2009). However, the relationship between cell wall proteinases of lactobacilli, proteolysis in cheese, and stretchability is far from being elucidated. This may be related to the high biodiversity exhibited by *L. helveticus* in terms of proteases gene content. Two cell envelope proteinases (CEP), PrtH and PrtH2, have been described in the *L. helveticus* CNRZ32 strain (Pederson et al., 1999; Smeianov et al., 2007) and extensively studied in 29 strains from various biotopes; PrtH2 has been shown to be ubiquitous, whereas the presence of PrtH appears to be strain dependent (Genay et al., 2009). In terms of activity, the presence of 1 or 2 proteases does not increase the overall extent of proteolysis on milk or synthetic substrate. However, several regions of the pure α_{s1} -CN are hydrolyzed only by strains exhibiting both proteases at minimum, suggesting a synergistic effect (Sadat-Mekmene et al., 2011). Recently, Broadbent et al. (2011) published an extensive screening of PrtH genes in *L. helveticus* showing that 1 to 4 proteases genes

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can be present per strain. Genes encoding PrtH2 and PrtH3 are highly similar, with about 70% homology, whereas PrtH and PrtH4 are clearly different proteases, with a conserved region of 45% similarity at the amino acid level. Further study is still required to fully explain how, in cheese, the texture and functionalities can vary when different proteolytic systems are involved.

The aim of this work was to investigate, in experimental Swiss-type cheeses, the proteolytic activity of *L. helveticus* in 2 strains possessing different CEP content: ITGLH77 (PrtH2 only) and ITGLH1 (PrtH2 and PrtH, at least; Genay et al., 2009). The peptide fraction was analyzed by different methods, including identification of cleavage sites by tandem mass spectrometry (MS/MS). As peptidases released through autolysis can significantly modulate the size and number of peptides (Gagnaire et al., 1998; Valence et al., 1998), extent of lysis was verified using 2 intracellular markers. Except for the work of Richoux et al. (2009), no data exist in the literature regarding the dynamics of this stretchability for experimental Swiss-type cheeses. Stretchability was thus determined at different times of ripening. Principal component analysis was then performed in an attempt to clarify complex relationships between proteolysis due to *L. helveticus* and stretchability.

MATERIALS AND METHODS

Cheese Making

Two sets of cheeses were manufactured in parallel, using strain *L. helveticus* ITGLH77 or strain *L. helveticus* ITGLH1. Cheese making was repeated the next day from the same batch of milk. The pasteurized skim milk was micro-filtered and standardized with heat-treated cream (115°C for 20 s) using a standardized process as described by Richoux et al. (2008). One hundred kilograms of milk was matured at 32°C for 30 min after adding 0.03% (vol/wt) of a 50% calcium dichloride solution, 0.001% (wt/wt) of propionic starter (PAL-ITGP9, Standa, Caen, France), 0.12% (vol/wt) of *Streptococcus thermophilus* culture [PAL-ITG 20/87 (Standa) cultured at 43°C for 5 h in Marstar 412A (Danisco, Dangé Saint Romain, France)], and 0.03% (vol/wt) of *L. helveticus* culture (ITGLH1 or ITGLH77 cultured on Phagex Lb, Standa).

Milk was coagulated at 32°C by addition of 30 mL of calf rennet (Berthelot, ABIA, Beaune, France). After cutting the curd into grains with a mean diameter of 3.5 mm, the whey-curd mixture was cooked at 54°C for 25 min. The duration of stir-out was 30 min. The curd grains were then drained off and poured into 35-cm-diameter molds and pressed for 2 h in a thermo-regulated room at 36°C. The acidification step was conducted for

24 h. The cheeses (8 kg) were then cooled and salted for 18 h in NaCl-saturated brine at 11°C. The cheeses were cut into 8 portions (1 kg each), wrapped under vacuum in BK1L ripening bags (Cryovac, Epernon, France), and ripened at 12°C for 20 d (cold room) and then at 24°C for 21 d (warm room). Hereafter, the cheeses manufactured using *L. helveticus* ITGLH77 and ITGLH1 as starters are designated H77 and H1 cheeses, respectively.

Cheese Analysis

Emmental cheeses were analyzed for moisture (IDF, 2004), fat (Heiss, 1961), and calcium (Pearce, 1977), and enumeration of the *L. helveticus* flora was performed according to the methods described by Thierry et al. (1998). Under a sterile atmosphere, Emmental cheeses were collected at d 1 (after pressing), 2 (after brining), 6 and 13 (during ripening in the cold room at 12°C), 20 (when cheeses were transferred into the warm room), 23, 27, and 34 (during ripening in the warm room at 24°C), and 41 (at the end of the warm room period). Ten grams of grated Emmental cheese was dispersed in 90 mL of sodium citrate (20 g/L) in a bag and crushed in the Smasher (AES Laboratoire, Bruz, France) for 4 min; from this solution the bacterial cell counts were made. Cheese proteolysis was assessed by determination of total nitrogen (TN), pH 4.6 soluble nitrogen (SN), and 12% NPN according to Gripon et al. (1975) using the Kjeldahl method (IDF, 2001).

Measurement of the Cheese Stretchability

Cheese stretchability (3 replicates per sample) was assayed by a method involving vertical traction of the cheese melted at 82°C according to Richoux et al. (2001). The length (mm) of strands of heated cheese was measured at the breaking point of the stretched strand.

Microstructure of the Strands

The microstructure of the strands of Emmental cheeses was visualized using an inverted microscope Nikon Eclipse-TE2000-C1si (Nikon, Champigny sur Marne, France) allowing confocal laser scanning microscopy. Confocal experiments were performed using an argon laser operating at 488 nm excitation wavelength (emission was detected between 500 and 530 nm) and a helium-neon laser operating at 543 nm wavelength excitation (emission was detected between 565 and 615 nm). The observations were performed on 5 mm, newly formed strands using one 10× (numerical aperture 0.45) and one 40× (numerical aperture 1.33)

oil immersion objectives. Proteins were stained using Syto 9 (Invitrogen, Cergy Pontoise, France) and fat using Nile red (Invitrogen). Both stains were mixed together (Syto 9:Nile red, 3:1) and 10 μ L was added at the surface of the strands for 30 min at 20°C. Bacterial colonies were visualized by using the Live/Dead BacLight Bacterial Viability Kit using the Syto 9 dye and propidium iodide (Invitrogen).

Preparation of the Cheese Water-Soluble Extracts

Cheese aqueous phases were prepared by adding 40 g of distilled water to 10 g of grated Emmental according to the method of Kuchroo and Fox (1982); the mix was blended using an Ultra Turrax blender (IKAT18 basic, Staufen, Germany) for 3 min at 20,500 rpm and further stirred for 30 min at 40°C. Emmental samples were centrifuged at $6,000 \times g$ for 30 min at 4°C (Centrifuge Heraeus/Kendro Biofuge Primo R, Courtabœuf, France) to separate the aqueous phase from the pelleted caseins. Fat was eliminated and aqueous phase was filtered on Whatman filter paper 40 at 4°C and frozen at -20°C until further use.

Determination of the Casein Hydrolysis

Casein hydrolysis was evaluated by urea-PAGE of the insoluble pH 4.6 nitrogen fraction according to Collin et al. (1987). These fractions were expressed as the percentage of TN (determined using the Kjeldahl method).

The peptide pattern of the cheese water-soluble extracts (**WSE**) was analyzed by SDS-PAGE as described by Schagger and von Jagow (1987) using Protean II system (16 $\times$ 16 $\times$ 0.1 cm; BioRad, Marnes-la-Coquette, France) with SDS/Tris/Tricine buffer and a concentration gradient from 12 to 18% of acrylamide used according to Sadat-Mekmene et al. (2011).

Peptide Identification

Peptides contained in the cheese WSE were separated by reverse phase-HPLC (2695 separation module with 2487 detector, Waters Alliance, Saint Quentin en Yvelines, France) with a Vydac C18 column (4.6 mm i.d. $\times$ 250 mm), fitted with a precolumn containing the same phase. Separation was performed using solvent A [0.106% trifluoroacetic acid (**TFA**; vol/vol) in MilliQ purified water] and solvent B [0.1% TFA (vol/vol), 80% acetonitrile (vol/vol), in MilliQ purified water] under conditions of a linear gradient from 5 to 60% solvent B using a flow rate of 1 mL/min at 40°C and a double detection at 214 and 280 nm. One hundred microliters of the cheese WSE was injected onto the column and

3 fractions were collected as a function of the elution time: fraction A was collected from 2 to 22 min, fraction B from 22 to 42 min, and fraction C from 42 to 65 min. Each fraction was concentrated by speed vacuum before peptide identification by MS/MS.

The MS experiments were performed using a nanoRSLC Dionex U3000 system fitted to a QSTAR XL (MDS SCIEX, Ontario, Canada) equipped with a nanoelectrospray ion source (Proxeon Biosystems A/S, Odense, Denmark). The instrument was externally calibrated with a multi-point calibration using fragment ions that resulted from the collision-induced decomposition of a peptide from β -casein, β -CN(193–209). A preliminary sample concentration step was performed on a reverse phase column PepMap 100 [C18, 5 μ m, 300 μ m i.d. $\times$ 5 mm length; LC-Packings, Dionex, Amsterdam, the Netherlands]. Separation was performed on a reverse-phase column PepMap 100 (C18, 3 μ m, 75 μ m i.d. $\times$ 250 mm length; Dionex) at 35°C using solvent A [2% (vol/vol) acetonitrile, 0.08% (vol/vol) formic acid, and 0.01% (vol/vol) TFA in deionized water] and solvent B [95% (vol/vol) acetonitrile, 0.08% (vol/vol) formic acid, and 0.01% (vol/vol) TFA in deionized water]. A separation gradient was adapted for each fraction as a function of their hydrophobicity: fraction A, 5 to 30% of solvent B in 45 min and 30 to 85% in 2 min; fraction B, 5 to 15% of solvent B in 3 min, 15 to 40% in 42 min, and 40 to 85% in 2 min; fraction C, 5 to 25% of solvent B in 3 min, 25 to 60% in 42 min, and 60 to 85% in 2 min. Elution was performed at a flow rate of 0.3 μ L/min. Eluted peptides were directly electrosprayed into the mass spectrometer operated in positive mode. A full continuous MS scan was carried out followed by 3 data-dependent MS/MS. Spectra were collected in the selected mass range 350 to 2000 m/z for MS spectra and 60 to 2,000 m/z for MS/MS spectra. The first, second, and third most intense ions from the MS scan were selected individually for collision-induced dissociation (1+ to 4+ charged ions were considered for the MS/MS analysis). The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition using Analyst QS 1.1 software (MDS Sciex, Concord, ON, Canada).

To identify peptides, all data (MS and MS/MS) were submitted to MASCOT (v.2.2, Matrix Science Ltd., London, UK). The search was performed against a homemade database dealing with major milk proteins, which represents a portion of the UniProtID database (Artimo et al., 2012). No specific enzyme cleavage was used and the peptide mass tolerance was set to 0.3 Da for MS and 0.2 Da for MS/MS. Three variable modifications (phosphorylation on serine and threonine, oxidation of methionine, and deamidation of asparagines and glutamine residues) were selected. For each peptide

identified, a minimum MASCOT score corresponding to a *P*-value below 0.05 was considered as a prerequisite for peptide validation.

Calculation of Cleavage Scores Along the Protein Sequence

To estimate the distribution of the cleavage sites along the casein sequences during cheese aging, a particular data analyzing procedure was used. A series of cleavage scores were calculated for α_{s1} -, α_{s2} -, and β -CN on the 6 samples (3 times of aging $\times$ 2 strains) as follows. For each peptide identified by liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS), a score of 1 was attributed to the peptide bonds of the casein sequence that had to be hydrolyzed to release it; that is, the bonds that correspond to the N-terminal and C-terminal ends of the peptide—and a score of 0 was attributed to all other peptide bonds. This procedure was repeated for all the peptides identified, and the so-recovered series of scores (one for each peptide) were then added. At the end of the process, a series of scores representing the number of times (frequency) that a peptide bond had been identified as cleaved was thus obtained along the protein sequence. Results are presented by plotting the calculated scores according to the peptide bond positions of the studied protein. All these calculations were performed using homemade programs running with the Matlab software.

Estimation of Cell Lysis

Sodium citrate extracts of the cheeses were assayed for cell lysis by measuring the activity of aminopeptidases and lactate dehydrogenase (LDH) that are initially intracellularly located and can be released into the supernatant because of bacterial cell lysis during ripening. β -Naphthylamide (β -Na) derivatives were used according to Goldbarg and Rutenburg (1958): Lys β -Na for lysine aminopeptidase (PepN) and Phe-Pro β -Na for X-prolyl-dipeptidyl aminopeptidase (PepX). The total LDH activity was assayed using pyruvate as substrate, as previously described (Cogan et al., 1981).

Statistical Analyses

Unilateral Student's tests were performed to compare the mean values of cheeses data manufactured using both strains. Principal component analysis was performed by using the StatBox software version 6.30 (GrimmerSoft, Paris, France) on the following data: Stretching; amounts of β -, γ -, α_{s1} -, and α_{s1} -I-CN determined by urea-PAGE; area of chromatographic fractions A, B, and C; SN, NPN, and nitrogen ratio

(SN-NPN/SN); PepX and PepN activities; and pH and bacterial count. Two independent sets of cheeses were manufactured and, for each data measured, 2 or 3 determinations per sample were done according to the protocol used.

RESULTS

Chemical Analyses and Viability of Starters

The composition of the cheeses H1 or H77 at the end of ripening (Table 1) fit well with the data already published on Emmental cheeses (Richoux et al., 2001, 2009). No significant differences were noted between final composition of the H77 and H1 cheeses (*P* < 0.05) regarding the following parameters: TS (%), fat-in-DM (%), moisture in nonfat substances (%), and ratio of calcium to nonfat solids. Strains H77 and H1 were enumerated during cheese manufacture. Counts of *L. helveticus* decreased from 9.23 log₁₀ (cfu/g of cheese) at d 2 to 4.14 at d 41 for H77, and from 8.99 log₁₀ (cfu/g of cheese) at d 2 to 6.06 at d 41 for H1 (Table 1). The decrease in population of both strains was in agreement with data already observed by Valence et al. (2000).

Bacterial Cell Lysis

Bacterial cell lysis was measured by assaying intracellular LDH released into the cheese citrate extracts (Figure 1). Lower LDH activity was observed in H77 cheeses throughout ripening time as described by Valence et al. (2000). As a consequence of the lysis, other intracellular enzymes, such as aminopeptidases, were also released and 2 were assayed: PepX and PepN. Activity of PepX was 1.6 times lower in H77 cheese than in H1 cheese, whereas that of PepN was 1.5 times higher in H77 cheese than in H1 cheese. However, PepN activity was 3 to 7 times lower than PepX activity in H77 and H1 cheeses, respectively.

Extent of Proteolysis

The degree of cheese proteolysis was assessed by quantifying the nitrogen fractions; that is, TN, SN, and 12% NPN—these latter 2 expressed as percentage of the TN (Table 1). The content of SN increased from 4.87 to 17.93% from 0 to 41 d of ripening in H77 cheese, and from 5.52 to 19.52% in H1 cheese (i.e., 1.1 times lower in H77 cheeses). Higher differences in the content of small peptides were observed between both cheeses as shown by the ratio NPN/SN (Table 1). Thus, in H77 cheeses the small peptides represented 44% the soluble peptides produced at d 1 and increased to only 52.2% at d 41 of ripening. Consequently, the remaining

Table 1. Composition of Emmental cheeses manufactured using strains of *Lactobacillus helveticus* ITGLH77 or ITGLH1 as starter (n = 2) and evaluated after ripening for 41 d

Strain and parameter ¹	Cold room (12°C)				Warm room (24°C)				
	d 1	d 2	d 6	d 13	d 20	d 23	d 27	d 34	d 41
Strain ITGLH1									
pH	5.10 ± 0.01	5.21 ± 0.01	5.32 ± 0.03	5.34 ± 0.02	5.36 ± 0.03	5.33 ± 0.03	5.34 ± 0.01	5.41 ± 0.03	5.44 ± 0.01
Lactobacilli counts (Log ₁₀ cfu/g cheese)	—	8.99 ± 0.18	7.72 ± 0.21	7.26 ± 0.54	7.06 ± 0.12	7.01 ± 0.38	6.19 ± 0.15	6.16 ± 0.09	6.06 ± 0.03
SN/TN (%)	5.52 ^a ± 0.11	6.29 ^a ± 0.70	7.11 ^a ± 0.30	8.03 ^a ± 0.14	8.93 ^a ± 0.49	10.72 ^a ± 0.12	12.79 ^a ± 0.25	15.59 ^a ± 0.39	19.52 ^a ± 0.08
NPN/TN (%)	2.06 ^a ± 0.36	3.40 ^a ± 0.18	—	4.81 ^a ± 0.22	5.66 ^a ± 0.33	6.87 ^a ± 0.05	8.43 ^a ± 0.56	11.11 ^a ± 0.09	14.41 ^a ± 0.20
NPN/SN (%)	37.4 ^a ± 7.4	51.3 ^a ± 3.2	—	59.9 ^a ± 3.8	63.4 ^a ± 0.3	64.1 ^a ± 0.3	65.9 ^a ± 3.0	71.3 ^a ± 2.3	73.8 ^a ± 0.7
Total solid (%)									60.80 ^a ± 0.50
FDM (%)									46.00 ^a ± 0.40
MNFS (%)									54.30 ^a ± 0.70
CNFS (%)									3.10 ^a ± 0.04
Strain ITGLH77									
pH	5.11 ± 0.01	5.18 ± 0.02	5.31 ± 0.04	5.35 ± 0.02	5.38 ± 0.04	5.35 ± 0.03	5.31 ± 0.03	5.40 ± 0.03	5.42 ± 0.01
Lactobacilli counts (Log ₁₀ cfu/g cheese)	—	9.23 ± 0.05	8.88 ± 0.12	8.82 ± 0.42	8.26 ± 0.12	7.13 ± 0.38	6.81 ± 0.16	5.00 ± 0.06	4.14 ± 0.17
SN/TN (%)	4.87 ^a ± 0.19	5.59 ^a ± 0.48	6.77 ^a ± 0.66	6.86 ^b ± 0.05	7.78 ^b ± 0.65	9.18 ^b ± 0.24	10.57 ^b ± 0.65	14.30 ^b ± 0.61	17.93 ^b ± 0.24
NPN/TN (%)	1.42 ^b ± 0.00	2.26 ^b ± 0.17	—	2.95 ^b ± 0.02	3.52 ^b ± 0.32	4.09 ^b ± 0.36	4.95 ^b ± 0.40	7.39 ^b ± 0.01	9.37 ^b ± 0.43
NPN/CN (%)	29.2 ^a ± 1.2	40.4 ^b ± 0.4	—	43.0 ^b ± 0.6	45.2 ^b ± 0.3	44.5 ^b ± 2.8	46.8 ^b ± 0.8	51.7 ^b ± 2.3	52.3 ^b ± 3.1
Total solid (%)									60.60 ^a ± 0.50
FDM (%)									45.80 ^a ± 0.04
MNFS (%)									54.50 ^a ± 0.07
CNFS (%)									2.90 ^a ± 0.03

^{a,b}Means comparing H1 and H77 cheese composition at each ripening time with different superscripts differ ($P < 0.05$).

¹SN/TN = pH 4.6-soluble N as a percentage of total N; NPN/TN = 12% TCA-soluble N as a percentage of total N; NPN/SN = 12% TCA-soluble N as a percentage of pH 4.6-soluble N; FDM = fat-in-DM; MNFS = moisture in nonfat substances; CNFS = calcium in nonfat solid.

Figure 1. Changes in intracellular enzyme activities released during ripening in citrate cheese extracts of the experimental Emmental cheeses using the strains of *Lactobacillus helveticus* ITGLH1 and ITGLH77: (A) lactate dehydrogenase activity (LDH) and (B) aminopeptidase (PepX and PepN) activities.

peptides, 47.8%, were composed of peptides insoluble in TCA 12%, which corresponded to hydrophobic peptides having a size over 20 amino acids, according to Yvon et al. (1989). Conversely, the NPN/SN ratio in H1 cheeses varied greatly, from 37% at d 1 up to 73.8% at d 41 (Table 1), showing a higher production of NPN in this cheese and indicating a different general pathway for the casein degradation.

Casein Hydrolysis

Primary proteolysis of α_{s1} - and β -CN, estimated by urea-PAGE, is shown in Figure 2. More than half of the α_{s1} -CN was degraded at the end of ripening (i.e., 54 and 52% in H77 and H1 cheeses, respectively; Figure 2A), whereas β -CN was less degraded, only 39 and 43% in H77 and H1 cheeses, respectively (Figure 2B). The α_{s2} -CN was completely hydrolyzed in both cheeses at

the end of ripening (data not shown). No differences were observed between the kinetics of β -CN hydrolysis for both types of cheese. In contrast, differences were shown for α_{s1} -CN with a linear decrease in H1 cheeses and a 2-step hydrolysis in H77 cheeses that can be described by the 2 following equations: $Y_{1(\alpha_{s1}\text{-CN})\text{H77}} = -0.07 x_1 + 38.42$ ($r^2 = 0.30$) between d 1 and d 20 (cold room), and $Y_{2(\alpha_{s1}\text{-CN})\text{H77}} = -0.88 x_2 + 52.33$ ($r^2 = 0.96$) between d 20 and d 41 (warm room). However, such a difference was not significant anymore at d 41 in terms of residual percentage of α_{s1} -CN in both cheeses.

Peptide Analyses

Insoluble Peptides. The production of α_{s1} -I-CN was detected by urea-PAGE after 20 d of ripening (Figure 2A) and was similar for both cheeses. Such a low production of α_{s1} -I-CN could be explained first by the inactivation of the rennet activity, which is the main enzyme hydrolyzing the bond Phe₂₃-Phe₂₄, and second by a higher pasteurization temperature (78°C), which could have also reduced the residual activity of the milk

Figure 2. Changes in casein hydrolysis and oligopeptide production during ripening of Emmental cheeses manufactured using the strains of *Lactobacillus helveticus* ITGLH1 and ITGLH77: (A) solid line = α_{s1} -casein; dashed line = α_{s1} -I-casein; (B) solid line = β -casein; dashed line = γ -casein.

endogenous acid proteinase, the cathepsin D (Hayes et al., 2002). Therefore, the production of α_{s1} -I-CN could be mainly attributed to the lactobacilli starters.

As expected in a plasmin-type cheese, γ -CN were already present at the beginning of ripening (Figure 2B). Their concentration increased from 8 to 20% from the beginning of ripening in H77 cheeses, two times more than that observed in H1 cheeses. As the same milk was used for both types of cheeses, the lower γ -CN concentration in H1 cheeses could be due to further degradation of these fragments by either proteinases or peptidases of H1.

Soluble Peptides. The peptides present in WSE of cheese using both strains of *L. helveticus* were analyzed by Tris/Tricine SDS-PAGE (Figure 3) and reversed phase chromatographic profiles (Figure 4).

For the peptides in the range from 2.5 to 25 kDa of apparent molecular mass (Figure 3), main bands were common to both cheeses, exerting various intensities. Bands labeled a, b, and c were only present in cheese WSE of H77 cheeses, bands labeled d and e were only present in water soluble extracts of H1 cheeses (Figure 3). However, the main difference observed between both profiles was the higher intensity of some bands in the aqueous phases of cheese made with H77 throughout ripening, such as large-sized peptides (bands a, f, g, and

h; Figure 3). As previously reported by Gagnaire et al. (2004), bands >30 kDa represent whey proteins (such as lactoferrin and BSA) and intracellular contents of bacterial cells, which appeared to be slightly more intense in the aqueous phases of H1 cheeses throughout ripening, in agreement with higher level of LDH released in cheese.

The RP-HPLC profiles of the cheese WSE showed changes throughout the ripening time (Figure 4, part A and B). The most hydrophilic peptides were the first eluted from the column (fraction A), and the most hydrophobic peptides were eluted in fraction C; peptides in-between were eluted in fraction B. At d 1, fraction C (hydrophobic peptides) represented more than a half of the total area for H1 cheeses and two-thirds for H77 cheeses (Figure 4, part C). The percentage of area of this fraction decreased throughout the ripening time for both cheeses, but still remained above 45% for H1 cheeses and 60% for H77 cheeses at the end of ripening (Figure 4, part C). A subsequent increase of both fractions A and B was found for both cheeses. Nevertheless, regarding the H77 cheeses, fraction A did not exceed 15% throughout the ripening time, whereas fraction B reached 25%, suggesting a higher production of peptides with average hydrophobicity. In the case of H1, fraction A increased to 29% of the total peak area, whereas fraction B reached 25% of the total peak area at the end of the ripening, showing a greater production of hydrophilic peptides in agreement with the percentage of NPN found (Table 1).

Figure 3. Tris/tricine SDS-PAGE electrophoregrams of water-soluble extract of Emmental cheeses manufactured using the strains *Lactobacillus helveticus* ITGLH1 and ITGLH77. MM = molecular mass marker. Letters a to h represent bands that were differentially present in the water-soluble extracts of Emmental cheeses: bands d and e were more intense or present only in H1 cheeses and the other lettered bands in H77 cheeses.

Peptide Identification

Peaks from fractions A, B, and C of cheese WSE at d 1, 13, and 41 were collected and identified by RP-HPLC coupled online with MS/MS (Supplemental Tables S1 to S3; available online at <http://www.journalofdairyscience.org/>). Very few peptides were identified in fraction A of cheese WSE, which contained mainly amino acids or peptides <350 Da corresponding to the minimum selected masses. More than 100 peptides were identified in fraction B, and only around 30 were identified in fraction C of both cheeses. The peaks in fraction C are usually not well separated by RP-HPLC and therefore more difficult to identify by mass spectrometry.

The number of total peptides identified increased during ripening for both cheeses. At d 1 in H1 cheeses 70% of the identified peptides arose from β -CN, whereas 19.8% were from α_{s1} -CN, and 9.3% from α_{s2} -CN. This distribution changed during ripening, as β -CN represented 58.8%, α_{s1} -CN 27.5%, and α_{s2} -CN 13.7%. In the case of H77 cheeses, β -CN was also the casein from which the identified peptides mainly derived (i.e., 54%

Figure 4. Reverse phase-HPLC profiles of water-soluble extracts during ripening of cheese manufactured with *Lactobacillus helveticus*: (A) strain ITGLH1; (B) strain ITGLH77; and (C) changes in areas of fractions (Fr) A, B, and C represented as percentage at d 1 and 41 of ripening for both types of cheeses. Rt = retention time.

at d 1); nevertheless, the number of identified peptides from α_{s1} -CN was higher compared with H1 cheeses, and varied from 28.1% at d 1 to 37.7% at d 41. The number of peptides derived from α_{s2} -CN was higher in H77

cheeses but remained constant throughout aging time (i.e., 18% at d 1 and 16% at d 41).

Regardless of casein origin and aging time, the number of the large peptides containing more than 20 ami-

no acid residues was 1.33 times higher in H77 cheeses. Interestingly, even less numerous compared with those from β -CN, the peptides containing more than 20 amino acid residues arising from α_{s1} - and α_{s2} -CN were only present in H77 cheeses. Half of the large peptides arising from β -CN were present in both cheeses.

Cleavage Sites on Caseins

Profiles of the peptide splitting sites greatly varied between both types of cheeses in terms of regions in which peptide bonds were cleaved. Thus, peptide bonds that were split were distributed all along the β -CN sequence (Figure 5, A and B) at d 1, 13, and 41, for both types of cheese. As expected, the highest number of common splitting sites was found for plasmin sites at amino acids Lys₂₈, Lys₂₉, Lys₁₀₅, and Lys₁₀₇, but more surprisingly at Phe₅₂, Asn₆₈, Gln₇₂, Leu₁₉₂, and Tyr₁₉₃, which correspond to splitting sites specific for proteinases of *L. helveticus* (Sadat-Mekmene et al., 2011). Their frequency of cleavage was higher in H77 cheeses at d 1 and 13. At d 41 the number of splitting sites increased, reflecting the activity of other types of enzymes, such as aminopeptidases released through lysis of bacterial cells.

Identified peptides derived from α_{s1} -CN were present into more limited zones of the casein sequence, mainly in the N- and C-terminal regions in both cheeses; only a few peptides arose from the region between Arg₉₀ and Gly₁₂₆. Regarding the occurrence of the splitting sites at the beginning of the ripening (Figure 5, C and D), the most frequent peptide bond split was observed between the amino acid residues Phe₂₃-Phe₂₄, corresponding to the main action of the coagulant, cathepsin D, and proteinases of *L. helveticus*. Some splitting sites that can be attributed to proteinases of *L. helveticus* are located between Leu₉₂ and Lys₁₂₄. Some differences were observed in this region between H1 and H77 cheeses, with the presence in H77 cheeses of α_{s1} -CN(93–102) at d 1 and the presence of α_{s1} -CN(114–123), α_{s1} -CN(11–22), α_{s1} -CN(112–123), α_{s1} -CN(10–22), and α_{s1} -CN(112–124) at d 13 and not in H1 cheeses; only α_{s1} -CN(108–114), α_{s1} -CN(32–40), and α_{s1} -CN(106–114) were identified in H1 cheeses at d 13.

Peptides were identified mainly in the C-terminal end (between Tyr₁₀₀ and Leu₂₀₇) of α_{s2} -CN in both cheeses (Figure 5 E and F). The frequency of splitting was below 5 per peptide bond, confirming the few number of peptides identified from this casein in the WSE. However, the number of splitting sites observed in the C-terminal part of the α_{s2} -CN in the H77 cheeses was directed against 16 amino acids, whereas only 9 were concerned in H1. Splitting sites were slightly different

between both cheeses, with α_{s2} -CN(67–74) observed only in H1 cheeses at d 1, whereas α_{s2} -CN(14–24) and α_{s2} -CN(6–24) were only identified in H77 cheeses.

Surprisingly, even if most of the cleavage sites were common between both cheeses, only 33, 24, and 9 peptides from β -, α_{s1} -, and α_{s2} -CN, respectively, were the same in the aqueous phase of both cheeses at the end of ripening (Supplemental Tables S1 to S3; available online at <http://www.journalofdairyscience.org/>). The cleavage sites in common were distributed throughout the sequence in the case of β -CN, whereas they were only found in the N-terminal end of α_{s1} -CN and the C-terminal end of α_{s2} -CN.

Dynamic of Stretchability of Emmental Cheese During Ripening

The dynamic of stretchability of Emmental cheese was monitored throughout the ripening time (Figure 6A). The curve has a U-shape for both strains up to d 27 of ripening and reached a plateau thereafter. The scattering of the measures from d 1 to 6 for both cheeses could be due to the heterogeneity in the casein network at the beginning of ripening. During the aging in warm room, stretchability progressively increased during ripening for both types of cheeses. Moreover, stretching was significantly higher in H77 after cheeses were moved to the warm room. At the end of ripening (d 41), strands were 100 mm longer for the H77 cheeses.

Confocal Microstructure of the Strands of Emmental Cheeses

After stretching, the newly formed strands were visualized by confocal laser scanning microscopy at d 1 and 41 of ripening for both cheeses. Representative views of the strands are shown at d 1 for H77 cheeses (Figure 6B, A and B) and for both cheeses at d 41 (Figure 6B, C and D). Sections of the strands showed that paracasein matrix (in green/light gray strands) formed the continuous phase and was in the form of linear fibers. The fat (red/dark grey roughly ovoid objects) occurred as elongated pools trapped between the paracasein fibers, exhibiting a similar orientation as the paracasein matrix. Such pools of fat are in agreement with the coalescence of liquid fat droplets, formed as a consequence of damage to the fat globule membrane during Emmental cheese making process (Lopez, 2005; Lopez et al., 2007). Most of the fat coalesced and was exuded during heating and melting of the cheeses before stretching. Some fat melting and rising to the surface was also noticed when the strands were submitted to the laser of the confocal microscope. Bacterial colonies

Figure 5. Distribution of cleavage sites along the casein sequences during cheese aging. For each peptide identified by liquid chromatography-electrospray ionization-tandem MS, each time a peptide bond is cleaved a score of 1 is given to the amino acid corresponding to the N or C terminal end of the peptide produced, whereas the score of 0 is given to all other amino acids in the casein sequence. The number of times that a peptide bond has been identified as cleaved is summed and represented on the figure along the casein sequence: A and B = β -casein; C and D = α_{s1} -casein; and E and F = α_{s2} -casein, where A, C, and E show results for the H1 cheeses and B, D, and F for the H77 cheeses.

Figure 5 (Continued). Distribution of cleavage sites along the casein sequences during cheese aging. For each peptide identified by liquid chromatography-electrospray ionization-tandem MS, each time a peptide bond is cleaved a score of 1 is given to the amino acid corresponding to the N or C terminal end of the peptide produced, whereas the score of 0 is given to all other amino acids in the casein sequence. The number of times that a peptide bond has been identified as cleaved is summed and represented on the figure along the casein sequence: A and B = β -casein; C and D = α_{s1} -casein; and E and F = α_{s2} -casein, where A, C, and E show results for the H1 cheeses and B, D, and F for the H77 cheeses.

Figure 5 (Continued). Distribution of cleavage sites along the casein sequences during cheese aging. For each peptide identified by liquid chromatography-electrospray ionization-tandem MS, each time a peptide bond is cleaved a score of 1 is given to the amino acid corresponding to the N or C terminal end of the peptide produced, whereas the score of 0 is given to all other amino acids in the casein sequence. The number of times that a peptide bond has been identified as cleaved is summed and represented on the figure along the casein sequence: A and B = β -casein; C and D = α_{s1} -casein; and E and F = α_{s2} -casein, where A, C, and E show results for the H1 cheeses and B, D, and F for the H77 cheeses.

Figure 6. (A) Changes in stretchability during ripening of Swiss-type cheese manufactured using 2 strains of *Lactobacillus helveticus*: ITGLH1 and ITGLH77; (B) microstructures of the strands of Emmental cheeses manufactured using the strains of *L. helveticus* ITGLH1 and ITGLH77 by confocal laser scanning microscopy. Protein network is represented in green (light gray) strands, fat in red (dark gray) roughly ovoid objects inside the protein network, and bacterial colonies in light green (very light gray) small spheres located around the fat pools. Strands newly formed on d 1 were visualized with a 40× objective: (A) H77 cheese, (B) 4× zoom of a zone of the same cheese. Strands were visualized again at d 41 with a 10× objective: (C) H77 cheese, and (D) H1 cheese. Color version available in the online PDF.

were found at d 1 of ripening surrounding or in close contact with fat (Figure 6B, part B).

Principal Component Analysis on Emmental Cheeses

Principal component analysis allowed us to summarize the information in 2 dimensions, accounting 86% of the total variation. Figure 7A shows the scores of the individuals and Figure 7B a correlation circle of the variables. Principal component 1 explained most of the variation (78%) and was related to cheese aging (Figure 7A). The young cheeses scored negatively and were correlated with the high presence of caseins (Figure 7B). The old cheeses scored positively and were well separated on the principal component 2 axis, which explained only 8% of the variation and may reflect the change in the peptide composition. At the end of ripening, H77 cheeses were correlated with the higher content of γ -CN and hydrophobic peptides (fraction C); H1 cheeses were correlated with smaller and hydrophilic peptides (% NPN, fraction A) and a greater extent of autolysis. When stretching values were projected as supplementary data (available online at <http://www.journalofdairyscience.org/>), a positive correlation was observed with γ -CN, fractions B and C (large/medium and hydrophobic peptides) in agreement with the composition of H77 cheeses; and a negative correlation with PepX activity, the NPN/TN ratio, and fraction A (small peptides and amino acids) corresponding to H1 cheeses on the principal component 2 axis (Figure 7B).

DISCUSSION

A previous study on commercial cheeses (Gagnaire et al., 2001) showed that the thermophilic lactobacilli play a significant role in the Emmental proteolysis through the action of their CEP. Moreover, lactobacilli were shown to change the functional properties of the cheese (Oommen et al., 2002). More recently, the activities and specificities of a panel of 15 strains of *L. helveticus* were characterized under in vitro conditions, taking into account the number of proteinases synthesized by the strains (Genay et al., 2009). Rapid hydrolysis of pure β -CN was observed for all tested strains having 1 (Prth2) or 2 CEP (Prth and Prth2), and the kinetics of pure α_{s1} -CN hydrolysis was enhanced in presence of both CEP (Sadat-Mekmene et al., 2011). The number of casein-derived peptides identified when lactobacilli were grown in milk was severely reduced compared with that observed under in vitro conditions (Sadat-Mekmene et al., 2011). Therefore, knowing the distribution of the CEP and their specificities, that

Figure 7. Principal component (PC) analysis showing the first 2 principal components of proteolytic data of Emmental cheeses manufactured using the strains of *Lactobacillus helveticus* ITGLH1 and ITGLH77: (A) individual attributes, and (B) variables. SN = pH 4.6-soluble nitrogen; PepN and PepX= activity of intracellular aminopeptidase from *L. helveticus* released in cheese water-soluble extract. Stretching data (gray dot) are projected as supplementary data (available online at <http://www.journalofdairyscience.org/>).

study aimed to investigate the in situ proteolytic activity of *L. helveticus* for 2 strains having, respectively, 1 or 2 CEP: ITGLH77 (Prth2) and ITGLH1 (Prth2 and Prth at least; Broadbent et al, 2011) in experimental Swiss-type cheeses.

As lactobacilli, specifically *L. helveticus*, were shown to be responsible for enhanced stretching properties of Swiss-type cheeses (Richoux et al., 2009), an attempt was made to clarify the complex relationships between proteolysis due to *L. helveticus* and stretchability. Proteolysis was 1.5 times less extensive in cheese manufactured using H77 (having 1 CEP) as starter than using H1 (having 2 CEP), as shown by the NPN/TN ratio. This lower extent of proteolysis was especially noticeable for α_{s1} -CN compared with β -CN. Proteolysis was restricted to accessible parts of the casein sequences, as shown in Figure 5.

The lower extent of proteolysis in H77 cheeses also resulted in the accumulation of hydrophobic peptides, present in fractions B and C of the chromatographic profile, in the urea-PAGE (γ - and α_{s1} -I-CN or derived), and in Tris-tricine SDS-PAGE, where some oligopeptides >10 kDa were observed. Regardless of the casein origin, the number of the peptides identified by MS/MS containing more than 20 AA was 1.3 times higher in H77 cheeses, and the peptides containing more than 20 amino acid residues arising from α_{s1} - and α_{s2} -CN were only present in H77 cheeses.

However, differences between H1 and H77 cheeses cannot be attributed solely to the CEP activity, but also to the overall proteolytic enzymes. In fact, lysis of the bacterial cells was found as early as d 2 of aging, releasing part of the intracellular-located enzymes into the cheese aqueous phase. This lysis was much lower for the H77 cheeses than for the H1 cheeses, coherent with the lower amount of the fraction NPN/TN and the area of the peaks in the chromatographic fraction A, as well as the lower aminopeptidase and LDH activities observed.

Regarding the peptides produced, the cleavage sites were mainly restricted to those that were common for both strains and shown in a previous study (Sadat-Mekmene et al., 2011). The fact that more peptides were identified in H77 cheese could be related to the lesser degree of lysis, which prevented peptides from being further hydrolyzed to amino acids. This was clearly shown in α_{s1} -CN derived peptides (Supplemental Table S2; available online at <http://www.journalofdairyscience.org/>).

High stretchability was observed in both cheeses regardless of *L. helveticus* strains used, as seen in other studies (Guinee, 2002; Richoux et al., 2009). However, the dynamics of stretchability showed significant differences between both cheeses after d 20 of ripening—the H77 cheeses having the longest length of strands. The changes in nitrogen composition and proteolysis coincided with marked increases in the stretching properties of both cheeses; thus, the higher the proteolysis, the lower the stretching properties, as reported previously

for mozzarella and Kashakaval (Guinee, 2002). However, a balance in the extent of proteolysis should also be reached to obtain longer strand length, as shown by the increase in stretchability observed during aging of H77 cheeses. Confocal laser scanning microscopy penetrates the cheese surface to visualize thin optical sections without disturbing the internal structure. It was the first time, to the best of our knowledge, that confocal microscopy was applied to strands of Emmental cheeses. The strands showed similar microstructure as mozzarella strands. Their features were not different enough to discriminate both cheeses at the optical level. Only differences with aging were found, with the fibers becoming more voluminous likely because of changes in peptide composition and, therefore, in hydration of the paracasein matrix.

The principal component analysis reinforced the initial hypothesis that stretchability was correlated with a peculiar type of peptides (i.e., hydrophobic peptides present in fractions C and B). Thus, these hydrophobic peptides, among them large peptides from α_{s1} - and α_{s2} -CN only found in the H77 cheeses, could be involved in forming the fibers of caseins through hydrophobic bonds between intact caseins and large peptides (Richoux et al., 2009). Stretchability was also correlated to a lower value of proteolysis, which can be attributed to the presence of 1 CEP of the lactobacilli strains or lower release of intracellular peptidases into the cheese aqueous phase. As both phenomena cannot be distinguished, it remains difficult to definitively establish the role of the CEP of lactobacilli in the stretching properties of the Emmental cheeses. The role of CEP in the production of peptides, responsible for this technofunctional property, could be ascertained only by the use of mutants deficient in 1 or 2 CEP, which are not available at the present time.

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