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Production of peptides and free amino acids in a sterile extract describes peptidolysis in hard-cooked cheeses

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ABSTRACT

Hard cooked cheeses are mostly manufactured with lactic starters of *Lactobacillus helveticus*, which constitute a major proteolytic agent in the food. In this work, we assessed the proteolysis produced by enzymes of two strains of *L. helveticus* in a new cheese model, which consisted of a sterile substrate prepared with hard-cooked cheeses, and identified the time of ripening when main changes in proteolysis are produced. The extract, a representative model of the aqueous phase of the cheeses, was obtained from Reggianito cheeses of different ripening times (3, 90, and 180 days) made with starters composed of the strains tested, either SF138 or SF209. To obtain the substrate, the cheese was extracted with water, then centrifuged and the aqueous phase was sterilized by filtration through membrane (0.45 μm). The substrates were incubated at 34 °C during 21 days; samples were taken at 0, 3, 7, 14, and 21 days. Sterility was verified by plating samples on skim milk agar and incubating at 37 °C for 48 h. Proteolysis was determined by liquid chromatography of soluble peptides and free amino acids. Great variation in peptide profiles was found as incubation progressed in cheese extracts, which evidenced that proteases and peptidases from the starter were active and able to degrade the proteinaceous material available in the extracts. The extracts derived from cheeses with *L. helveticus* SF138 showed low production of peptides and a notable increase in free amino acids content during incubation. *L. helveticus* SF209, on the contrary, caused an increase on soluble peptides, but the free amino acids accumulation was lower than in the first case, which suggested that *L. helveticus* SF209 had either a low peptydolitic activity or produced an intense amino acids breakdown. This trend was more evident for extracts prepared with 90-day-old cheeses. It was concluded that the strains of *L. helveticus* assayed showed potentially complementary proteolytic abilities, as SF209 was able to provide a continuous replenishment of peptides during incubation, while SF138 increased their hydrolysis to free amino acids. The extract was an appropriate medium to model hard cooked cheese ripening in short periods of time.

1. Introduction

Hard-cooked cheeses were introduced in Argentina in the XIX century by Italian immigrants. Unlike "generic parmesan" or "parmesan-type" cheeses made worldwide, Argentinean hard cheeses, such as Reggianito, have reached some degree of differentiation (Zannoni, Bertozzi, & Hunter, 1994). This accomplishment is mainly due to milk composition, autochthonous microbiota, and changes in cheese-making technology (Candioti et al., 2002; Zalazar, Meinardi, & Hynes, 1999). Differentiation from commodity cheeses may help to valorize and protect Argentinean cheeses, as it has been already done for other food products, for example, Spanish Cavas wine.

In this way, efforts have been made to isolate and characterize lactic acid bacteria strains from the whey cultures traditionally used in the production of Reggianito, in which *Lactobacillus helveticus* is the dominant species, in order to protect the product's identity and to provide suitable and diverse starter cultures. Several strains have been identified and tested by *in vitro* and *in situ* experiments. Quiberoni, Tailliez, Quénee, Suárez, and Reinheimer (1998) isolated and identified 25 strains of *L. helveticus* by biochemical and molecular methods; these strains were also characterized by the authors in *in vitro* studies to describe their genetic diversity and technological features, like proteolytic and acidifying activities, salt tolerance, and phage sensitivity. The best performing strains were also tested as starters in cheese-making trials, both as single cultures or in two- or three-strain mixed cultures (Candioti et al., 2002; Hynes, Bergamini, Suárez, & Zalazar, 2003; Perotti, Bernal, Meinardi, & Zalazar, 2005).

L. helveticus is a major proteolytic agent in hard cheeses and can produce a high level of middle-sized and small peptides, as well as

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Table 1
Composition of crude extracts of Reggianito cheeses (means ± standard deviation of all types of extracts).

	Soluble extract
pH	5.20 ± 0.05
NaCl (%)	2.17 ± 0.11
Total protein (%)	7.07 ± 0.16

free amino acids, during cheese ripening (Chopard, Schmitt, Perreard, & Chamba, 2001; Hannon, Kilcawley, Wilkinson, Delahunty, & Beredsford, 2007; Hynes et al., 2003; Mäyrä-Mäniken & Bigret, 1998). This contribution varies highly from one strain to another; *L. helveticus* strains differentiate not only in the intensity of their caseinolytic activities but also in the specificity of their endopeptidases (Jensen, Vogensen, & Ardö, 2009). In fact, strains with equivalent proteolytic activities assessed by quantitative non-specific tests have shown significantly different hydrolysis patterns on peptides such as α_{s1}(f1-23) (Oberg, Broadbent, Strickland, & McMahon, 2002).

In agreement with this heterogeneity in proteolytic activity of *L. helveticus*, it is known that cheeses made with diverse starter strains can show very different levels and patterns of proteolysis (Chopard et al., 2001; Hynes et al., 2003; Kenny, FitzGerald, O'Cuinn, Beresford, & Jordan, 2006). However, it is less known when in ripening the proteolysis processes of cheeses made with differently proteolytic *L. helveticus* strains begin to set apart. Besides, the influence of *L. helveticus* strains on proteolytic and peptidolytic patterns of hard cooked cheeses has been less studied than their effect on the overall degree of proteolysis. Both aspects of the proteolytic changes during ripening of hard cooked cheese are interesting, as proteolysis provides substrates for flavor formation and contribute to texture (Fox, 2003, Hannon et al., 2007). In this way, it would be helpful to know which kind of flavor precursors a given *L. helveticus* strain is able to provide and at which step of ripening do their concentrations increase.

Even though cheese-making trials are often required to make a decision about desirable starters, they are quite long experiments, as 180 days is the minimum time required for Reggianito cheese ripening (Código Alimentario Argentino, 2006). Different approaches have been proposed to model cheese ripening and study biochemical activities of lactic cultures in simple, reliable ecosystems; most accepted models are miniature semi-hard or Cheddar cheeses, cheese slurries, and Ch-easy®, among others (Farkye, Madkor, & Atkins, 1995; Hynes, Ogier, & Delacroix-Buchet, 2000; Muehlenkamp-Ulate & Warthesen, 1999; Shakeel-Ur-Rehman, McSweeney, & Fox, 1998, Smit, Braber, Van Spronsen, Van Den Berg, & Exterkate, 1995). Cheese slurry systems have been proposed to rapidly evaluate the influence of starters or enzymes in cheeses. The incubation of slurries for short times (1–2 to 15–20 days) at higher temperature than that of cheese ripening (ca. 30 °C) is usually carried out to promote accelerated biochemical changes (Farkye et al., 1995; Muehlenkamp-Ulate & Warthesen, 1999). Several methods for slurry preparation have been assayed: they have been obtained from fresh curds of cheeses traditionally manufactured (Muehlenkamp-Ulate & Warthesen, 1999), from starter-free curd (Farkye et al., 1995, Lacroix, St-Gelais, Champagne, Fortin, & Vuillemard, 2010), or from curd made with UHT-treated milk (Wijesundera, Roberts, & Limsowtin, 1997). For hard cheeses, examples of model systems are less common. A few studies have been carried out in miniature hard-cooked cheeses (Hynes, Aparo, & Candioti, 2004; Mucchetti, Locci, Massara, Vitale, & Neviani, 2002). A soluble extract that represents the aqueous phase of the food, sterilized by direct UHT method, has been proposed for Cheddar cheese (Crow, Curry, & Hayes, 2001). A Parmigiano Reggiano cheese extract has also been obtained to provide an appropriate culture medium for bacterial species occurring in long ripened cheeses and to assess their aminopeptidase activities (Gatti et al., 2008; Neviani, De Dea Lindner, Bernini, & Gatti, 2009).

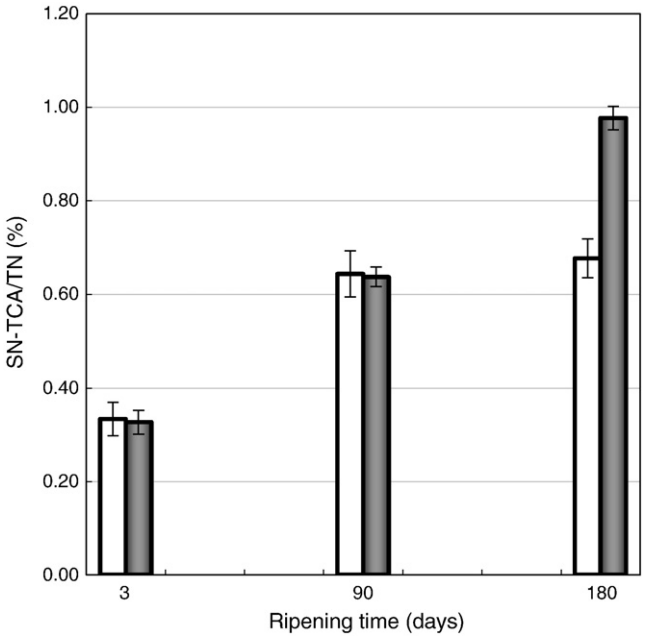


Fig. 1. Nitrogen content in fraction of cheese extracts soluble in tricholoracetic acid 12% (SN-TCA), expressed as the proportion of total N (TN), at 3, 90, and 180 days of ripening, before incubation. (□): extracts derived from cheeses manufactured with *L. helveticus* 138, (■) extracts derived from cheeses manufactured with *L. helveticus* 209. Values are mean ± standard deviation of three replicates.

In the present work, we assessed a soluble extract of hard-cheese sterilized by a non-thermal method, as a new cheese model. In comparison with previous extracts, it has the advantage of being free of microorganisms both from starter and non-starter origin, but proteins, peptides, and calcium salts are not exposed to the changes caused by strong heat treatments. In addition, the enzymes occurring in the cheese are expected to remain active.

To validate the use of this new model, we tested soluble extracts of cheeses of different age and made with different starter strains of *L. helveticus*. We investigated the changes in peptide profiles and free amino acids accumulation during incubation of the substrate.

2. Materials and methods

2.1. Strains and cheese making

L. helveticus SF138 and SF209 belong to the collection of Instituto de Lactología Industrial (INLAIN). They were isolated from whey cultures

Table 2
pH and microbial counts of total mesophilic counts of soluble extracts of Reggianito cheeses during incubation at 34 °C (means ± standard deviation of all types of extracts).

Incubation time (days)	0	3	7	14	21
pH	5.18 ± 0.04	5.15 ± 0.02	5.15 ± 0.01	5.16 ± 0.01	5.17 ± 0.02
Total mesophilic counts (CFU mL^{-1})	<10	<10	<10	<10	<10

from two different dairy plants and showed different proteolytic activities in cheese making and *in vitro* experiments (Candioti et al., 2002; Quiberoni et al., 1998).

Reggianito cheeses were obtained in the pilot plant in INLAIN according to the standard cheese-making technology. Lactic starter consisted of single cultures of *L. helveticus* SF138 or SF209 incubated in sterile whey as previously described by Candioti et al. (2002). Two 4-kg cheeses were obtained from each batch.

For each strain tested, half cheese was reserved at day 3 after cheese making, after pressing, brining, and surface-drying (young cheese, *y*). The remaining cheese was also cut in two parts, and all cheese halves were ripened at 12 °C and 80% relative moisture. At day 90, a second half was taken (mid-ripened cheese, *m*). The remaining pieces were maintained 180 days (full-ripened cheeses, *f*).

2.2. Extract

Young, mid-, and full- ripened cheeses, made with starter strains *L. helveticus* SF138 or SF209, were crushed in a manual screw mill and frozen at −20 °C up to use.

Extracts were prepared by mixing cheese and water (1:1) using a blender, after which the mix was centrifuged (20 min, 3000×*g*). The aqueous phase was recovered, filtered through paper (Whatman Nº 42) then through glass wool disposable pre-filters (Millipore, Sao Paulo, Brasil). For each type of extract, the preparation was repeated three times, and the obtained extracts were combined before their analysis and standardization. Protein content and salt concentration of the extracts were assessed by the Kjeldahl method (IDF, 1993) and Mohr titration (Kolthoff, Sandell, Meehan, & Bruckenstein, 1988), respectively; pH was measured using a pHmeter. Salt and pH were adjusted to the target values 5% and 5.20, respectively, to approach the composition of Reggianito cheese aqueous phase. The extract was sterilized by filtration through membranes of 0.45 μm of pore diameter (Millipore, Sao Paulo, Brasil).

Blank extracts were obtained, in which enzymes were inactivated by heating at 70 °C 30 min in a water bath.

2.3. Incubation, sampling and analysis

Extracts were incubated in sterile bottles at 34 °C in order to accelerate the changes during incubation and were sampled under controlled microbiological conditions at 0, 3, 7, 14, and 21 days. Sterility was checked by plating samples on skim milk agar and incubating at 37 °C for 48 h (Bude-Ugarte, Guglielmotti, Giraffa, Reinheimer, & Hynes, 2006).

Initial proteolysis of the extracts was assessed by soluble nitrogen fractions; the extracts were fractionated by the addition of trichloroacetic acid to a final concentration of 12%, and phosphotungtic acid (2.5%)–H_2SO_4 (6%), to estimate medium- to small-sized peptides nitrogen (SN-TCA) and oligopeptides and free amino acids nitrogen (SN-PTA), respectively, according to Candioti et al. (2002). Analysis was performed in triplicate.

To describe the evolution in peptide profiles and free amino acids accumulation during incubation of the extracts, peptide profiles were obtained by liquid chromatography according to Hynes et al. (2003) for all sampling times (0, 3, 7, 14, and 21 days). Individual contents of free amino acids were quantified as described by Bergamini, Hynes, Candioti, and Zalazar (2009) in samples of 0, 3, 7, and 14 days of incubation as preliminary assays showed little changes in FAA accumulation after this period.

2.4. Statistics

One-way ANOVA was applied to compare means of nitrogen values and total free amino acids content. Chromatographic data were analyzed by multivariate methods, including a fuzzy approach for data preprocessing for RP-HPLC chromatograms of peptide profiles (Piraino, Parente, & McSweeney, 2004). After that, principal components analysis (PCA) was applied to reduce dimensionality of peptide and free amino acids profiles. All tests were performed using the SPSS 10.0 software (SPSS Inc., Chicago, USA).

3. Results

3.1. Chemical composition, pH, and sterility of the extract

Nitrogen content, salt concentration, and pH of the crude extracts are shown in Table 1. The pH was near the target value and was not adjusted. Salt concentration was increased up to 5.00% with NaCl. During incubation, pH remained constant in all extracts and mesophilic bacterial development was not detected (Table 2).

3.2. Basal (Initial) proteolysis in the extracts

Figs. 1 and 2 show SN-TCA and SN-PTA, expressed as proportions of total soluble nitrogen, in the soluble extracts before incubation (time 0). Nitrogen content in both soluble fractions increased with cheese age and also was strain-dependent. Until 90 days of ripening, SN-TCA was similar in cheeses made either with *L. helveticus* SF138 or SF209, but at 180 days, cheeses with SF209 showed a significantly higher ($\alpha \leq 0.05$) content of nitrogen in this fraction (Fig. 1), which contains medium-sized to small peptides, amino acids, and smaller N compounds, such as amines/urea, and ammonium (Ardö, 1999). A similar trend was observed for SN-PTA: cheeses with *L. helveticus* SF209 had significantly higher values than *L. helveticus* SF138, at increasing ripening times (Fig. 2). SN-PTA includes very small peptides, amino acids, and smaller N compounds other than dibasic amino acids and ammonia (Ardö, 1999).

The profiles of soluble peptides of the extracts before incubation represent the basal proteolysis in the cheeses of different age; they showed the same trend verified for SN-TCA: cheeses made with *L. helveticus* SF209 had more complex profiles than cheeses made with *L. helveticus* SF138, and peak areas increased with ripening time for the former while decreased with ripening time in the later case (Fig. 3).

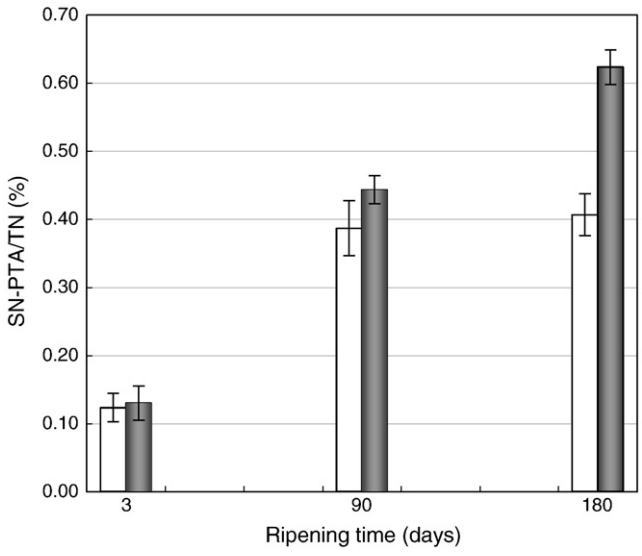


Fig. 2. Nitrogen content in fraction of cheese extracts soluble in phosphotungstic acid 2.5% (SN-PTA), expressed as the proportion of total N (TN), at 3, 90, and 180 days of ripening, before incubation. (□): extracts derived from cheeses manufactured with *L. helveticus* 138, (■) extracts derived from cheeses manufactured with *L. helveticus* 209. Values are mean ± standard deviation of three replicates.

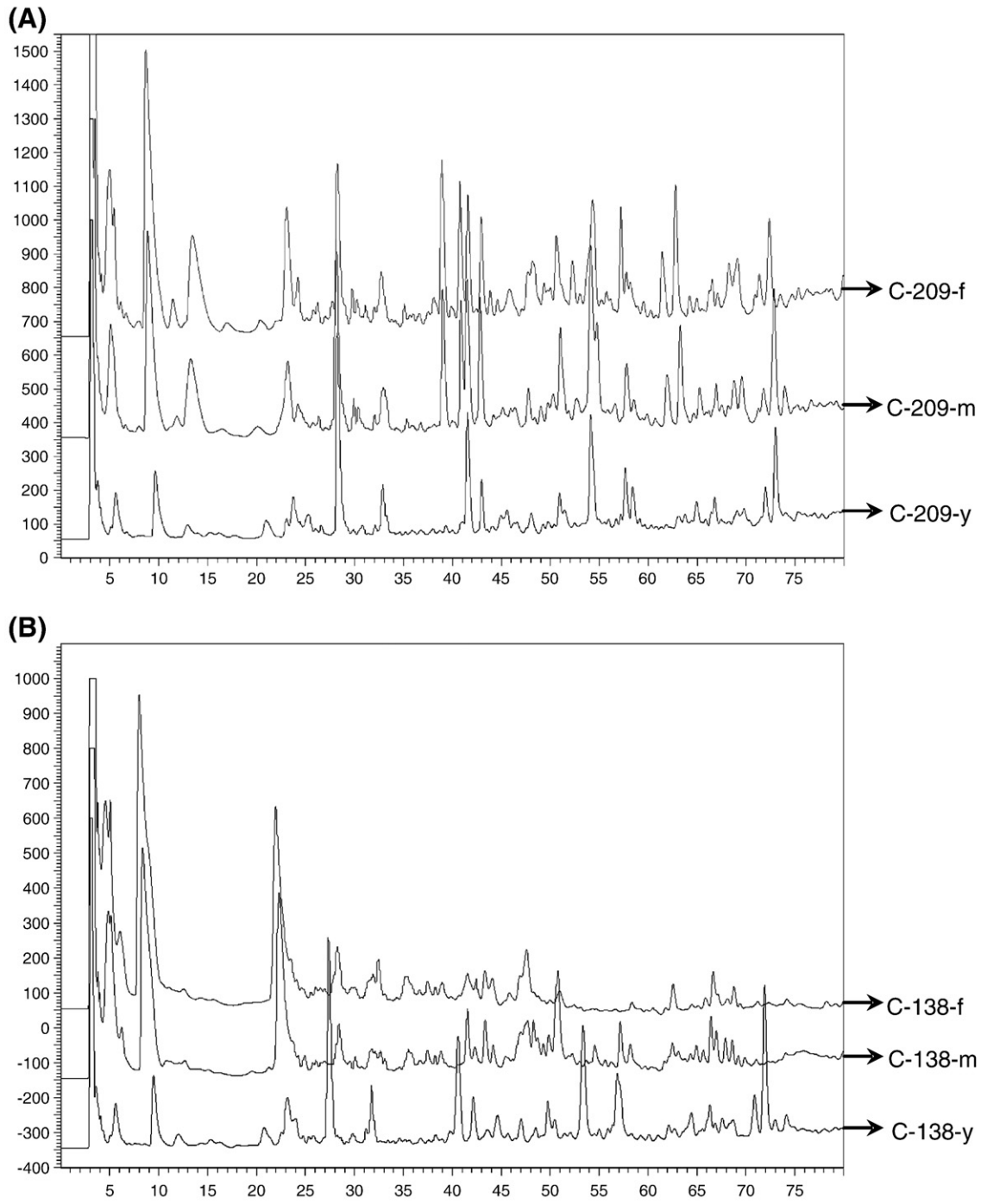


Fig. 3. Peptide profiles of extracts of cheeses manufactured with *L. helveticus* 209 (A) and *L. helveticus* 138 (B) during ripening: 3 (y), 90 (m), and 180 (f) days, before incubation.

Free amino acids total content, however, followed the opposite trend verified for SN-PTA as mid- and full-ripened *L. helveticus* SF138 cheeses had a total FAA content significantly higher than *L. helveticus* SF209, and increased more during ripening (Fig. 4). Level of total FAA in extracts from cheeses with *L. helveticus* 138 after 90 days of ripening was more than twice of that of cheeses with *L. helveticus* 209.

3.3. Peptidolysis during incubation of the extracts

Peptide profiles of blank extracts, which were heated to stop enzymatic reactions, did not show any changes during incubation, except for a group of peaks whose elution times were comprised between 65 and 77 min (Fig. 5). The increase in the areas of these hydrophobic peptides is most likely due to the action of the milk indigenous protease, plasmin (Kelly & O'Donnell, 1998). On the other hand, peptide profiles of non-heated extracts changed remarkably during incubation at 34 °C, which indicates that the proteases and peptidases from *L. helveticus* SF138 and SF209 continued to be active in the substrates (an example is shown in Fig. 6).

Evolution of peptidolysis in the non-heated extracts incubated for 0, 3, 7, 14, or 21 days was described by PCA of chromatograms of young (Fig. 7A), mid- (Fig. 7B), and full- ripened cheeses (Fig. 7C). In

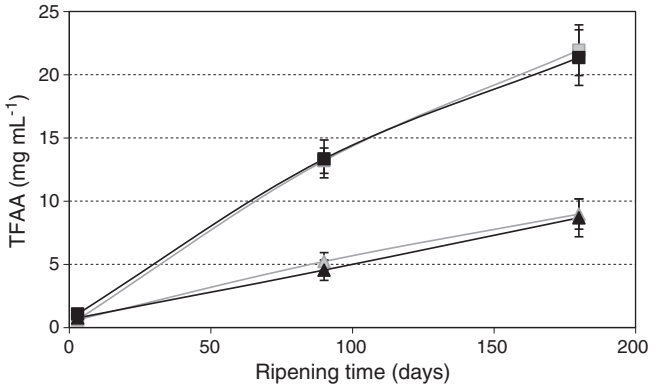


Fig. 4. Total amount of FAA (mg mL^{-1}) in soluble extracts before incubation, derived from cheeses manufactured with *L. helveticus* 209 (▲) and *L. helveticus* 138 (■) during ripening. Blanks (thermally treated): grey symbol and line. Extracts (non-thermally treated): black symbol and line. Values are mean ± standard deviation of three replicates.

order to compare changes in peptide profile during extract incubation and cheese ripening, scores of initial extracts were included in all the graphs. Also, evolution of peptidolysis during incubation and ripening is shown with arrows in the figures. The differences between the starter strains of *L. helveticus* were much more evident by incubating extracts of mid- and full-ripened cheeses (Fig. 7B and C). Although incubation caused large changes in the proteolysis of the extracts of young cheeses, peptide hydrolysis in both extracts evolved similarly. Still, after 14 days of incubation, samples of cheeses with *L. helveticus* 138 were distanced from those of *L. helveticus* 209 (Fig. 7A). In the case of mid- and full-ripened cheeses, the initial proteolysis for *L. helveticus* 138 and 209 was different, and also the evolution of peptidolysis diverged during incubation, with sample scores for *L. helveticus* 209 and 138 moving principally along PC1 and PC2, respectively (Fig. 7B and C). On the other hand, the evolution of peptidolysis during incubation of extracts was very similar to that verified during cheese ripening for both strains. All extracts of *L. helveticus* 209 evolved similarly during incubation or ripening, with scores moving principally toward negative values on PC1 at higher incubation or ripening times (Fig. 7A, B, and C). In the case of *L. helveticus* 138, some differences were found in the evolution of peptidolysis of samples of different age. Incubation of young extracts showed that scores moved toward negative values on PC1 and positive values on PC2. This was in agreement with the evolution of peptidolysis during cheese ripening up to 90 days (Fig. 7A). On the contrary, incubation of mid-ripened cheeses was characterized by scores moving principally toward negative values on PC2, as well as the evolution during cheese ripening between 90 and 180 days (Fig. 7B). Finally, the extracts of full-ripened cheeses of both strains followed the same trends than those of mid-ripened cheeses (Fig. 7C).

As for free amino acids, total content in blank extracts remained constant, while it increased in the unheated extracts at a rate that was related to cheese age and starter strain (Fig. 8). Extracts of young cheeses made with either *L. helveticus* 138 or 209 had a similar increase in total free amino acids (Fig. 8A). In mid-ripened cheeses, the initial content of free amino acids was higher in the extract of *L. helveticus* SF138 cheeses and the increase regarding the respective blank extracts was also much higher than that in the extracts of *L. helveticus* SF209 cheeses (Fig. 8B). As for extracts of full-ripened cheeses, the differences were mainly in the initial level of FAA, and then the increase was rather similar in both types of extracts (Fig. 8C). The increase of total FAA in all the extracts of cheeses with *L. helveticus* 209 was very similar to that verified during cheese ripening. In effect, the levels of total FAA in extracts from y- and m-ripened cheeses after 14 days of incubation were similar to the values of extracts of mid-

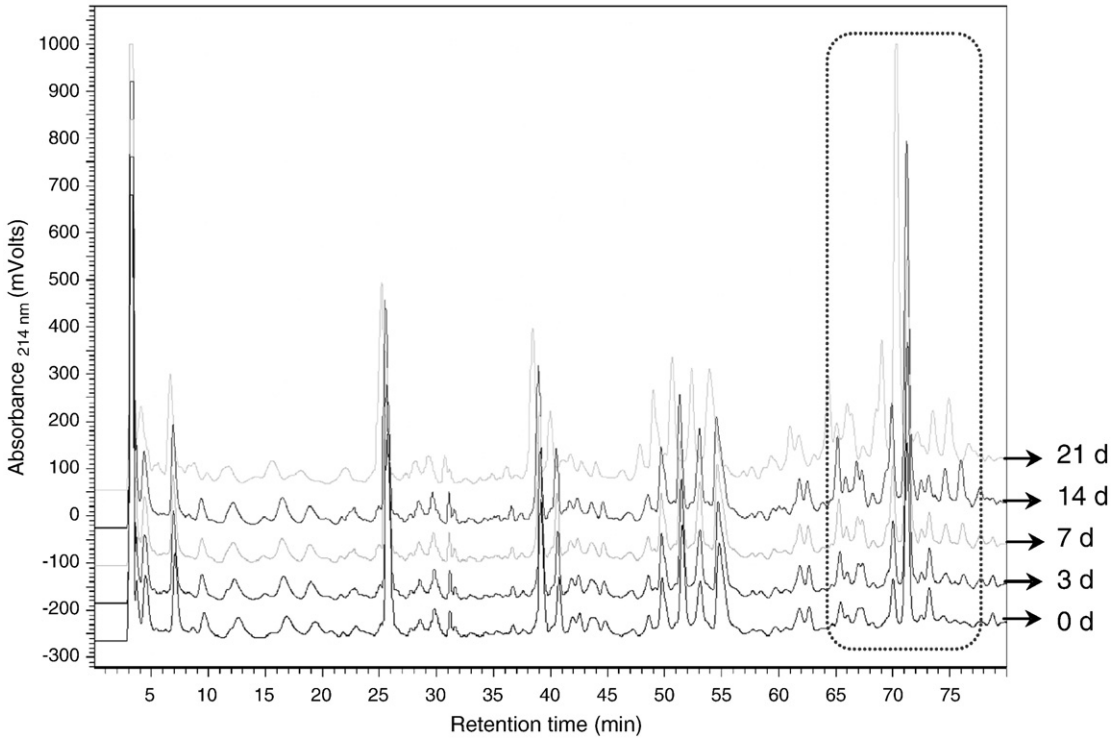


Fig. 5. Peptide profiles of blanks extracts (thermally treated), obtained from young-ripened Reggianito cheese manufactured with *L. helveticus* 209, during incubation time. The rectangle encloses the group of peaks that showed changes during incubation.

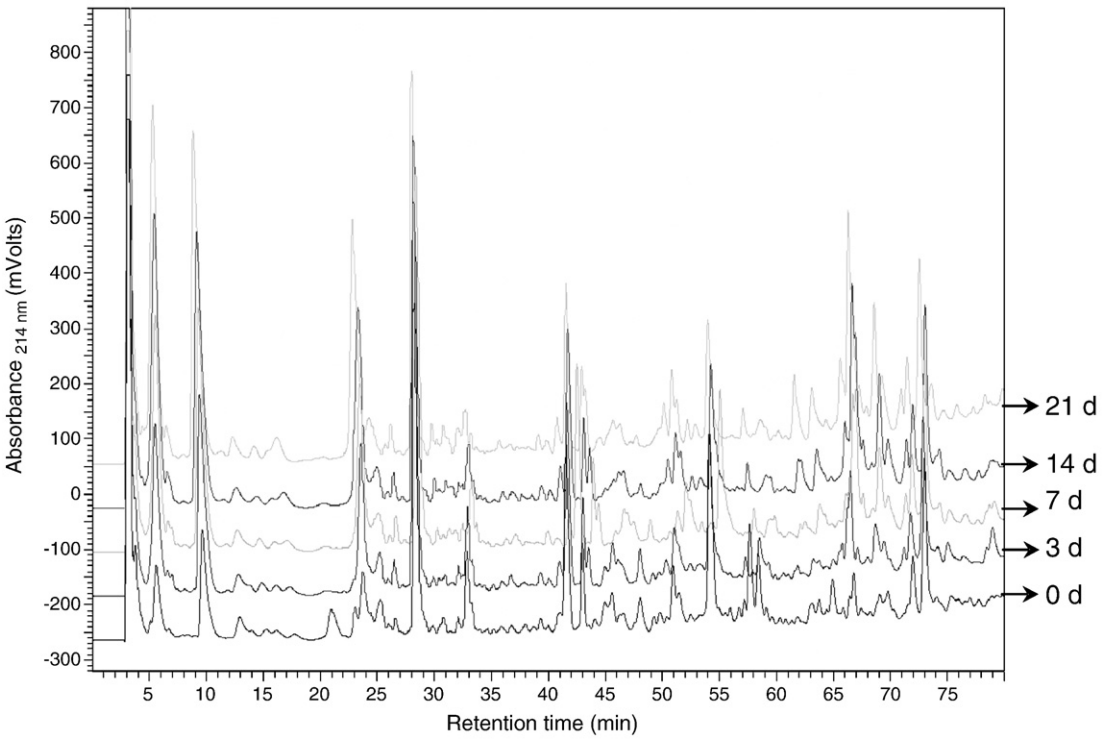


Fig. 6. Peptide profiles of soluble extracts of young-ripened Reggianito cheese manufactured with *L. helveticus* 209 during incubation time.

and full-ripened cheeses at 0 day of incubation, respectively. Similar results were obtained for *L. helveticus* 138, but only for extracts of mid-ripened cheeses. The increase of FAA in cheeses with *L. helveticus* 138 was very pronounced between 0 and 90 days of ripening, change that was not reflected during incubation of young cheese extracts.

PCA was applied on the FAA profiles in order to describe the changes during incubation of the extracts and detect their correlation with the changes during cheese ripening. Scores plots are presented as for peptide profiles (Fig. 9). Evolution of FAA profiles during incubation of extracts of young ripened cheeses was similar for both *L. helveticus* strains (Fig. 9A). On the contrary, the initial FAA profiles and also the evolution during incubation of mid- and full-ripened cheeses were different for *L. helveticus* 138 and 209 (Fig. 9B and C). These results are similar to those obtained for peptide profiles. On the other hand, the evolution of FAA profiles during cheese ripening and extract incubation was very similar for all extracts of *L. helveticus* 209. With regard to *L. helveticus* 138, the correlation between incubation and ripening was verified principally for mid-ripened cheeses, which is in agreement with results obtained for total FAA.

4. Discussion

Young cheeses provided the extracts whose proteolysis patterns varied the most during incubation; in quantitative terms, these extracts showed the highest variation in peptide production. Free amino acids total content increase 3 to 4 times compared to blanks (Fig. 8A). However, evolution of proteolysis in both kind of extracts assayed was rather similar, regardless the strain of *L. helveticus* used as primary starter in cheese making (Figs. 7A, 8A and 9A). This is probably due to the fact that basal proteolysis was very low in young cheeses at the beginning of the ripening, and the enzymes occurring in both extracts performed similar basic hydrolysis. In addition, the level of peptidases was probably low in these extracts because these intracellular enzymes are released into the medium after cellular lysis, which was almost certainly very low in cheeses at the beginning of ripening (Kenny et al., 2006, Savijoki, Ingmer, & Varmanen, 2006). Only little differentiation by starter strain was observed in peptide profiles of young cheese extracts at the end of incubation (Fig. 7A).

On the contrary, changes in peptide profiles during incubation were strain-related in soluble extracts made with mid-ripened cheeses (Fig. 7B). This observation suggested that the peptidases in the extracts had different activities upon the initial pool of peptides available, which was also diverse for cheeses made with *L. helveticus* SF138 or SF209 (Fig. 7B). Also, the evolution of FAA content was different in extracts of mid-ripened *L. helveticus* SF138 or *L. helveticus* SF209 cheeses (Fig. 9B). Before incubation, the total content of FAA was almost 3 times as high in the *L. helveticus* SF138 extract as in the *L. helveticus* SF209 substrate, and then it also increased more during incubation—ca. 9 mg mL^{-1} against ca. 6 mg mL^{-1}, respectively (Fig. 8B). These results are probably due to the different enzymatic machineries of each strain and the amount of peptidases released into the medium at 90 days of ripening.

At the end of ripening, the initial proteolysis of *L. helveticus* SF138 or SF209 cheese extracts was very different; incubation of the extracts, in this case, would model an over- ripening period. Peptide profiles, and free amino acids concentrations, showed that while proteolysis in *L. helveticus* SF209 extract progressed during incubation, in *L. helveticus* SF138 extract only minor changes were observed (Figs. 7C and 8C). In comparison with changes during incubation of mid-ripened cheese extracts, the rate of accumulation of free amino acids in full-ripened cheese extracts decreased for *L. helveticus* SF138 cheese extract, while it increased for *L. helveticus* SF209, even if the absolute values were always lower for the latter (Fig. 8B and C).

Peptide profiles and FAA accumulation in the extracts highlight the importance of two facts: the time in ripening when differences

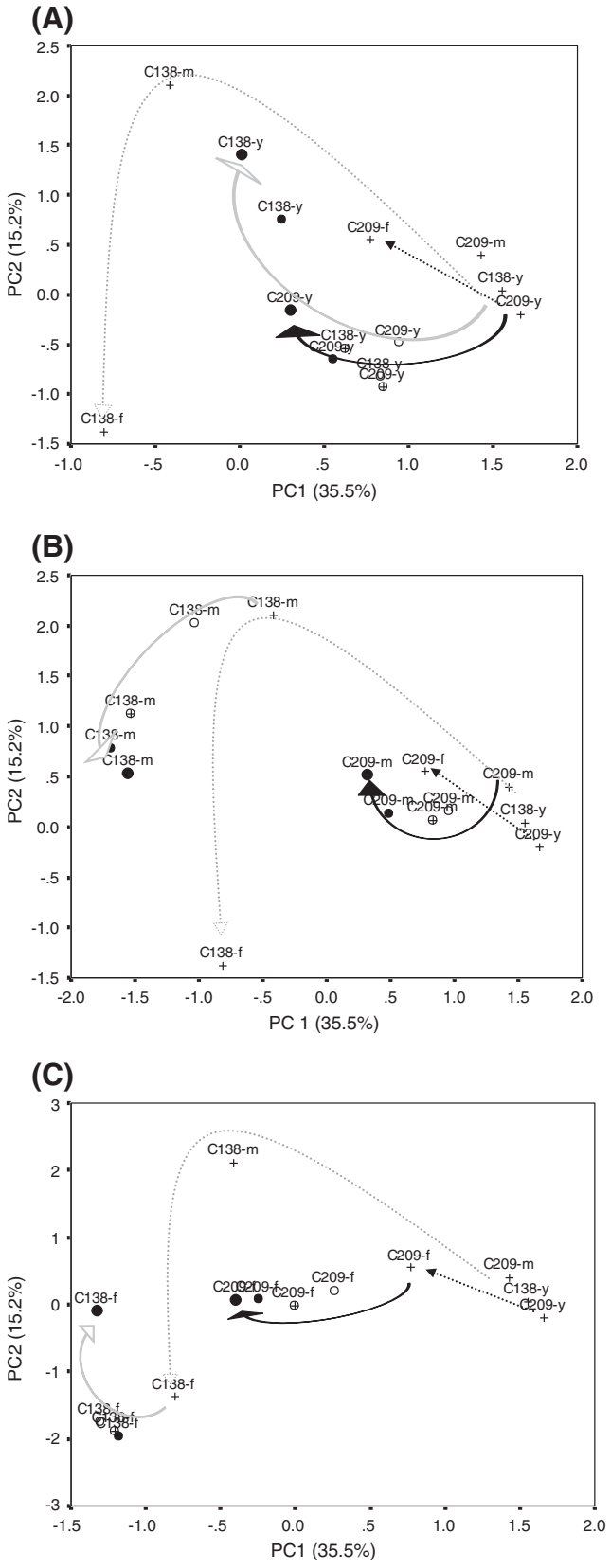


Fig. 7. Principal components score plot (PC1 vs. PC2) of peptide profiles of extracts during incubation. (A) Young cheese extracts, (B) mid-ripened cheese extracts, (C) full-ripened cheese extracts. Scores of all extracts before incubation were included in the three plots. Full and dotted arrows indicate incubation and ripening, respectively. Black and grey arrows correspond to extracts derived from cheeses manufactured with *L. helveticus* 209 and *L. helveticus* 138, respectively. Time of incubation: 0 (+), 3 days (o), 7 days (⊕), 14 days (●) and 21 days (●).

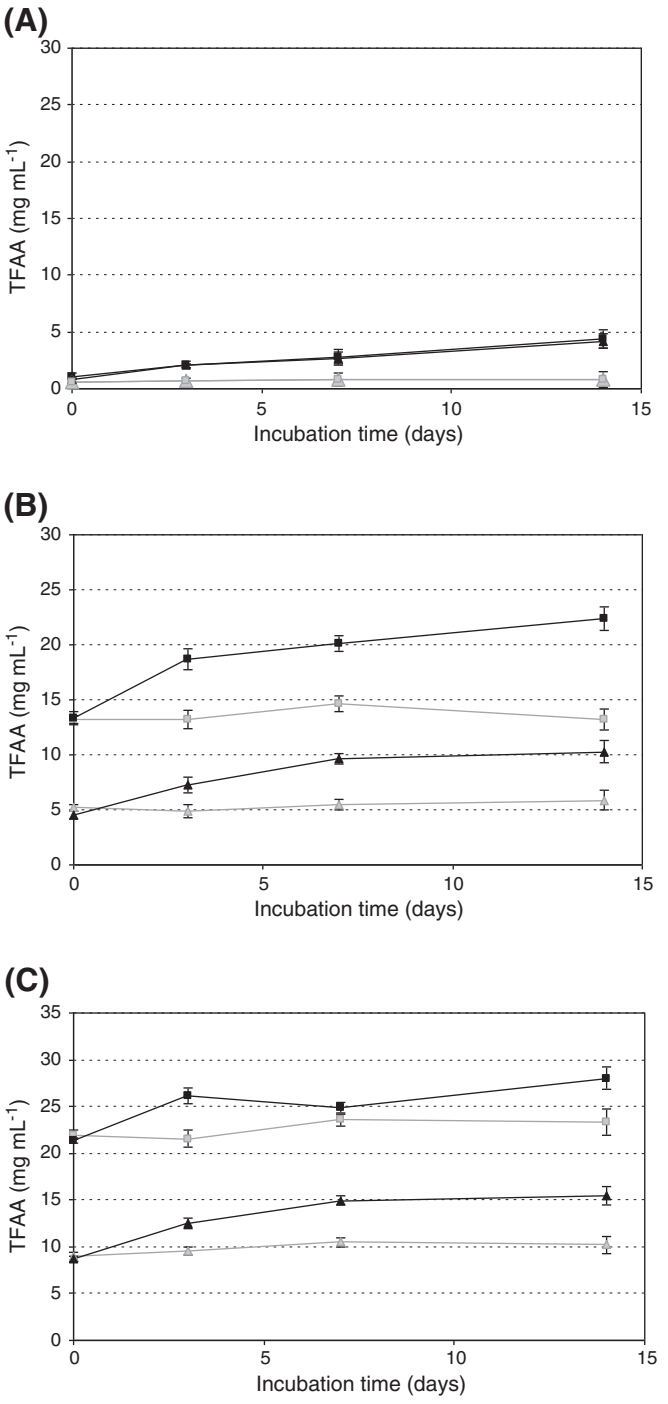
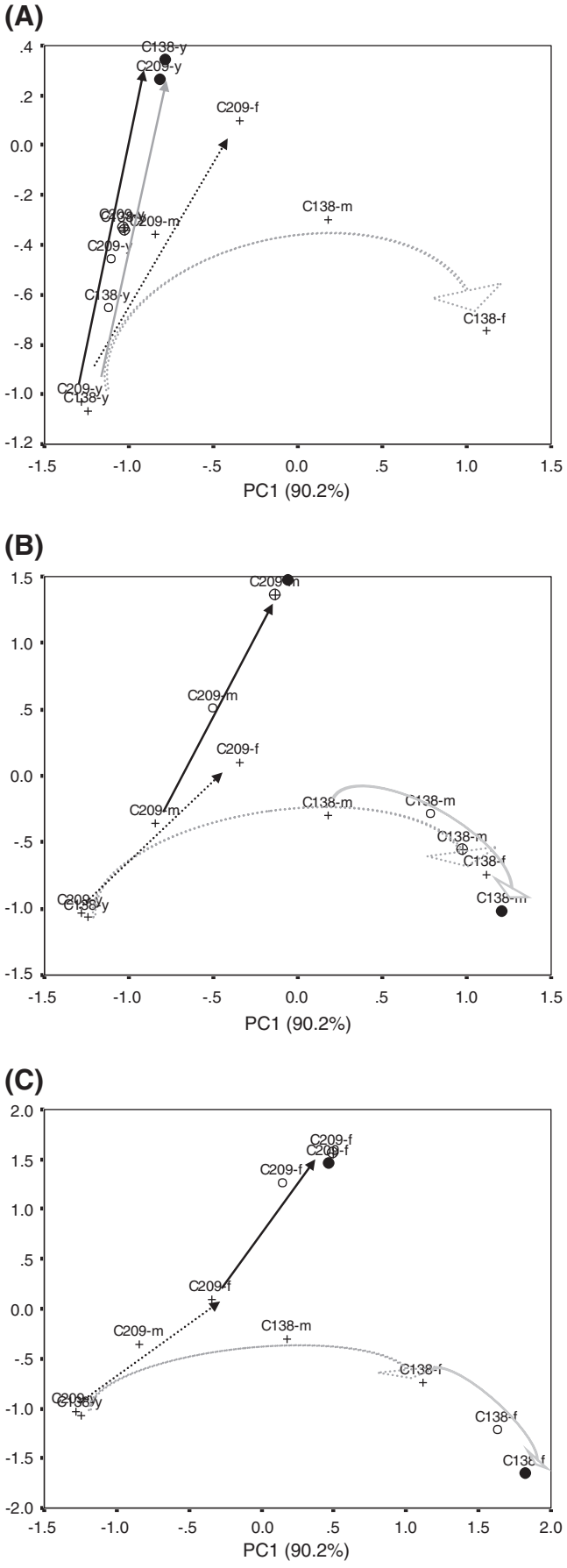


Fig. 8. Evolution of total amount of free amino acid (TFAA) (mg mL^{-1}) in soluble extracts derived from cheeses manufactured with *L. helveticus* 209 (▲) and *L. helveticus* 138 (■) at 3 (A), 90 (B) and 180 (C) days of ripening, after 0, 3, 7 and 14 days of incubation at 34 °C. Blanks (thermally treated): grey symbol and line. Extracts (non thermally treated): black symbol and line. Values are mean ± standard deviation of three replicates.

in proteolysis due to *L. helveticus* begin to be apparent in cheeses and how do they impact in further hydrolysis of peptides during incubation. According to our results, proteolytic ability of the starter strains had an impact in hard cooked cheeses after several weeks of ripening, and differentiation between strains was better detected when tested in a medium in which some proteolysis had already developed and in which probably there was a higher level of the intracellular enzymes of



starters. This is an important finding as proteolytic activity of lactic acid bacteria strains aimed at constituting starters is usually screened in milk (Quiberoni et al., 1998) or studied on intact caseins (Jensen et al., 2009) or intermediate-sized peptides (Oberg et al., 2002). Unfortunately, *in vitro* studies about these strains only assessed global proteolysis by Hull method (Quiberoni et al., 1998) and no data on their caseinolytic activities are available; consequently, is impossible to determine if a relation between caseinolytic patterns and proteolytic activity in cheese can be drawn.

During incubation, the evolution of the proteinaceous material in extracts of cheeses made with the two strains of *L. helveticus* assayed was different. *L. helveticus* SF209 was considered the most proteolytic strain according to previous results, issued mostly of unspecific methods (Candioti et al., 2002; Quiberoni et al., 1998). Its enzymes actually showed a more intense effect in producing soluble peptides (Figs. 1, 2, and 3A), but a slower increase in the concentrations of free amino acids was verified (Figs. 4 and 8). This may be due to a poor ability to hydrolyze small peptides into free amino acids because of a low level of aminopeptidases or, as results of SN-PTA suggest (Fig. 2), to a higher transformation of FAA into smaller nitrogen compounds. In the extract of *L. helveticus* SF138 cheeses, on the contrary, neither an increase of peptides areas nor a more complex profile was caused by cheese ripening or extract incubation; on the contrary, peaks disappeared or their areas decreased with time (Fig. 3B). Concomitantly, free amino acids accumulation increased steadily, especially during incubation of mid-ripened cheese extract (Fig. 8). These results suggest that proteolytic–peptidolytic machinery of *L. helveticus* SF138 was not able to produce small peptides from intermediate-sized peptides in the extract, but only to hydrolyze those already available at early steps of ripening into free amino acids.

5. Conclusions

In this work, we provided a new cheese model suitable to characterize biochemical activities during ripening of hard-cooked cheeses in a short period of time and evidenced that proteolytic abilities of two *L. helveticus* strains were better differentiated when tested in mid-ripened cheese products.

Incubation of extracts of mid-ripened (3-month-old) hard cooked cheeses for 14 to 21 days at 34 °C led to similar proteolysis patterns that continuing the ripening of the cheeses up to 6 months, and it was useful to characterize the activities of peptide formation and free amino acids accumulation of two strains of *L. helveticus*. In this way, both strains of *L. helveticus* showed potentially complementary proteolytic activities, as SF209 was able to provide a continuous replenishment of peptides during incubation, while SF138 increased their hydrolysis into free amino acids.

In addition to characterize thermophilic starters, this new cheese model will be applied to assess the impact of couples of starter and adjunct cultures on peptidolysis and volatile compounds production.

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Fig. 9. Principal components score plot (PC1 vs. PC2) of FAA of extracts during incubation. A) Young cheese extracts, B) mid-ripened cheese extracts, C) full-ripened cheese extracts. Scores of all extracts before incubation were included in the three plots. Full and dotted arrows indicate incubation and ripening, respectively. Black and grey arrows correspond to cheese extracts with *L. helveticus* 209 and *L. helveticus* 138, respectively. Time of incubation: 0 d (+), 3 days (o), 7 days (⊕) and 14 days (●).

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