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Propionibacteria and facultatively heterofermentative lactobacilli weakly contribute to secondary proteolysis of Emmental cheese

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Abstract — Proteolysis is a major event during cheese ripening. In Emmental cheese, the contribution of plasmin and thermophilic lactic starters to proteolysis has been well established. Our aim was to assess the contribution of each ripening flora to secondary proteolysis. With this aim, we used the aqueous phase (or juice) of Emmental cheese as a culture medium, which contains the enzymes released by thermophilic starters. Crude juice (containing all cheese flora) and cell-free juice either inoculated with two propionibacteria (PAB) strains or uninoculated (control), were incubated under anaerobiosis at 24 °C for 20 d. Free amino groups and free amino acids, bacterial growth and organic acids were followed throughout the incubation time. Facultatively heterofermentative lactobacilli (FHL) represented the main flora growing in crude juice up to 10 d incubation. FHL and PAB respectively reached 4.0×10^8 and 1.0×10^{10} cfu·mL^{-1} of juice and consumed citrate and lactate, in accordance with that already observed in cheese. The level of amino groups increased linearly during incubation time by a factor of 1.9 in crude juice, 1.7 in cell-free juice and only 1.5 in juice inoculated with PAB strains, showing that the enzymes of thermophilic starters are the main contributors to secondary proteolysis. FHL growth resulted in a 16% increase in free amino acids compared to cell-free juice and in exhaustion of Arg and His. Both PAB strains caused a 18–27% decrease in free amino acids and also modified the amino acid distribution, with consumption of Asp, Gly, Glu, Ser and Ile.

secondary proteolysis / *Propionibacterium* / facultative heterofermentative lactobacilli / Emmental cheese / free amino acid

Résumé — **Les lactobacilles hétérofermentaires facultatifs et les bactéries propioniques ont une faible contribution à la protéolyse secondaire de l'Emmental.** La protéolyse est un phénomène majeur dans l'affinage du fromage. Dans l'Emmental, la contribution de la plasmine et des levains thermophiles à la protéolyse secondaire a été bien établie. Le but de cette étude était de

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déterminer la contribution de chacune des flores d'affinage à la protéolyse secondaire. Pour cela, nous avons utilisé de la phase aqueuse d'Emmental (ou jus), qui contient les enzymes relarguées par les levains thermophiles lors de leur lyse, comme milieu de culture. Le jus de fromage brut (contenant toutes les flores du fromage), le jus exempt de cellules bactériennes utilisé soit tel quel (témoin), soit ensemencé avec deux souches de bactéries propioniques (PAB) ont été incubés en anaérobiose à 24 °C pendant 20 j. Les groupements aminés libres et les acides aminés libres ainsi que les paramètres de croissance et de fermentation ont été suivis durant l'incubation. Les lactobacilles hétérofermentaires facultatifs (FHL) représentaient la flore principale poussant dans le jus brut jusqu'à dix jours d'incubation. Les FHL et les PAB atteignaient respectivement $4{,}0 \times 10^8$ and $1{,}0 \times 10^{10}$ ufc·mL^{-1} de jus et consommaient le lactate et le citrate, en accord avec les résultats déjà observés dans le fromage. Les taux de groupements amines libres augmentaient linéairement pendant l'incubation par un facteur 1,9 dans le jus brut, 1,7 dans le jus témoin et seulement 1,5 dans le jus ensemencé avec les souches de PAB, montrant que les enzymes des levains thermophiles contribuent majoritairement à la protéolyse secondaire. La croissance des FHL résultait en un accroissement de 16 % des acides aminés libres par rapport au jus témoin, et une disparition de Arg et His. Chacune des deux souches de PAB entraînait une chute de 18–27 % en acides aminés libres et modifiait également la distribution en acides aminés, en consommant Asp, Gly, Glu, Ser et Ile.

protéolyse secondaire / *Propionibacterium* / lactobacille hétérofermentaire facultatif / Emmental / acide aminé libre

1. INTRODUCTION

During cheese ripening, proteolysis is one of the most significant phenomena which gives the appropriate characteristics of texture and to a lesser extent of flavour to the final product. The proteolytic agents involved in the primary proteolysis of the caseins in Swiss-type cheeses are well-known at the present time, with plasmin involved to a greater extent than the added coagulant, rapidly inactivated by heating of the curd [34]. In contrast, the contribution of the different flora to the proteolysis in such cheeses is only partially understood. The available information mostly concerns the thermophilic lactic acid bacteria used as starters (*Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *lactis* and *Streptococcus thermophilus*). Besides their primary function to produce lactic acid from lactose, they also hydrolyse caseins into peptides necessary for their growth occurring during the pressing of the cheese. Moreover, thermophilic lactobacilli, especially *L. helveticus*, lyse rapidly during the subsequent ripening in a cold room (temperature about 15 °C for a couple of weeks) [40] and release active peptidases in the cheese. The starters consequently have a great contribution to the production of peptides and free amino acids throughout the ripening [14, 29, 39].

Two main ripening flora of Swiss-type cheese could also contribute to proteolysis: propionic acid bacteria (PAB) and non-starter lactic acid bacteria. The former are mainly represented in Emmental cheese by *Propionibacterium freudenreichii* species. They are added to the milk as secondary starter and grow, when the cheeses are placed in a warm room (18–24 °C, for about one month). The latter are mainly composed of facultatively heterofermentative lactobacilli (FHL), which grow from the beginning of ripening in a cold then in a warm room. The contribution of each of these flora to proteolysis remains unclear, and their proteolytic systems are incompletely described. PAB proteinases are weakly implicated in the breakdown of caseins [20]. Nevertheless, PAB possess nu-

merous intracellular peptidases active on casein peptides [15]. However, their lysis has been shown to be late and rather limited in cheese [40]. FHL are in a dynamic state during ripening of Swiss-type cheeses with *L. paracasei* being the main species. *L. plantarum* and *L. rhamnosus* have also been found, as well as minor levels of strict heterofermentative lactobacilli such as *L. brevis* [3, 37]. Like PAB flora, FHL possess low proteolytic activity in milk and have numerous intracellular peptidases [3]. Peptidase activity of *L. paracasei* is lower than that of *L. helveticus* and higher than that of *L. delbrueckii* ssp. *delbrueckii* and ssp. *lactis* [33]. Lysis of *L. casei* and *L. plantarum* has not yet been extensively studied but was close to *L. helveticus* under conditions prevailing in cheese [12]. Their intracellular peptidases could therefore contribute to increasing proteolysis in cheese. Experiments in cheese have shown that raw milk flora, composed of FHL, PAB and enterococci, increased the proteolysis of Swiss-type cheese [4, 10] but the respective contributions of the different flora has not been clearly determined, due to the difficulty of tightly controlling the cheese ecosystem. Moreover, it is also difficult to dissociate in situ the action of each proteolytic system from that of the thermophilic LAB, which previously grew and lysed.

The objective of this study was to evaluate the contribution of PAB and FHL to the secondary proteolysis of Emmental cheese by simulating their condition of growth in cheese, i.e. with a medium that mimics as closely as possible the physico-chemical and microbiological characteristics of the cheese. For this purpose, we used the aqueous phase of Emmental cheese, which can be extracted by hydraulic press and is called juice [31], as a culture medium to grow PAB and FHL. Juice, extracted during cheese ripening, contains in particular soluble peptides and amino acids as well as active peptidases released from thermophilic starters [14]. Two PAB strains were inoculated as pure cultures in cell-free juice, whereas concerning non-starter lactic acid bacteria, we chose to incubate crude juice of cheese, which contained the indigenous cheese flora.

2. MATERIALS AND METHODS

2.1. Extraction of juice from Emmental cheese

Emmental cheese, representative of the French industrial Emmental production, was taken before the warm room ripening from one factory in Brittany corresponding to 13 d of ripening. The juice was extracted as described by Salvat-Brunaud et al. [31]. Its complete composition was given by Salvat-Brunaud et al. [32] under the name X_1. Emmental juice (about 150 mL) was filtered through Whatman paper 541 (Prolabo, Bruchet Dano, Rennes, France) leading to a fraction called crude juice. A fraction of crude juice was successively filtered through several cellulose acetate membrane filters with 1.2, 0.45 and 0.22 μm pore size (Sartorius, Palaiseau, France) to obtain sterile juice, which will be called cell-free juice thereafter.

2.2. Strains and growth conditions in Emmental juice

Two strains, *Propionibacterium freudenreichii* ssp. *freudenreichii* TL33 and *P. freudenreichii* ssp *shermanii* TL34 were taken from the TL collection (LRTL, INRA, Rennes, France). They were stored at –80 °C in Yeast Extract Lactate (YEL) broth [26] containing 15% (v/v) glycerol. They were successively transferred twice into YEL broth first at 1% (v/v) and second at 0.5% (v/v) and then into cell-free Emmental juice for 48 h at 30 °C at 0.5% (v/v), before finally being transferred at

0.5% (v/v) into 40 mL of freshly extracted and cell-free Emmental juice.

2.3. Incubation of Emmental juices under warm room conditions

Three media were compared: crude juice (containing all cheese flora), cell-free juice inoculated with two PAB strains and uninoculated cell-free juice used as a control. For each, 40 mL were distributed among tubes containing 2.5 mL of culture each. The media were then statically incubated under anaerobiosis (anaerocult A, Merck, Nogent-sur-Marne, France) at 24 °C for 20 d, conditions prevailing during the ripening of Emmental in the warm room.

2.4. Microbiological analyses

Dilutions of each sample were performed with salted peptone water containing 1 g·L^{-1} peptone (Tryptone Biokar, Beauvais, France) and 8.5 g·L^{-1} NaCl (Merck, Nogent-sur-Marne, France). All the microbiological measurements were made in duplicate by means of a Spiral system apparatus (DS model Interscience, Saint-Nom-la-Bretèche, France), except PAB, added into the agar plates. PAB were enumerated on LGA medium after 6 d incubation at 30 °C under anaerobiosis (anaerocult A, Merck) [36]. Flora growing during crude juice incubation were determined as follows: total mesophilic aerobic flora was assessed on Plate Count Agar (Biokar) after incubation for 3 d at 30 °C. FHL were enumerated on FH medium incubated for 3 d at 37 °C under anaerobiosis [17]. Ten colonies were picked up from the FH plates corresponding to the end of incubation time, purified and further characterised (morphology, Gram, catalase test and fermentation pattern using API 50CH (Biomérieux, Marcy-l'Étoile, France). In addition, thermophilic lactic starters present in crude juice were tentatively enumerated on M17 medium under anaerobiosis for 2 d at 43 °C for streptococci [35] and on MRS pH 5.4 for 2 d at 43 °C under anaerobiosis for lactobacilli [16]. As both the latter media are not selective, several colonies were examined by optical microscopy to control the type of flora growing on these media. The absence of contamination of cell-free Emmental juice and PAB cultures was checked by numerating the mesophilic aerobic microflora on Plate Count Agar (Biokar) after incubation for 3 d at 30 °C.

PAB growth curves were fitted with the Gompertz model modified by Zwietering et al. [41] to evaluate the lag time, the doubling time and the maximal population value reached, as described by Salvat-Brunaud et al. [32].

2.5. Physicochemical analyses

After growth and microbiological analyses, all the samples were centrifuged at $2400 \times g$ for 50 min at 10 °C (Centrifuge CR4 11, Jouan, Saint Nazaire, France) and the supernatants were filtered through 0.22 µm and stored at –20 °C until further biochemical analyses.

The pH was measured during incubation time with a pHmeter CG 837 (Schott, Prolabo).

2.5.1. Organic acids

Citric, lactic, propionic, acetic and formic acids were analysed by cation exchange chromatography according to the elution conditions of Salvat-Brunaud et al. [31].

2.5.2. Measurement of proteolysis in Emmental juice

* Total proteolysis in the juice was measured according to the determination of free amino groups by the *O*-phtaldialdehyde method of Church et al. [6]. Samples were

diluted to 1:50 with distilled water before determination and the results were expressed as mmole·L^{-1} equivalent methionine (Sigma, Saint-Quentin Fallavier, France) used as a standard.

* Total free amino acid determination. Total free amino acids were determined using Cd-ninhydrin reagent, according to Baer et al. [2], with modification of the sample preparation. Duplicate samples were precipitated with 900 mL·L^{-1} absolute ethanol. After 1 h at room temperature, the mixture was centrifuged at 2400 × *g* for 15 min. The supernatants were diluted 1:50 with distilled water. A sample (50 µL) was mixed with 0.55 mL of distilled water and 1.20 mL of the Cd-ninhydrin solution was added. The results were expressed as mmol·L^{-1} equivalent methionine used as a standard.

* Free amino acid analysis. The ethanol precipitation performed above was also used for the amino acid analysis. Ethanol supernatant (0.80 mL) was dried in a Speedvac evaporator, then submitted to derivatisation with phenylisothiocyanate (Pierce, Touzart & Matignon, Vitry-sur-Seine, France). The duplicate determination of the amino acid derivatives was performed by RP-HPLC on a Picotag C_{18} column (150 × 3.9 mm ID; Waters, Saint-Quentin-en-Yvelines, France) according to Bidlingmeyer et al. [5].

3. RESULTS

3.1. Growth of PAB in cell-free Emmental juice

Figure 1a shows the growth of both inoculated PAB during 20 d culture in cell-free Emmental juice. With an inoculation level initially of 6.3×10^7 and 2.5×10^7 cfu·mL^{-1}, respectively, for TL33 and TL34, both strains had similar characteristics of growth: their lag phase was short (44.2 ± 0.5 h for TL33 and 38.9 ± 2.4 h for TL34), they reached a high level of maximal population (1.3×10^{10} and 0.8×10^{10} cfu·mL^{-1} of juice for TL33 and TL34 respectively) and had doubling times of 15.8 ± 1.5 h for TL33 and 13.9 ± 0.2 h for TL34.

Most lactate was consumed with a concomitant production of propionate and acetate from 4 d of cultivation up to 12 d of incubation (Figs. 1b and 1c). Two thirds of citrate (10.7 mmol·L^{-1}) was also used after 20 d incubation.

The pH of the juices (Figs.1b and 1c) increased from pH 5.44 to pH 5.78 for TL33 and to pH 5.74 for TL34 during PAB growth up to 12 d incubation and remained constant during the stationary phase, whereas the pH of the cell-free juice remained constant throughout the incubation time to an average value of pH 5.39 ± 0.03.

3.2. Growth of FHL in crude juice

FHL population was at 3.2×10^6 cfu·mL^{-1} before incubation of the crude juice. It increased to 4.0×10^8 cfu·mL^{-1} at the end of the incubation time (Fig. 2a), with a doubling time of about 13.5 h. Among the 10 isolated clones, all were short bacilli, Gram-positive and catalase-negative, none produced gas from glucose. Identification from their fermentation pattern showed 8 *L. paracasei* and 2 *L. curvatus*. FHL formed the main population up to 12 d of incubation, then the PAB present in crude juice reached a population over 10^9 cfu·mL^{-1}. Apart from FHL and PAB, no other flora was detected, as indicated by the fitting of curves obtained using PCA and FH media. Thermophilic lactobacilli and streptococci could not be detected throughout incubation time because only colonies of short bacilli (most likely hetero-fermentative lactobacilli capable of growing at 45 °C) were found on the media used.

The pH of the crude juice (Fig. 2b) rose two distinct phases, one during the growth

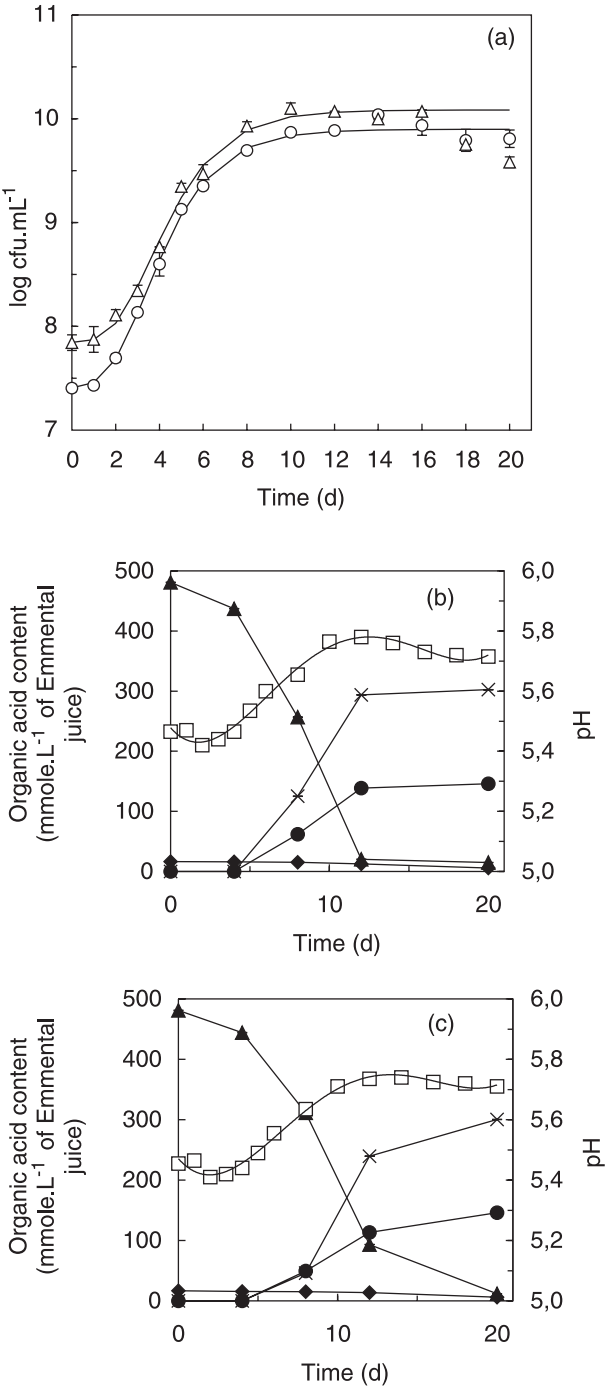

Figure 1. (a) Growth of two strains of propionibacteria in a cell-free juice, extracted from Emmental cheese entering the warm room, and incubated at 24 ºC under anaerobiosis: *Propionibacterium freudenreichii* TL33 (△) and *Propionibacterium freudenreichii* TL34 (○), (——) represents the growth curve calculated from the Gompertz model; the evolution of organic acid concentrations and pH (□) in juice throughout the incubation time: lactate(▲), propionate (×), acetate (●) and citrate (◆), (b) for the TL33 and (c) TL34 strains.

of FHL and a second during the growth of PAB which led to a pH of 0.2 units higher than pH of the pure culture of PAB at the end of the incubation time. However, as PAB had just reached the stationary phase, pH did not reach the plateau as previously observed for PAB pure cultures. FHL exhausted citrate (16.6 mmol·L^{-1}) during their growth and subsequently produced acetate (32.8 mmol·L^{-1}) and formate (24.5 mmol·L^{-1}) after 10 d of incubation, before PAB reached a population level high enough (~3×10^6 cfu·mL^{-1}) to significantly modify the organic acid levels (Fig. 2b). The formate level remained constant later on whereas acetate concentration increased due to PAB fermentation.

3.3. Time course of the proteolysis in the different juices

Global proteolysis in the juices was determined throughout the 20 d incubation time using two indicators: first the concentration of free amino groups, including those from peptides and free amino acids, and second the concentration of free amino

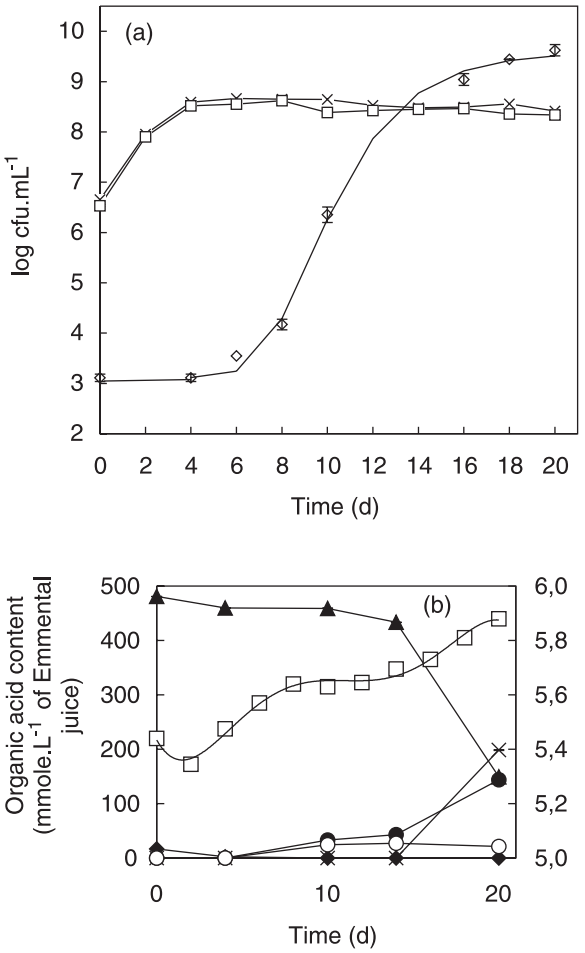

Figure 2. (a) Growth of bacteria in crude juice during the incubation at 24 °C under anaerobiosis: mesophilic aerobic microflora (×); facultatively heterofermentative lactobacilli (□) and propionibacteria (◇), (——) represents the growth curve calculated from the Gompertz model. (b) Evolution of the organic acids and the pH (□) in juice throughout the incbation time: lactate (▲), propionate (×), formate (○), acetate (●) and citrate (◆).

acids (Tab. I). The concentration of free amino groups increased almost linearly as a function of time for the three types of juices throughout the 20 d incubation time: by a factor of 1.9 in crude juice, 1.7 in cell-free juice and only 1.5 in juice inoculated with PAB strains. The increase of the proteolysis indexes in the cell-free juice, which remained sterile throughout incubation time, was only due to the action of the enzymes released from lysis of the thermophilic starters in the Emmental cheese as previously described [14]. It clearly appears that these enzymes were the main contributors of the increase in the proteolytic indexes in the four juices (Tab. I).

In order to distinguish the action of the released enzymes from thermophilic starters, from that of FHL and PAB, we calculated the ratio of the amount of free amino groups in crude juice or in juices inoculated with PAB to the amount of free amino groups in the cell-free juice, considered as control. During the first part of incubation, this ratio was similar in all juices (Fig. 3a). Thereafter, from 8 d till the end of incubation in PAB cultures, the concentration of free amino groups became lower (–11%) than in the cell-free juice, showing an utilisation of nitrogenous compounds by both PAB strains. In the crude juice, the rate of free amino group release was higher (+13%) than in the cell-free juice from 4 to 12 d of incubation, showing the activity of FHL which represented the major flora during this phase. Thereafter, the difference between crude and cell-free juice diminished, probably due to the consumption of peptides and free amino acids by PAB, which reached a population of over 10^9 cfu·mL^{-1} during this second phase.

Concerning free amino acids, the same ratio of their amount in crude juice or in PAB cultures to their amount in cell-free juice was calculated. These ratios showed a similar evolution to the ratio for amino groups, with higher differences between the juices containing ripening flora and control (Fig. 3b). Hence, from 8 d of incubation in PAB cultures, free amino acids were 18 and 27% less than in cell-free juice with TL34 and TL33 respectively. This suggests that PAB consumed preferably amino acids rather than peptides during

Table I. Concentration of free amino groups and free amino acids in Emmental juice, either crude (i.e. containing cheese flora), cell-free, or inoculated with propionibacteria, before and after their incubation at 24 °C under anaerobiosis.

	Concentration of amino groups (mmol·L^{-1})	Concentration of free amino acids (mmol·L^{-1})
Cell-free juice before incubation	58.0 ± 1.6	33.1 ± 3.6
Juices after 20 d incubation:		
cell-free juice	97.4 ± 4.0	66.5 ± 6.1
crude juice	108.3 ± 3.4	57.4 ± 4.5
juice + *Propionibacterium freudenreichii* TL33	87.5 ± 1.9	48.6 ± 7.0
juice + *Propionibacterium freudenreichii* TL34	86.7 ± 3.2	54.5 ± 2.5

The results are expressed by means ± standard deviation with 4 and 6 replications, respectively, for free amino groups and free amino acids.

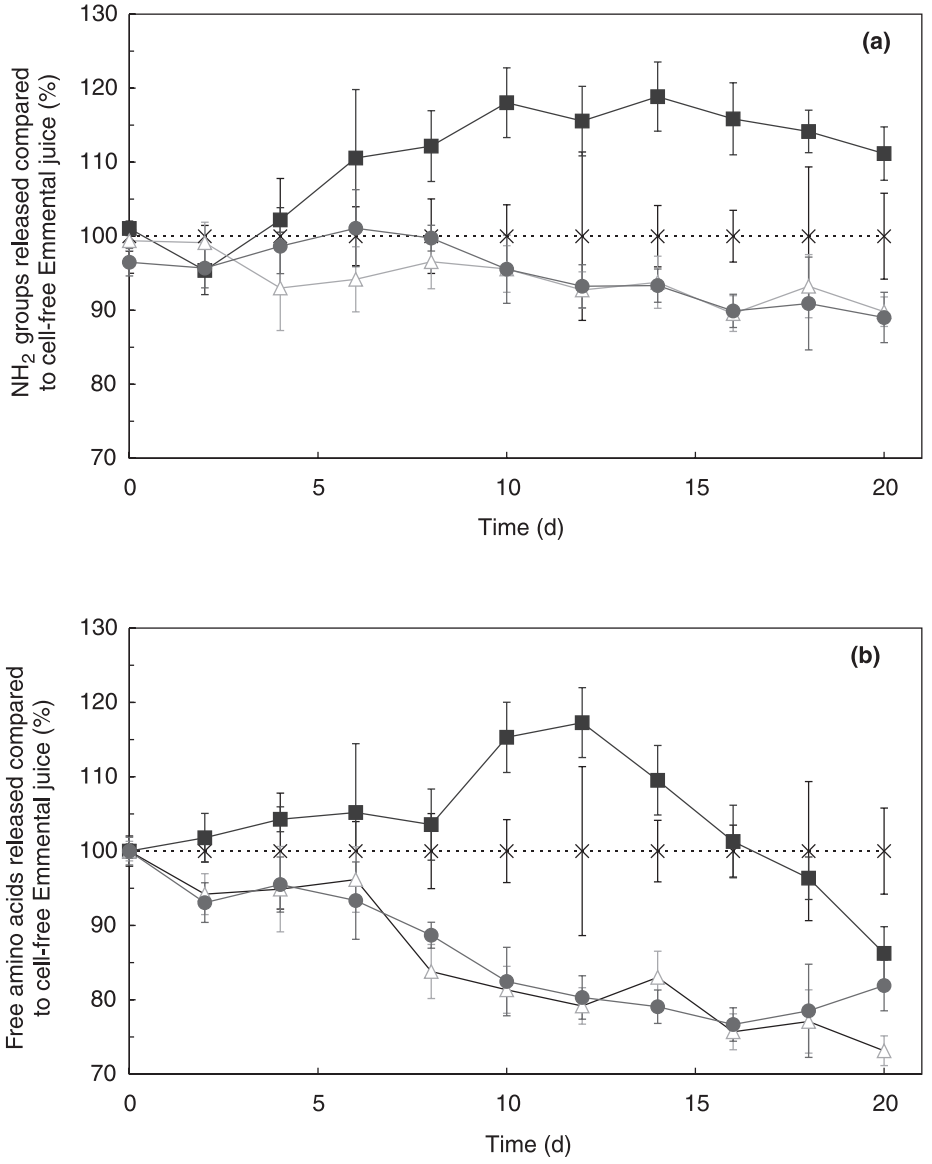

Figure 3. Concentration of free amino groups (a) and of free amino acids (b) during the culture of *Propionibacterium freudenreichii* TL33 (△) and TL34 (●) in cell-free juice and during the incubation time of the crude juice (■), at 24 ºC under anaerobiosis. The results are presented as a comparison with the cell-free Emmental juice (---×---) that has been adjusted to a base value of 100. The error bars show the variation of the measures for the free amino groups (n = 4) and the free amino acids (n = 6).

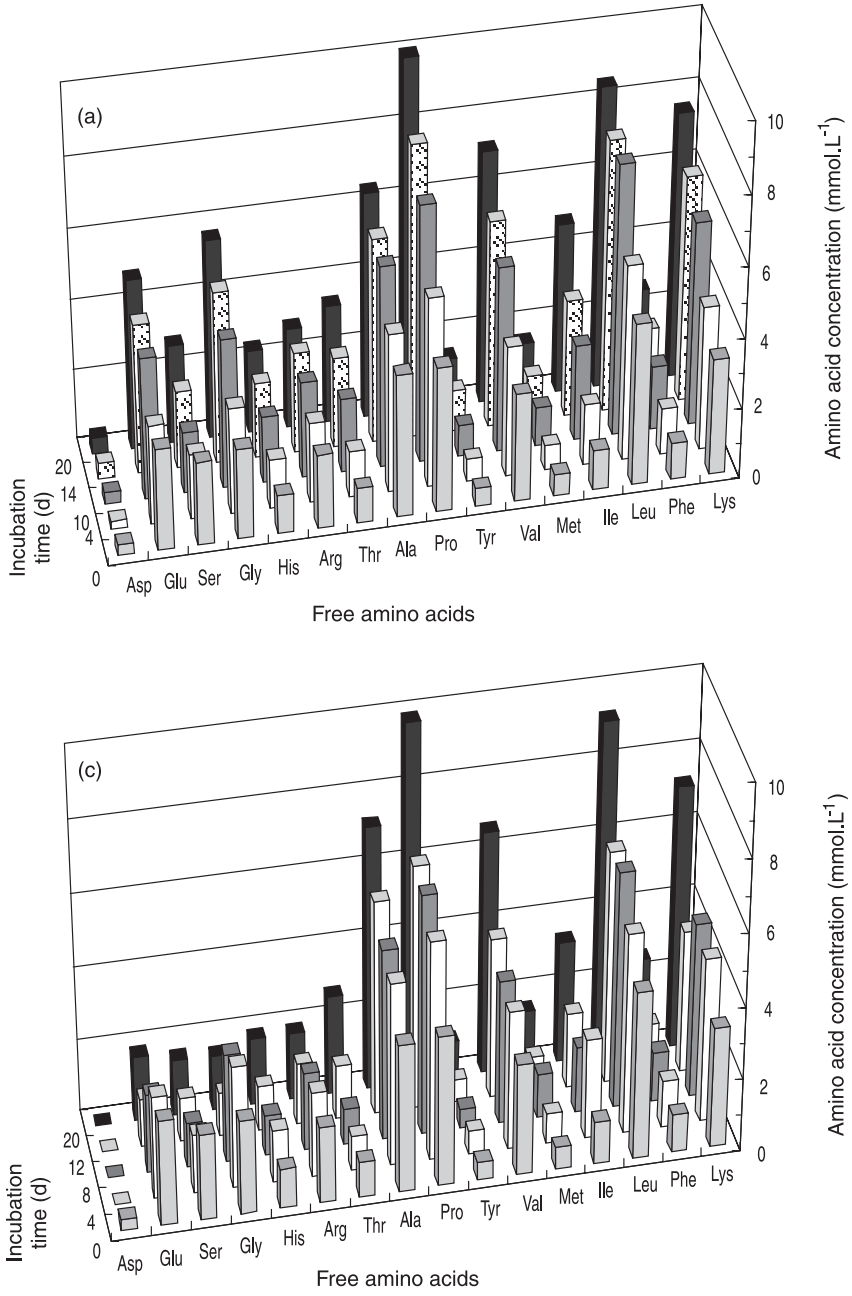

Figure 4. Evolution of free amino acids distribution in the cell-free juice (control) (a), crude juice (b) and juice inoculated with TL33 (c) and TL34 (d) strains of propionibacteria incubated at 24 ºC under anaerobiosis.

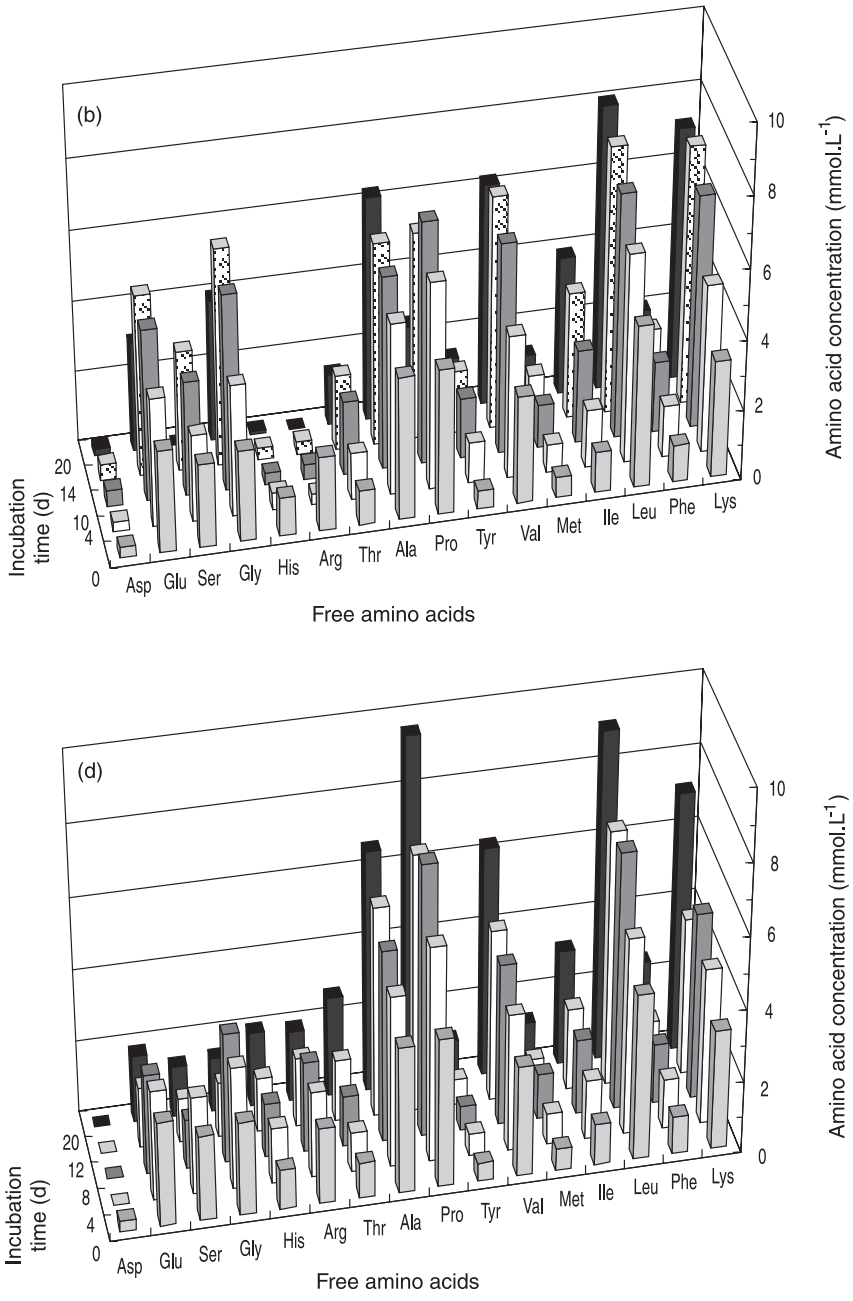

Figure 4 (suite).

their stationary phase. In crude juice, free amino acids were first 16% higher than in cell-free juice due to the action of FHL and then 31% lower from 12 d to the end of incubation time, probably due to PAB activity.

The distribution of free amino acids during the incubation of the different juices is shown in Figure 4. Although the amount of free amino acids increased in the cell-free juice, the distribution of the free amino acids remained unchanged throughout incubation time. Thus, the main released amino acids after 20 d incubation were Glu, Gly, Ala, Pro, Val, Leu and Lys, whereas Asp, Tyr and Met represented less than 2 mmol·L^{-1} (Fig. 4a). In juices containing ripening flora, specific modifications of the amino acid distribution were observed at the end of incubation. The effect of FHL on amino acid distribution was evaluated by comparing the composition of crude juice and cell-free juice up to 10 d incubation. During this period, all the amino acids were at a higher concentration in crude juice, except Arg and His which drastically decreased (Fig. 4b). PAB metabolism resulted in an exhaustion of Asp as early as 4 d of incubation, whereas the proportion of Gly, Glu, Ser, Arg and Ile was also significantly lower than in cell-free juice for the two tested strains (Figs. 4c and 4d).

4. DISCUSSION

Emmental juice represents cheese's aqueous phase, in which all the flora are growing during cheese making and ripening. Such a medium contains not only the carbon and nitrogen sources essential for the growth of the ripening flora, but also other potential activators or even inhibitors of growth as well as metabolites, pH, ionic environment and enzymatic systems released by cell lysis of the different flora and especially the thermophilic starters. Most of them remain still unknown at the present time. Emmental juice has already been used as a culture medium for growing PAB [18, 32]. The use of such a culture medium offers a means to test the activity of ripening flora in conditions as close as possible to those of cheese. Moreover, involvement of each ripening flora in secondary proteolysis can be distinguished in juice in contrast to cheese experiments, where the complete absence of FHL has up to now been difficult to achieve [1, 4].

The growth parameters of both PAB strains in Emmental cheese juice were in agreement with those previously observed by Salvat-Brunaud et al. [32], and close to those found in experimental cheese [36] and in French industrial Emmental [37]. Moreover, kinetics of lactate consumption fitted well with the molar ratio given by the Fitz equation [13]. Results also confirm a co-consumption of citrate and lactate during incubation time as shown by Deborde et al. [9]. PAB reached high cell counts in juice (~10^{10} ufc·mL^{-1}). This value is at the top of those generally observed in cheese (1 to 5×10^9 ufc·g^{-1}), which correspond to 0.2 to 1×10^{10} ufc·mL^{-1} of the aqueous phase.

Concerning FHL, to our knowledge, they had never been grown in cheese juice. Like PAB, FHL reached high juice cell counts, corresponding to the highest values observed in Emmental cheese (4.0×10^8 ufc·mL^{-1}) [37]. In our case, the FHL flora growing in juice was mainly composed of *L. paracasei* species, according to the results observed in Emmental cheese as well as in most semi-hard or hard cheeses [3, 11, 24, 37]. FHL growth in Emmental juice was concomitant with citrate consumption and subsequent formate and acetate production, in molar ratio close to that observed for *L. rhamnosus* by Jimeno et al. [18]. Some FHL strains, notably those citrate-negative, can also use other energy sources, such as ribose [30]. Due to the high populations reached in juice by both flora, an enhanced effect on proteolysis can be

expected in juice compared to cheese. Moreover, cultures in cheese juice can be extended until substrate exhaustion, which will favour cell lysis [25]. Nevertheless, this study shows that the enzymes released from the thermophilic starters contribute markedly more to secondary proteolysis than PAB and FHL.

Growth of both PAB strains resulted in a similar decrease in peptides and free amino acids throughout incubation time, although these strains differ in vitro in their peptidase and autolytic activity, with TL34 being slightly higher than TL33 [23] for both types of activities [22]. *P. freudenreichii* TL33 showed a viability loss of 67% of the population but our results suggest that either no lysis occurred or that it was limited, as observed in Emmental cheese [40]. Our results are in agreement with the generally admitted view that PAB do not contribute to cheese proteolysis [34], although they have been proposed by some authors as possible contributors within raw milk flora [4]. PAB have been shown to synthesise Pro under in vitro conditions mimicking the Emmental environment [19], but our results show that PAB were not significantly involved in the production of Pro in the juice since this amino acid was already released at a high level in cell-free juice by the pool of proteolytic enzymes. In contrast, they modified the amino acid profile by consuming large amounts of Asp, Ser, Gly and Glu, according to the results obtained in laboratory media [7, 28].

FHL contributed to the increase in amino groups (+13%) and free amino acids (+16%) in juice, which indicate that at least a part of the bacterial cells lysed and subsequently released active peptidases in juice. Cells counts on FH medium did not decrease during incubation, but it could hide a viability decrease of some strains among the population, as already observed in cheese [8]. FHL possess high autolytic activity [3, 12] and peptidase activity [33, 38]. The raw milk flora of Swiss-type cheese, composed of FHL and PAB has been shown to generally increase proteolysis indexes during ripening, i.e. from 20 to 80% increase in small peptides and amino acids depending on the origin of the raw milk [10]. This ability is highly strain-dependent, as shown by numerous experiments using pure or mixed cultures of FHL added as starter adjuncts in Cheddar cheese [27]. The moderate increase in proteolysis that we observed during crude juice incubation is the result of the combined effect of two lactobacilli species and very probably several strains for each species.

In conclusion, we showed that FHL more than PAB can contribute to the increase in Emmental proteolysis towards its basal level due to starter enzymes. Each type of ripening flora, however, gives its proper qualitative contribution to the free amino acid profile of juice and both flora are required to have a distribution close to that found in a ripened Emmental, characterised by low amounts of Arg and Asp [21].

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