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Autolysis and intracellular enzyme release from cheese related dairy lactobacilli*

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Abstract — The ability of *Lactobacillus helveticus, Lb. delbrueckii* subsp. *bulgaricus, Lb. delbrueckii* subsp. *lactis, Lb. casei, Lb. plantarum, Lb. fermentum* and *Lb. brevis* to lyse under various conditions of temperature, sodium chloride concentration and pH was investigated. Intracellular enzymes released from the cells in vitro and in a cheese system were also considered. A temperature close to the optimun temperature for growth, a pH varying from 5.5 to 6.5 and a sodium chloride concentration varying from 0.5 to 1.0 M as well as a freezing and thawing treatment seem to be the optimal conditions for cell autolysis. Enzymes release in vitro and in the cheese were found to be related to the rate of autolysis of the cells. © Inra/Elsevier, Paris

autolysis / *Lactobacillus* / aminopeptidase / ripening / cheese

Résumé — Autolyse et relargage des enzymes intracellulaires des lactobacilles apparentés au fromage. L'autolyse des bactéries *Lactobacillus helveticus, Lb. delbrueckii* subsp. *bulgaricus, Lb. delbrueckii* subsp. *lactis, Lb. casei, Lb. plantarum, Lb. fermentum* et *Lb. brevis* a été étudiée dans des conditions différentes de température, pH, et concentration en chlorure de sodium. Le relargage des activités enzymatiques in vitro et dans un fromage modèle a aussi été pris en considération. Les résultats obtenus montrent qu'une température proche de la température optimale de croissance de la bactérie, un pH variant de 5,5 à 6,5 selon les souches, ainsi qu'un traitement de congélation/décongélation sembleraient être les conditions optimales pour l'autolyse des cellules de *Lactobacillus* étudiées. Il a aussi été démontré que les souches montrant une forte autolyse relarguent leurs enzymes intracellulaires plus rapidement dans le caillé. © Inra/Elsevier, Paris

autolyse / *Lactobacillus* / aminopeptidase / affinage / fromage

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1. INTRODUCTION

Autolysis could be defined as the spontaneous desintegration of the bacterial cell, this process is of great importance during cheese ripening because it leads to the release of the intracellular enzymes that are now known to play a key role in the textural changes occurring during ripening and, for the development of the characteristic flavour of cheese.

Studies on the autolytic properties of cheese related micro-organisms have started in the early forties [16] but, it is only in the late eighties that real interest was directed towards the autolysis process and its possible impact on cheese ripening [4, 8, 14].

Several authors determined the rate of autolysis at different temperatures [2, 15, 21, 24, 31, 34]; the general conclusion indicates that the optimum temperature for autolysis varies according to the organism tested. In a limited number of studies, high rates of autolysis were measured at low temperatures [34].

As far as the influence of pH on the rate of autolysis was concerned, an optimum pH close to neutrality was observed on several occasions [26, 28]. Optimum autolysis in the acidic range of pH were also reported [9].

Enzyme release in cheese was first reported by Law et al. [18] who described the release of intracellular dipeptidase from starter lactococci during Cheddar cheese ripening.

Twenty years later, Wilkinson et al. [35] found higher enzyme release in cheeses ripened at 10 °C when compared to cheeses ripened at 4 °C.

El Soda et al. [13] demonstrated that strains showing high autolytic properties in vitro will release their intracellular enzymes at a faster rate in a cheese system. Chapot-Chartier et al. [5] noticed significant differences in the rate of enzyme release in cheese made with *Lactococcus lactis* subsp. *cremoris* AM2 and *L. lactis* subsp. *lactis* NCDO763. In fact the release of enzyme from *L. lactis* subsp. *cremoris* AM2 was significantly higher when compared to *L. lactis* subsp. *lactis* NCDO763.

Attention was also given to the enzymes involved in the autolytic process. A detailed description of the first attempts to characterize the autolysins of lactic acid bacteria can be found in Chapot-Chartier [4]. Polyacrylamide gel electrophoresis was recently used to compare strains according to the numbers of autolysis bands. Using this method, Valence and Lortal [33] detected seven autolytic activity bands in *Lb. helveticus* ISCL5. Partial purification of the enzymes was also described. A similar study was also conducted by Østlie et al. [30] on *Lactococcus* strains where two to five lytic bands were detected. Glycosidase as well as N-acetyl muramyl L-alanine amidase activities were present in some of the strains.

The aim of the present contribution is to compare the autolytic properties of different *Lactobacillus* species and to determine the different factors affecting the process. Release of intracellular enzymes during autolysis in vitro as well as in a cheese system were also considered.

2. MATERIALS AND METHODS

2.1. Cultures and growth conditions

The micro-organisms used in this study were: *Lactobacillus helveticus* CNRZ 32 and 303, *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 369 and 418, *Lb. delbrueckii* subsp. *lactis* CNRZ 250 and 242, *Lb. plantarum* CNRZ 425 and 73, *Lb. fermentum* CNRZ 229 and *Lb. brevis* CNRZ 423. *Lb. casei* CNRZ 62 and *Lb. casei* UL1 were obtained from Université Laval, Quebec, Canada.

For the preparation of cells, 1 000 mL of MRS [10] were inoculated with 3 % of an active culture of the *Lactobacillus* strain. Cell growth phases were monitored by measuring the absorbance at 650 nm using an LKB Pharmacia Nova Spectrophotometer II. After 8–12 h, early stationary

phase cells were harvested by centrifugation at 2 800 *g* for 20 min at 4 °C. The optical density and times corresponding to the early stationary phase cells are as indicated in *table I*. The pellet was then washed twice with 0.01 M potassium phosphate buffer pH 7.0. The resulting bacterial pellet was resuspended in 0.2 M potassium phosphate buffer pH 7.0.

2.2. Measurement of the rate of autolysis

The cell suspension was set at an optical density of 0.8 to 1.0. The percentage decrease in optical density at 650 nm after incubation for different time intervals was used as a measure of the autolytic activity. The experiments were run in triplicate.

2.3. Influence of the physiological age of the cells on their autolytic properties

Cells grown in MRS broth at 30 °C for *Lb. plantarum, Lb. brevis* and *Lb. casei* or at 37 °C for *Lb. helveticus, Lb. delbrueckii* subsp. *bulgaricus, Lb. delbrueckii* subsp. *lactis* and *Lb. fermentum* were harvested by centrifugation at different stages of growth: exponential growth phase, early stationary phase and stationary phase. The harvested cells were washed twice in 0.01 M potassium phosphate buffer pH 7. The rate of autolysis was then determined as described previously.

2.4. Effect of temperature on the rate of autolysis

Experiments were carried out in potassium phosphate buffer 0.2 M pH 7.0 at the following temperatures: 10, 20, 30, 40 and 50 °C.

2.5. Effect of sodium chloride concentration on the rate of autolysis

Experiments were carried out in potassium phosphate buffer 0.2 M pH 7.0 at 30 °C for *Lb. plantarum, Lb. brevis* and *Lb. casei* or at 37 °C for *Lb. helveticus, Lb. delbrueckii* subsp. *bulgaricus, Lb. delbrueckii* subsp. *lactis* and *Lb. fermentum* at the following concentrations of sodium chloride: 2, 1, 0.5 and 0.1 M.

2.6. Effect of pH on the rate of autolysis

Experiments were carried in potassium phosphate buffer 0.2 M at 30 °C for *Lb. plantarum, Lb. brevis* and *Lb. casei* or at 37 °C for *Lb. helveticus, Lb. delbrueckii* subsp. *bulgaricus, Lb. delbrueckii* subsp. *lactis* and *Lb. fermentum* at the following pH values: 4.5, 5.5, 6.5, 7.5 and 8.5.

Table I. The optical density and times corresponding to the early stationary phase of several lactobacilli.

Tableau I. Densités optiques et temps correspondants au début de phase stationnaire de différentes souches de lactobacilles.

Strains	Optical density	Time (h)
Lb. helveticus CNRZ 32	1.45	10
Lb. helveticus CNRZ 303	1.40	12
Lb. casei CNRZ 62	1.35	9
Lb. casei CNRZ RD1	1.25	10
Lb. plantarum CNRZ 425	1.55	8
Lb. plantarum CNRZ 73	1.40	9
Lb. delbrueckii subsp. *bulgaricus* CNRZ 369	1.50	9
Lb. delbrueckii subsp. *bulgaricus* CNRZ 418	1.35	12
Lb. delbrueckii subsp. *lactis* CNRZ 250	1.25	11
Lb. delbrueckii subsp. *lactis* CNRZ 242	1.30	12
Lb. fermentum CNRZ 229	1.20	11
Lb. brevis CNRZ 423	1.35	9

2.7. Influence of lysozyme concentration on the rate of autolysis

In order to follow the influence of lysozyme concentration on the rate of autolysis, the cells were resuspended to give a final optical density of 0.8 to 1.0 in the appropriate conditions predetermined for each strain and in the presence of 5 or 10 mg lysozyme·mL^{-1} of the cell suspension. The mixture was incubated at the desired temperature and the rate of autolysis measured as previously described.

2.8. Measurement of protein and enzyme release during cell autolysis

Aminopeptidase, dipeptidylaminopeptidase and protein release during cell autolysis were measured as follows: aliquots of the autolysing cell suspensions set at the desired conditions of buffer molarity, pH and sodium chloride concentration and incubated at the desired temperature were collected at different time intervals and centrifuged at 2 800 *g* for 10 min at 4 °C. The resultant supernatants containing the intracellular enzymes were stored at –20 °C, then used to determine the protein concentration of the released cell material and to measure the aminopeptidase and dipeptidylaminopeptidase activities.

2.9. Aminopeptidase and dipeptidylaminopeptidase activities

The aminopeptidase activity was measured according to the procedure described by El Soda and Desmazeaud [12]. The substrate used was L-leucyl paranitroanilide (Leu-*p* NA). The same procedure was also used to follow the dipeptidylaminopeptidase activity. The substrate used for this purpose was Arg-Pro-paranitroanilide (Arg-pro-*p* NA). One unit of enzymatic activity was defined as that amount of enzyme producing a variation of 0.01 unit·min^{-1} of absorbance under the present assay conditions. The specific activity was defined as the number of activity units·mg^{-1} of protein which is present in the supernatant.

2.10. Protein determination

The protein concentration was estimated according to the method of Lowry et al. [23].

2.11. Cheese manufacture

2.11.1. Ras cheese curd

Ras cheese was manufactured using laboratory scale cheese making equipments according to Abd-El-Tawab [1]. However, no starter was added to the cheese milk and lactic acid was used to secure acid development during cheese making (65 mL /48 L milk to reach a pH of 5.76). Early stationary phase cells were collected from l L of MRS broth, the resultant pellet was washed twice in 0.01 M phosphate buffer pH 7.0 and then resuspended in a volume of the same buffer equivalent to 1/10 of the volume of the growth media. The bacterial suspension which was frozen and thawed twice was added to 8 L of the milk.

In order to study the influence of lysozyme, 7 g of commercial lysozyme preparation (Genencor USA) per 8 kg of milk were added to the cell suspension prior to their addition to the milk. The cheese was ripened at 12 °C for 45 days.

2.11.2. Determination of aminopeptidase in the cheese extracts and in the whey

The aminopeptidase activity present in cheese was measured in extracts of ground cheese prepared by the method described by El Abboudi et al. [11]. Cheese (20 g) was mixed with 40 mL of phosphate buffer (0.01 M pH 7.0) in a mortar. The samples were then centrifuged for 30 min at 5 000 *g* at 4 °C. After removing the upper solid fat layer, the soluble fraction was recovered and centrifuged again under the same conditions. The resulting cheese extract was used for determination of the peptidase activities.

Whey samples were collected after cutting and scalding for the determination of aminopeptidase activity.

3. RESULTS AND DISCUSSION

3.1. Effect of several factors on the autolytic properties of different Lactobacillus species

The results concerning the influence of several factors on the autolytic properties of twelve strains of lactobacilli (*figure 1a, b, c, d*) indicate that in most cases maximum

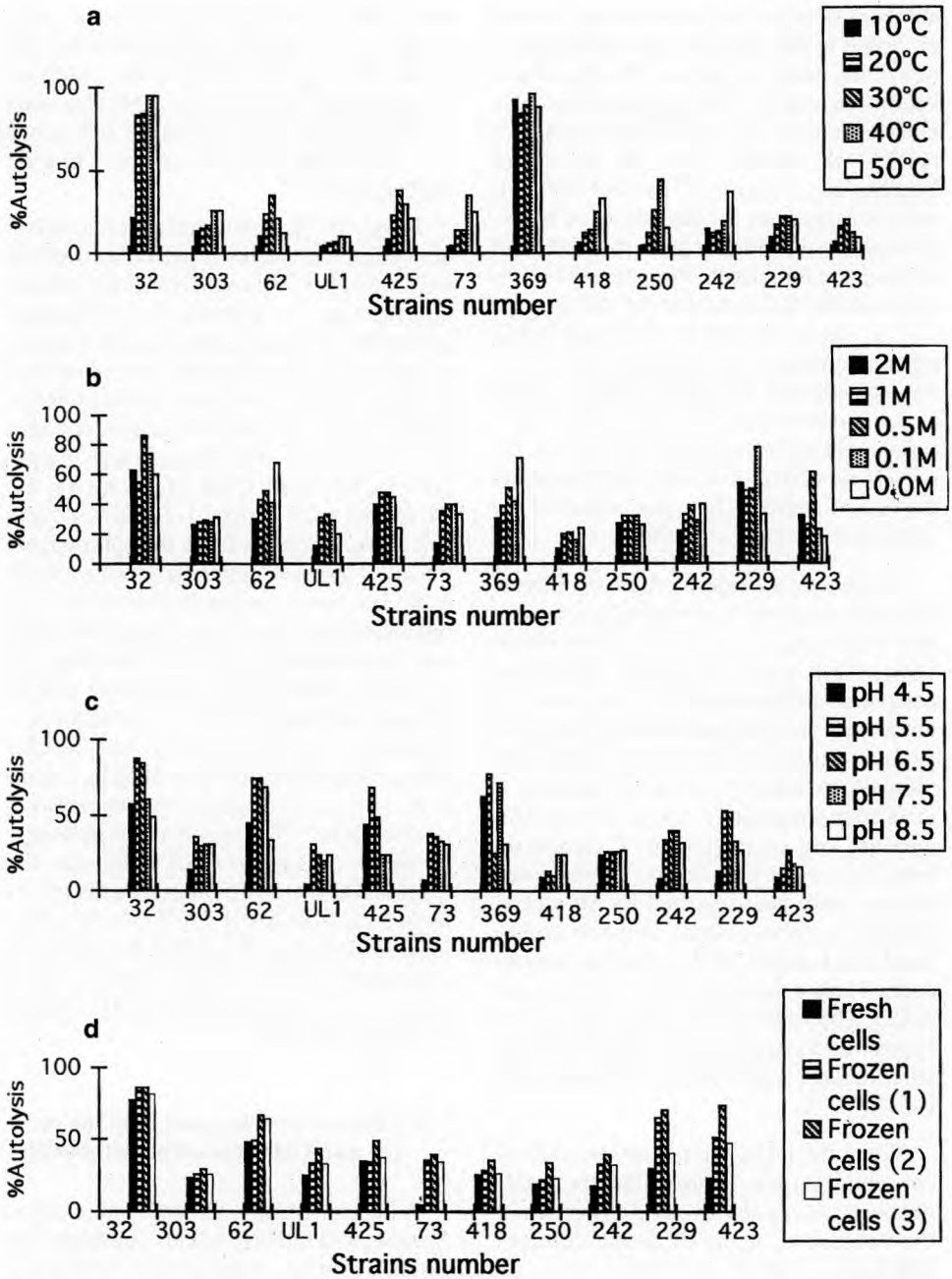

Figure 1. Effect of temperature (**a**), Sodium chloride concentration (**b**), pH (**c**) and freezing and thawing (**d**) on the autolysis of several lactobacilli after 24 hours.

Figure 1. Influence de la température (**a**), de la teneur en chlorure de sodium (**b**), du pH (**c**) et des cycles de congélation, décongélation (**d**) sur l'autolyse de plusieurs souches de lactobacilles après 24 heures d'incubation.

autolysis occurred at a temperature similar or closed to the optimum growth temperature of the micro-organisms. Similar observations were also made by Lortal et al. [21] in the case of *Lb. helveticus* CNRZ 414. Our results however differ from the findings of Neujahr and Logardt [25] where the optimum temperature for the autolysis of *Lb. fermentum* was higher than the optimum temperature for growth. From *figure 1a*, it is also possible to notice that the rate of autolysis is strain dependent. Although maximum autolysis for *Lb. helveticus* was 40 °C, 95 % of strain *Lb. helveticus* CNRZ 32 lysed at this temperature while only 26 % autolysis could be measured in the case of *Lb. helveticus* CNRZ 303; other differences in the rate of autolysis between strains of the same species can also be seen.

In general, the rate of autolysis increased with increases in incubation temperature in the range of 10–40 °C. All the strains showed little autolysis at 10 °C, while their behaviour differed at 50 °C. Our results in this respect are comparable to the findings of Bie and Sjöstrom [2], who measured an increase in autolysis with the increase in incubation temperature. They also could not measure any autolysis at 6 °C during the first 5 days of the experiment. Similar observations were also reported by Mou et al. [24] for *L. lactis* subsp. *cremoris* and by Sund and Linder [32] for viridans streptococci. Higher autolysis at 40 °C were also detected by Lemee et al. [19] for *Propionibacterium freudenreichii* CNRZ 725. The latter authors also noticed very little autolysis at 10 °C.

When the influence of sodium chloride concentration was investigated, the results obtained (*figure 1b*) revealed differences in the behaviour of the different cultures. Although *Lb. helveticus* CNRZ 32, *Lb. casei* UL1, *Lb. plantarum* CNRZ 425, *Lb. fermentum* CNRZ 229 and *Lb. brevis* CNRZ 423 showed higher levels of autolysis in the presence of sodium chloride, the rest of the strains were either not influenced by the presence of salt or autolysed at lower rates when sodium chloride was added to the cell suspension. In their work on the autolysis of lactococci, Vegarud et al. [34] also noticed differences in the autolytic behaviour of their cultures towards sodium chloride concentration.

Figure 1c illustrates the rate of autolysis of the different strains at pH values varying from 4.5 to 8.5. The results obtained indicate that the rate of autolysis is significantly influenced by the pH value. It is for instance possible to notice that for *Lb. helveticus* CNRZ 32, the rate of autolysis measured at pH 4.5 was 57 % while the value reported at pH 5.5 was 87 %. The obtained rate of autolysis for *Lb. casei* CNRZ 62 were 45, 74, 74, 68 and 34 % at pH 4.5, 5.5, 6.5, 7.5 and 8.5, respectively. As far as the optimum pH for autolysis was concerned, most strains exhibited maximum autolysis at pH 5.5. An optimum pH for autolysis in the acidic range was also reported by Coyette and Ghuysen [7] for *Lactobacillus acidophilus* and by Ohmiya and Sato [29] for *Lactobacillus acidophilus, L. helveticus* and *L. casei*. Similar observations were also described by Lemee et al. [19] for *Propionibacterium freudenreichii* CNRZ 725. *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 369 was distinguished from the other strains by its higher autolysis at pH 7.5 (*figure 1c*). *Lactobacillus delbrueckii* subsp. *bulgaricus* is comparable in that respect to the *L. lactis* subsp. *cremoris* strains HP and ML studied by Mou et al. [24].

3.2. Effect of freezing and thawing on the autolysis of several lactobacilli

As a general rule, the cells subjected to freezing and thawing cycles autolysed at a faster rate when compared to fresh cells. The differences in the rate of autolysis between fresh cells and cells subjected to freezing and thawing differed according to the strain tested (*figure 1d*). In the case of *Lb. fermentum* CNRZ 229, 30 % autolysis could

be measured with fresh cells after 48 h of incubation,while the value was 71 % when the cells were subjected to two cycles of freezing and thawing. The corresponding values in the case of *Lb. casei* CNRZ 62 were 48 % and 67 % respectively. Our findings in that respect are comparable to the work of Ohmiya and Sato [29] who demonstrated that the rate of autolysis of *Lb. helveticus* stored at –20 °C overnight and then thawed was 45 % higher when compared to cells stored at 3 °C for the same period of time. Freezing and thawing cycles had little effect on the rate of autolysis of *Lb. helveticus* CNRZ 32 and 303.

When the number of freezing and thawing cycles were compared, it clearly appeared that cells subjected to two cycles of freezing and thawing autolysed faster than cells subjected to one or three cycles of freezing and thawing. The decrease in the rate of autolysis after the third cycle may be due to a partial inhibition of the autolysis due to the physical treatment.

3.3. Influence of the physiological age of the cells on the rate of autolysis for different strains of lactobacilli

The effect of harvesting *Lb. helveticus* CNRZ 32, *Lb. casei* CNRZ 62, *Lb. plantarum* CNRZ 425 and *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 369 at different stages of growth on the rate of autolysis is shown in *figure 2*. The results obtained indicate an increase in autolysis during exponential growth reaching maximum activity before transition to the stationary phase, this was then followed by a marked decrease in the autolytic activity. For instance, 80 % autolysis could be measured in the case of the cells of *Lb. helveticus* CNRZ 32 harvested during exponential growth, while only 16 % autolysis was detected in the cells harvested during late stationary phase. Similar trends were also observed for *Lb. casei* CNRZ 62, *Lb. plantarum* CNRZ 425 and *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 369. Our results on the effect of the physiological age of the cells on the rate of autolysis are comparable to the results of Neujahr and Logardt [25] Lortal et al. [22] and Ohmiya and Sato [29] on lactobacilli and Mou et al. [24] and Niskasaari [26] on *L. lactis* subsp. *cremoris*. Similar observations were also described by Lemee et al. [19] for *Propionibacterium freudenreichii* CNRZ 725. In contrast, strains of viridans streptococci showed the identical autolysis in both exponential and stationary phases of growth [32]. It should, however, be noticed that autolysin activity was detected in both log and stationary cells of *Lb. acidophilus* [7] but, the action of the enzyme on stationary phase cells was inhibited.

3.4. Autolytic properties of several lactobacilli under optimum conditions and cheese ripening conditions

The combined action of pH, temperature, NaCl concentration and freezing and thawing were studied in the presence and absence of lysozyme at the optimal conditions for cell lysis. A similar study was also undertaken at conditions close to cheese ripening, which were a pH of 5.5, 0.5 M NaCl and an incubation temperature of 10 °C.

Figure 3a illustrates the rate of autolysis of the four *Lactobacillus* species under optimum conditions for cell autolysis and indicates a gradual increase in the rate of autolysis of the different strains as a function of time, which was highest in the case of *Lb. plantarum* CNRZ 425, followed by *Lb. helveticus* CNRZ 32, *Lb. casei* CNRZ 62 and *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 369. In their comparative study on the autolytic properties of the lactobacilli, Ohmiya and Sato [29] also noticed higher rates of autolysis in *Lb. helveticus* when compared to *Lb. casei*.

In the presence of lysozyme, higher rates of autolysis could be measured after 1 h of incubation. The values were 89 % and 68 %

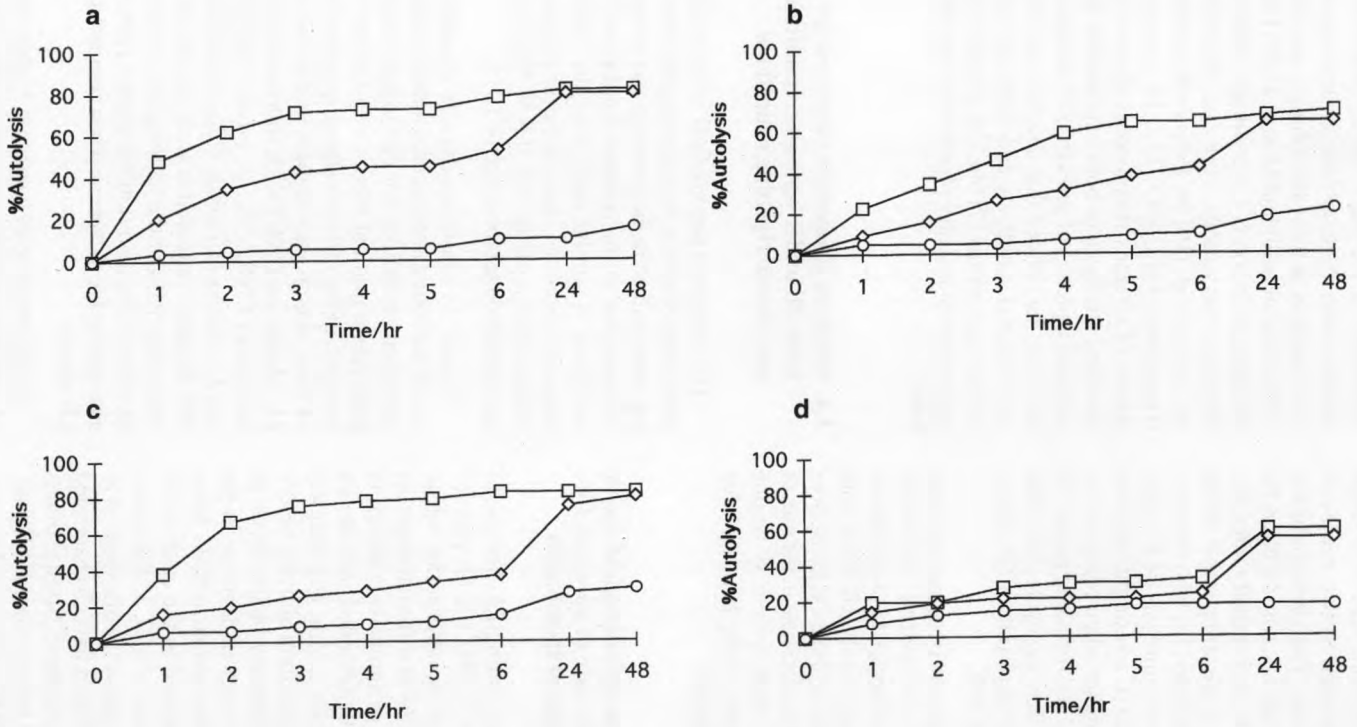

Figure 2. Influence of the physiological age of the cells on the rate of autolysis for different strains of lactobacilli: *Lb. helveticus* CNRZ 32 (**a**), *Lb. casei* CNRZ 62 (**b**), *Lb. plantarum* CNRZ 425 (**c**) and *Lb. bulgaricus* CNRZ 369 (**d**). Cells were incubated at 35 °C in 0.2 M phosphate buffer pH 5.5 containing 0.5 M sodium chloride (frozen cells 1 cycle). □ Exponential phase cells; ◇ early stationay phase cells; ○ late stationary phase cells.

Figure 2. Influence de l'âge physiologique des cellules sur la vitesse d'autolyse des différentes souches de lactobacilles : *Lb. helveticus* CNRZ 32 (**a**), *Lb. casei* CNRZ 62 (**b**), *Lb. plantarum* CNRZ 425 (**c**) et *Lb. bulgaricus* CNRZ 369 (**d**). Cellules incubées à 35 °C en tampon de phosphate 0,2 M et pH 5,5 contenant 0,5 M de chlorure de sodium (un cycle de congélation/décongélation). □ Phase exponentielle ; ◇ début de la phase stationnaire ; ○ fin de la phase stationnaire.

for *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 369 and *Lb. helveticus* CNRZ 32, respectively. The autolysis then reached a plateau, maintained during the rest of the incubation time. The same trend, but to a lower extent, could also be observed for *Lb. plantarum* CNRZ 425 and *Lb. casei* CNRZ 62 (*figure 3b*).

When the cell suspensions were incubated under cheese ripening conditions very little or no autolysis could be measured during the first 6 h of incubation. This was then followed by a gradual increase in the % autolysis, which reached 79 % for *Lb. helveticus* CNRZ 32, 54 % for *Lb. casei* CNRZ 62, 34 % for *Lb. plantarum* CNRZ 425 and 17 % for *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 369 after 120 h (*figure 4a*). As a general rule, the two strains following the thermobacterium group were more sensitive to lysozyme when compared to *Lb. casei* CNRZ 62 and *Lb. plantarum* CNRZ 425. When lysozyme was added to the cell suspension (*figure 4b*), autolysis could be measured from the first hour of incubation in both *Lb. helveticus* CNRZ 32 and *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 369 and increased gradually during the 120 h of incubation used for the experiment. For the two other strains, the lag phase observed in the absence of lysozyme was also noticed in the presence of the lytic enzyme. Positive effect

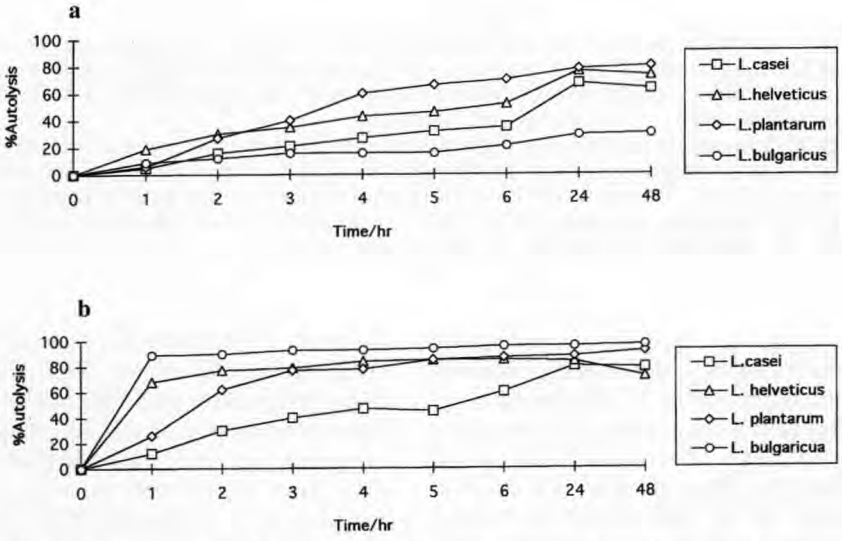

Figure 3. Autolytic properties of several lactobacilli (optimum conditions for autolysis). Frozen and thawed cells (2 cycles) were incubated at 30 °C (*L. casei* 62 and *L. plantarum* 425) or 40 °C (*L. helveticus* 32 and *L. delbrueckii* subsp. *bulgaricus* 369) in 0.2 M phosphate buffer pH 5.5 (*L. helveticus* 32, *L. delbrueckii* subsp *bulgaricus* 369 and *L. plantarum 425*) or pH 6.5 (*L. casei* 62) containing 0.5 M sodium chloride (*L. helveticus* 32 and *L. delbrueckii* subsp *bulgaricus* 369 and *L. casei 62*) or 1 M sodium chloride (*L. plantarum* 425). **a**. In the absence of lysozyme. **b**. In the presence of 5 mg/mL lysozyme.

Figure 3. Propriétés autolytiques de différentes souches de lactobacilles (conditions optimales de l'autolyse). Les cellules ont été congelées et décongelées (2 cycles) et incubées à 30 °C (*L. casei* 62 et *L. plantarum* 425) ou 40 °C (*L. helveticus* 32 et *L. delbrueckii* subsp. *bulgaricus* 369) en tampon de phosphate 0,2 M et pH 5,5 (*L. helveticus* 32, *L. delbrueckii* subsp. *bulgaricus* 369 et *L. plantarum 425*) ou pH 6,5 (*L. casei* 62) 0,5 M de chlorure de sodium (*L. helveticus* 32 et *L. delbrueckii* subsp. *bulgaricus* 369 et *L. casei 62*) ou 1 M de chlorure de sodium (*L. plantarum* 425). **a**. En l'absence de lysozyme. **b**. En présence de 5 mg/mL de lysozyme.

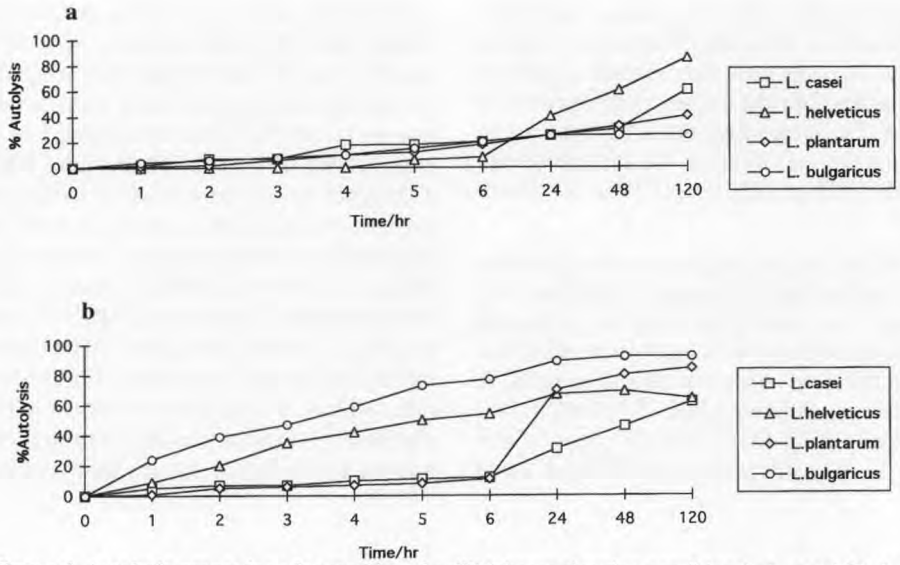

Figure 4. Autolytic properties of several lactobacilli (cheese ripening conditions): *L. casei* 62, *L. helveticus* 32, *L. plantarum* 425 and *L. bulgaricus* 369. Frozen and thawed cells (2 cycles) were incubated at 10 °C in 0.2M phosphate buffer pH 5.5 containing 0.5 M sodium chloride. **a**. In the absence of lysozyme. **b**. In the presence of 5 mg/mL lysozyme.

Figure 4. Influence de l'âge physiologique des cellules sur la vitesse d'autolyse des différentes souches de lactobacilles (conditions de l'affinage des fromages) : *L. casei* 62, *L. helveticus* 32, *L. plantarum* 425 et *L. bulgaricus* 369. Les cellules ont été congelées et décongelées (2 cycles) et incubées à 10 °C en tampon phosphate 0,2 M et pH 5,5 contenant 0,5 M de chlorure de sodium. **a**. En l'absence de lysozyme. **b**. En présence de 5 mg/mL de lysozyme.

of lysozyme on cell lysis was previously demonstrated in *L. lactis* subsp. *cremoris* by Niskasaari et al. [27]. The resistance of lactobacilli to lysozyme was also previously described in *Lb. fermentum* by Logart and Neujahr [20]. The resistance of a strain to lysis may be due to the absence of an autolytic system or effective regulation of the autolytic enzymes by membrane-associated lipoteichoic acid and related compounds [6].

3.5. Aminopeptidase and dipeptidylaminopeptidase release during the autolysis of several lactobacilli under optimum conditions and cheese ripening conditions

The results reveal the presence of active aminopeptidase and dipeptidylaminopeptidase activity in *Lb. helveticus* CNRZ 32, *Lb. casei* CNRZ 62 and *Lb. plantarum* CNRZ 425. *Lb. helveticus* CNRZ 32 is distinguished from the two other strains by its higher aminopeptidase and dipeptidylaminopeptidase activities; it showed 68.4 % and 43.6 % higher aminopeptidase activity when compared to *Lb. plantarum* CNRZ 425 and *Lb. casei* CNRZ 62, respectively. The corresponding values for dipeptidylaminopeptidase were 40.7 % and 37.5 %, respectively. *Lb. plantarum* CNRZ 425 was also slightly more active than *Lb. casei* CNRZ 62.

The release of the intracellular aminopeptidase and dipeptidylaminopeptidase enzymes from the bacterial cells was then studied in *Lb. helveticus* CNRZ 32, *Lb. casei* CNRZ 62 and *Lb. plantarum* CNRZ 425 under the optimum conditions for cell auto-

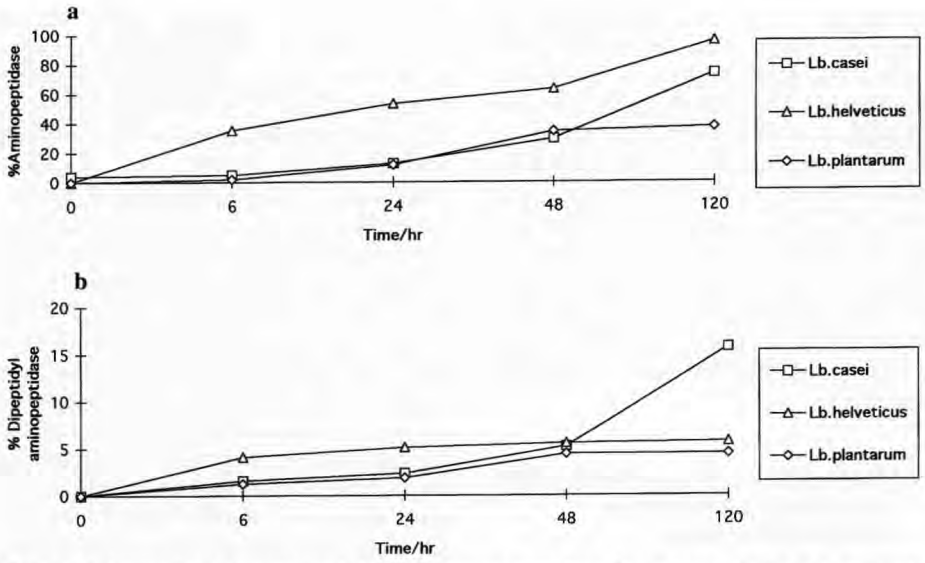

Figure 5. Aminopeptidase (**a**), and dipeptidylaminopeptidase (**b**), release during the autolysis of *L. casei* 62, *L. helveticus* 32, *L. plantarum* 425 and *L. bulgaricus* 369 under cheese ripening conditions: results are expressed as percent of the total extract obtained after grinding the cells.
Figure 5. Relargage de l'activité aminopeptidase (**a**) et dipeptidylaminopeptidase (**b**) pendant l'autolyse de *L. casei* 62, *L. helveticus* 32, *L. plantarum* 425 et *L. bulgaricus* 369 incubées dans les conditions de l'affinage des fromages. Les résultats sont exprimés comme un pourcentage de l'extrait total obtenu après le broyages de cellules.

lysis as well as under cheese ripening conditions (pH 5.5, 0.5 M sodium chloride and a temperature of 10 °C). The results illustrated in *figure 5a* indicate a gradual release of the aminopeptidase and dipeptidylaminopeptidase activities from the cells as a function of time. In the case of *Lb. helveticus* CNRZ 32, the released intracellular peptidase was detected from the first hour of incubation under both the optimum conditions for enzyme release and the cheese ripening conditions. On the other hand, a lag time vaying from 2 to 6 h was necessary before the detection of such enzymes in the case of *Lb. plantarum* CNRZ 425 or *Lb. casei* CNRZ 62. In the three strains tested, the rate of enzyme release was lower when measured in cells incubated under ripening conditions. It was, for instance, possible to measure 53 % aminopeptidase release from *Lb. helveticus* CNRZ 32 after 24 h when the cells were incubated at 10 °C; the corresponding value for cells incubated at 40 °C was 76 %. In the case of *Lb. casei* CNRZ 62, the enzyme released after 24 h was 13 and 84 % for cells incubated at 10 and 30 °C, respectively. A similar trend is also noticed for *Lb. plantarum* CNRZ 425.

Figure 5b also shows that the rate of aminopeptidase release is much higher when compared to the rate of dipeptidylaminopeptidase release in the three species tested. In fact in *Lb. helveticus* CNRZ 32, aminopeptidase release was 76 % after 24 h of incubation at 40 °C, the corresponding value for the dipeptidylaminopeptidase was only 5 %. In the case of *Lb. plantarum* CNRZ 425 and *Lb. casei* CNRZ 62, aminopeptidase release from cells incubated at 30 °C was 36 and 84 %, respectively, for the aminopeptidase, while it was 6 and 10 %, res-

pectively, for dipeptidylaminopeptidase. As a general rule, *Lb. helveticus* CNRZ 32 is distinguished from the other two strains by its higher rate of enzyme release. For cells incubated at 10 °C for 120 h, *Lb. helveticus* CNRZ 32 exhibited 96 % aminopeptidase release. The values obtained for *Lb. casei* CNRZ 62 and *Lb. plantarum* CNRZ 425 were 74 and 29 %, respectively. The percentage release of dipeptidylaminopeptidase under the same conditions for *Lb. helveticus* CNRZ 32, *Lb. casei* CNRZ 62 and *Lb. plantarum* CNRZ 425 were 6, 16 and 4 % respectively.

3.6. Characteristics of Ras cheese made with autolysing cells from Lactobacillus species

The chemical composition of control cheese and cheese with added frozen cells of *Lb. helveticus* CNRZ 32, *Lb. helveticus* CNRZ 32 + lysozyme, *Lb. casei* CNRZ 62, *Lb. plantarum* CNRZ 425 and *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 369 indicated that the different treatments did not affect the composition of Ras cheese through the ripening period (results not shown). The gross composition of the cheese made with *Lb. helveticus* CNRZ 32, *Lb. helveticus* CNRZ 32 + lysozyme, *Lb. casei* CNRZ 62, *Lb. plantarum* CNRZ 425 and *Lb. delbrueckii* subsp *bulgaricus* CNZR 369 treated cheese was rather close to that of control. The mean values for pH, dry matter %, fat % and protein % in the 6 weeks old cheese were 5.1, 69 %, 35.37 %, and 29.56 % respectively.

3.7. Release of peptidase during cheese ripening

The aminopeptidase activity of the different micro-organisms was selected as a marker enzyme for cell lysis. In the case of *Lb. helveticus* CNRZ 32, the aminopeptidase was detected early in the cheese extract. As a matter of fact, 49 aminopeptidase activity units per 100 g dry weight of cheese could be detected from the first day of manufacture. The mean value for the other strains were about 0.18/100 g dry weight of cheese. The corresponding values after one week were 23, 22 and 21/100 g dry weight of cheese for *Lb. casei* CNRZ 62, *Lb. plantarum* CNRZ 425 and *Lb. delbrueckii* subsp. *.bulgaricus* CNZR 369, respectively, while the value was 59/100 g dry weight of cheese for *Lb. helveticus* CNRZ 32. After 2 weeks of ripening, maximum release of the *Lb. helveticus* CNRZ 32 aminopeptidase was reached. Aminopeptidase activity in the cheese was then stable till the end of the ripening time (*figure 6*). A similar trend was also noticed in the other strains but, maximum release was reached after 3 weeks in *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 369, and 4 weeks in *Lb. casei* CNRZ 62 and *Lb. plantarum* CNRZ 425.

In the cheese made with *Lb. helveticus* CNRZ 32 cells treated with lysozyme, the values of the released aminopeptidase were very low and only slightly higher than those of control, while 85 % of the activity was detected in the whey (results not shown) which indicates a rapid release of the enzyme during the early stages of cheesemaking. This finding may explain the lower levels of aminopeptidase in the cheese made with lysozyme treated *Lb. helveticus* CNRZ 32.

The release of intracellular peptidase by autolysis from the cells was previously shown by several authors [17, 18]. Birkeland et al. [3] detected lactate dehydrogenase (LDH) and proline iminopeptidase (PIP) activity in the cheese matrix during the early stages of the ripening period.

El-Soda et al. [13] monitored the release of aminopeptidase activity from a highly autolytic and a poorly autolytic strain of *Lb. casei* in Cheddar cheese. The enzyme could be detected in the cheese manufactured with the highly autolytic strain after 48 h of ripening, while a week was necessary to detect the activity in the cheese made with the strain showing little autolysis.

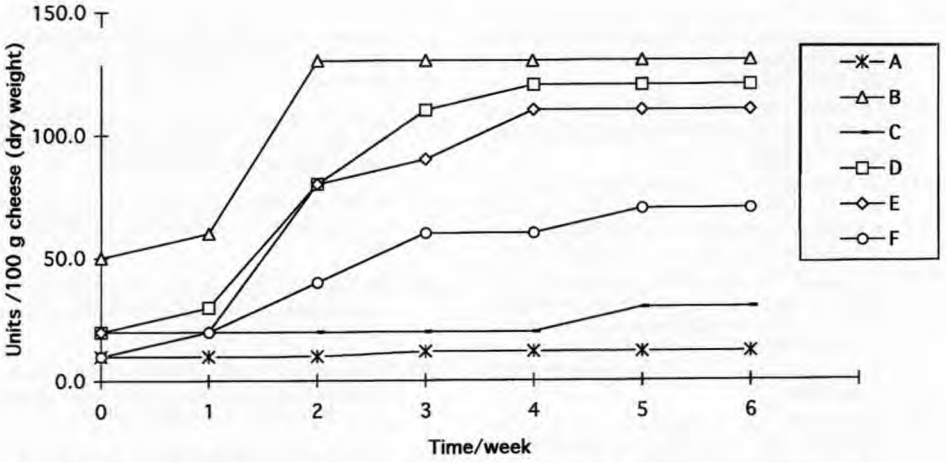

Figure 6. Release of aminopeptidase activity from several lactobacilli incorporated in a cheese system. **A**. Control. **B**. *Lb. helveticus* CNRZ 32. **C**. *Lb. helveticus* CNRZ 32 + 5 mg/mL lysozyme. **D**. *Lb. casei* CNRZ 62. **E**. *Lb. plantarum* CNRZ 425. **F**. *Lb. bulgaricus* CNRZ 369.

Figure 6. Relargage de l'activité aminopeptidase à partir de cellules de lactobacilles incorporées dans un caillé modèle. **A**. Témoin. **B**. *Lb. helveticus* CNRZ 32. **C**. *Lb. helveticus* CNRZ 32 + 5 mg/mL lysozyme. **D**. *Lb. casei* CNRZ 62. **E**. *Lb. plantarum* CNRZ 425. **F**. *Lb. bulgaricus* CNRZ 369.

More recently, Wilkinson et al. [35] noticed that the increase of salt in moisture levels in cheese in the range 0.4–5 % was accompanied by an increase in the activity of lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PDH) and a decrease in post-proline dipeptidyl aminopeptidase (PPDA). Holding the crude cell free extract containing marker intracellular enzymes in a cheese like environment indicated that LDH was significantly more stable than G6PDH or PPDA.

Chapot-Chartier et al. [5] showed that decrease of cell viability was accompanied by the observation of disrupted cells by electron microscopy and the release of intracellular peptidase in the cheese.

4. CONCLUSION

This work indicates the importance of selecting adjuncts' cells according to their autolytic properties and it also emphasizes the necessity to understand the conditions leading to cell autolysis.

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