Original article

Interactions between propionic acid bacteria and thermophilic lactic acid bacteria

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Abstract — The growth of propionic acid bacteria was directly dependent on the concentration of free amino acids in cheese, regardless of the strains of lactobacilli contained in the raw mixed cultures, suggesting that the growth of these bacteria was mainly dependent on the proteolytic activity of lactic acid bacteria. The stimulating effect of *S. thermophilus* was poor probably because this species produces only low amounts of amino acids in milk or cheese. Lactobacilli, which undergo cell lysis shortly after cheese manufacture, produce large amounts of amino acids. Facultatively heterofermentative lactobacilli were shown to reduce the risk of secondary fermentation in Emmental cheese. This interaction was not due to their production of diacetyl, formate or acetate. Propionibacteria able to grow at 14 °C have a greater tendency to induce secondary fermentation compared to those unable to grow at this temperature. © Inra/Elsevier, Paris.

propionic acid bacteria / lactic acid bacteria / facultatively heterofermentative lactobacilli / interaction / secondary fermentation

Résumé — Interaction entre les bactéries propioniques et les bactéries lactiques thermophiles.

L'étude présentée a porté sur l'interaction entre les bactéries propioniques (BP) et 23 levains lactiques, 30 de leurs souches isolées, 36 souches isolées de produits laitiers fabriqués hors de la Suisse, une culture de *S. thermophilus* ainsi que la flore naturelle de deux laits de mélange. La croissance des bactéries lactiques a été réalisée dans des expériences de simulation de fabrication fromagère dans du lait UHT, dont le pH a été maintenu au niveau de celui du fromage par l'ajout de carbonate de calcium. Les lactosérums obtenus ont été utilisés comme source d'azote pour la croissance des BP. Les résultats ont montré que l'intensité de la croissance des BP variait en fonction du lactosérum utilisé. Toutefois, les différences observées ne dépendaient pas spécifiquement de la souche des bactéries lactiques, mais uniquement de la concentration en acides aminés. Les différences entre les intensités de croissance ont pu être supprimées en uniformisant la concentration des acides aminés ajoutés, ce qui suggère que la croissance des BP dépend essentiellement de l'activité protéolytique des bactéries lactiques. L'effet stimulant de *S. thermophilus* sur la croissance des BP a été peu prononcé à cause de la faible concentration des acides aminés libérés. Par contre, les lactobacilles, dont la lyse cellulaire apparaît rapidement après la fabrication du fromage, produisent de grandes quantités d'acides aminés qui stimulent la croissance des BP. Il a été démontré que l'addition de lactobacilles hétérofermentaires

Oral communication at the 2nd Symposium on Propionibacteria, Cork, Ireland, June 25-27, 1998.

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facultatifs aux levains pouvaient réduire le risque de fermentation secondaire dans les fromages d'Emmental en diminuant l'intensité de la croissance des BP. Cette interaction n'est pas due à la production de diacétyl, formiate ou acétate par les lactobacilles hétérofermentaires. Les BP capables de croître à 14 °C ont tendance à induire la fermentation secondaire, ce qui n'est pas le cas des souches de BP incapables de croître à cette basse température. © Inra/Elsevier, Paris.

bactérie propionique / lactobacille thermophile / lactobacille hétérofermentaire facultatif / interaction / fermentation secondaire

1. INTRODUCTION

In Switzerland, Emmental cheese is manufactured exclusively from raw milk. The thermophilic raw mixed-cultures (RMC) used contain approximately the same numbers of *S. thermophilus* and *L. delbrueckii* ssp. *lactis*. These cultures were collected from naturally soured raw milks, used as starters in cheese factories 40 years ago and checked for their quality by manufacturing experimental cheeses. Subcultures of the best of these cultures, containing *S. thermophilus* and *L. delbrueckii* ssp. *lactis* only, were kept frozen or lyophilized. A liquid mixed-culture of propionic acid bacteria (PAB) is also added to the milk. PAB grow during ripening and confer the typical nutty taste to this type of cheese [11, 12, 16]. However, PAB are also responsible for two faults, brown spots and secondary fermentation, which are mostly encountered in cheese manufactured in winter. The cause of the appearance of brown spots is due to insufficient growth of PAB [1] and this fault has almost disappeared from Emmental cheese, whereas it is still observed in semi-hard, Swiss cheeses such as Appenzell, probably due to contaminating PAB in the milk. Secondary fermentation, thought to be caused by excessive growth of PAB during ripening [24, 26], occurs sporadically and alters the quality of Emmental cheese. Raw mixed cultures favouring late fermentation contain *L. helveticus*, suggesting that the high proteolytic activity of this bacterium produces nitrogenous compounds which accelerate the growth rate of PAB at the

beginning of ripening [14, 19, 20]. It has also been observed that the addition of facultatively heterofermentative lactobacilli (FHL) to the milk reduces the risk of secondary fermentation [23] and growth of PAB [15, 17]. These observations indicate that some lactic acid bacteria may have a specific influence on the growth of PAB.

The purpose of this work was to study the influence of the proteolytic activity of the natural milk flora, and single and mixed strains of thermophilic lactic acid bacteria (LAB) on the growth of PAB, during simulated cheese manufacture. These conditions are important since rennet participates in cheese proteolysis at the very beginning of cheese manufacture; the presence of residual chymosin activity has been shown in Emmental cheese, in spite of the high cooking temperature used [5]. Rennet can also influence the growth of raw mixed culture bacteria [3], contrary to our earlier results obtained with a raw mixed culture [2]. These experimental conditions were not taken into consideration in similar studies on the influence of lactic acid bacteria on PAB growth [7, 20, 22].

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were of analytical grade. Trypticase peptone was from Difco (Chemie Brunschwig, Basel, Switzerland). Other chemicals were from Merck (Merck-Schuchardt, Zürich, Switzerland). Sterile filters of 0.20 µm and 0.45 µm were from Sartorius (Sartorius AG, Göt-

tingen, Germany). Resazurin was purchased from Sigma (Division of Fluka Chemie, Buchs, Switzerland).

2.2. Bacteria

The PAB used are mixed cultures which have recently replaced the old PAB culture used since the end of the thirties. The PAB, as well as 23 RMCs containing approximately the same number of *S. thermophilus* and *L. delbrueckii* ssp *lactis* and the pure cultures of *S. thermophilus*, *L. rhamnosus*, and *L. casei* were obtained from our Department of Microbiology. The PAB and RMCs used are produced exclusively by the Federal Dairy Research Institute. Thirty pure cultures of *L. delbrueckii* ssp *lactis* and 3 strains of *S. thermophilus* were isolated from 10 RMCs. Thirty six strains of thermophilic lactic acid bacteria were isolated from various dairy products obtained from Italy, Portugal, Minorca, Turkey, Tadjikistan, Slovenia and Siberia.

2.3. Growth of PAB

PAB were grown in 10 mL of the defined medium, described by Crow [9], modified by omission of cysteine and $(\text{NH}_4)_2\text{SO}_4$ so as to avoid growth of PAB in the absence of a nitrogen source. The filter-sterilized wheys obtained from the simulation assay (1.0 mL/10 mL⁻¹ medium) were used as a nitrogen source and the growth of the bacteria was followed photometrically at 650 nm [2].

2.4. Simulation assay

Simulation assays using RMCs were performed in screw capped 50-mL vials, as described elsewhere [2, 3]. Simulation assays using pure cultures of lactic acid bacteria was modified as follows: 50-mL glass vials stoppered with butyl rubber stoppers to which 1.2 g of CaCO_3 and a magnetic bar were added and sterilized. The assay was performed in 40 mL UHT milk containing 100 µg filter-sterilized resazurin as a redox indicator. After addition of 8 µL liquid rennet, to coagulate the milk in approximately 26 min, and breaking the curd by vigorously shaking the vial, the head-space of the vial was flushed with sterile filtered CO_2 for 20 min. The temperature profile used in Emmental manufacture over the first 24 h, (heating to 53 °C in

35 min, maintaining this temperature for 45 min, and cooling to 30 °C in 22 h) was used. The vials were then incubated at 30 °C over 12 d. Except for the renneting phase, the simulations were stirred constantly during incubation.

2.5. Determination of the proteolytic activity

Free amino acids (FAA) were determined in the whey of the simulation assays using cadmium ninhydrin [4]. X-Prolyl dipeptidyl peptidase activity was determined according to Casey and Meyer [8], using Gly-Pro-AMC (Bachem AG, 4416 Bubendorf, Switzerland) as substrate.

3. RESULTS AND DISCUSSION

Since the growth of LAB in simulation assays can vary depending on the RMCs used, leading to the production of different amounts of lactic acid, it was important to ascertain that the pH value was similar to that of cheese at the beginning of ripening. This condition was obtained, even with the strongest lactic acid producing RMC, by adding CaCO_3 and mixing (figure 1).

3.1. Influence of the natural milk flora on PAB growth

The influence of the natural flora of raw milk was studied by incubating two bulk raw milks, to which rennet and CaCO_3 were added, at 30 °C for 4 d. After incubation, the microflora and the concentration of FAA and lactate were measured (table I). Whey from sample 1 stimulated the growth of PAB-A much more than that from sample 2 (figure 2A). When the volume of whey from sample 1 was adjusted in order to obtain the same final concentration of amino acids in each test tube, the two growth curves were very similar (figure 2B), and independent of the bacteria responsible for the production of amino acids. Sample 1 contained about 5 times more FAA than sample 2. These

Figure 1. Effect of CaCO_3 ($1.2 \text{ g} \cdot 40 \text{ mL}^{-1}$) on the pH values in the simulation assay. UHT milk to which no culture was added ($\blacklozenge$). UHT milk with rennet ($8 \mu\text{L} \cdot 40 \text{ mL}^{-1}$) and raw mixed culture (0.3%) with ($\blacktriangle$) or without ($\blacksquare$) CaCO_3 .

Figure 1. Effet du CaCO_3 ($1.2 \text{ g} \cdot 40 \text{ mL}^{-1}$) sur le pH dans les expériences de simulation. Lait UHT sans ajout de culture ($\blacklozenge$). Lait UHT additionné de présure ($8 \mu\text{L} \cdot 40 \text{ mL}^{-1}$) et de levain lactique ($0,3 \%$) avec ($\blacktriangle$) et sans ($\blacksquare$) CaCO_3 .

results suggest that the greater level of proteolytic activity of sample 1 is responsible for the difference observed between both milks.

3.2. Influence of raw mixed cultures on PAB growth

In order to determine whether raw mixed cultures have a specific influence on the growth of PAB, simulation assays were performed with 23 thermophilic raw mixed cultures used for manufacturing various types of cheeses in Switzerland. The results obtained differed markedly depending on the raw mixed culture used (data not shown). However, after adjustment of the amount of FAA in the whey added, $< 10 \%$ variation

in the growth rate was observed with the 23 RMCs analyzed. These results suggest that none of the cultures had a specific effect on the growth of PAB since only their proteolytic activity was responsible for the growth variations.

3.3. Influence of isolated lactic acid bacteria on PAB growth

The RMCs used in our experiments contain various unknown strains of *L. delbrueckii* ssp. *lactis*. Therefore, one cannot exclude that some strains may have a specific influence on the growth of PAB which could not be observed in the presence of the other strains. This possibility was examined by performing simulation assays with 30

Table I. Biochemical and microbiological composition of the two raw bulk milks used.

Tableau I. Composition biochimique et microbiologique des deux laits crus de mélange utilisés.

Milk	Lactobacilli cfu.g ⁻¹	FHL cfu.g ⁻¹	Salt tolerant organisms cfu.g ⁻¹	Aerobic mesophiles cfu.g ⁻¹	Enterococci cfu.g ⁻¹	D-Lactate mmol.L ⁻¹	L-Lactate mmol.L ⁻¹	FAA, mmol eq leucine
1	> 1 × 10 ⁶	9 × 10 ³	9.4 × 10 ³	> 10 ⁶ × 10	8.5 × 10 ³	307	114	36.5
2	> 1 × 10 ⁶	> 1 × 10 ⁶	4.5 × 10 ⁵	7 × 10 ⁶	8.3 × 10 ⁷	8	117	6.9

The milks were renneted (8 µL of liquid rennet/40 mL UHT milk) and CaCO₃ (1.2 g/40 mL⁻¹ UHT milk) added and incubated for 4 d at 30 °C before being analyzed.
Les laits emprésurés (8 µL de présure liquide / 40 mL lait UHT), contenant du CaCO₃ (1.2 g/40 mL⁻¹ de lait UHT) ont été incubés durant 4 j à 30 °C, avant d'être analysés.
FHL: Facultatively heterofermentative lactobacilli / FHL: Lactobacilles hétérofermentaires facultatifs
FAA: Free amino acids / FAA: Acides aminés libres

Lactobacilli were enumerated on MRS-agar at 38 °C for 3 d, under anaerobic conditions; FHL on FH-agar at 38 °C for 3 d, anaerobically; salt tolerant organisms on mannitol salts agar at 38 °C for 2 d, aerobically; aerobic mesophilic organisms on plate count agar at 30 °C for 3 d, aerobically; enterococci on kanamycin-aesculin-agar at 38 °C for 24 h under aerobic conditions / Les lactobacilles ont été dénombrées sur MRS gélosé à 38 °C, 3 j, en anaérobiose ; FHL sur FH gélosé à 38 °C, 3 j, en anaérobiose ; organismes halotolérants sur gélose mannitol-sel à 38 °C, 2 j, en aérobiose ; organismes mésophiles aérobies sur PCA à 30 °C, 3 j, en aérobiose ; entérocoques sur gélose kanamycine-aesculine à 38 °C, 24 h, en aérobiose.

pure cultures of *L. delbrueckii* ssp. *lactis* isolated from 10 RMCs. However, the growth of the lactobacilli was not satisfactory and gave nonreproducible results. Since satisfactory growth of lactobacilli was obtained with RMCs containing streptococci, 3 pure cultures of *S. thermophilus*, also isolated from the RMCs, were added to the pure cultures of lactobacilli to simulate the RMCs. The results obtained were similar to those obtained with RMCs. It was therefore assumed that the streptococci produced substances which favoured the growth of the lactobacilli. The lactobacilli were then grown in whey obtained after simulation assays performed with streptococci alone but the results again were not satisfactory as they were similar to those obtained in the absence of streptococci. Assuming that the effect of streptococci on the growth of lactobacilli may be due to an improvement in the anaerobic growth conditions produced by the NADH-oxidase of streptococci [21], simulation assays were performed by flushing the head space of the simulation vial with CO₂. Under these conditions, the growth of lactobacilli was similar to that obtained in the presence of streptococci, as measured by the concentration of D-lactate produced. The growth curves of PAB, using wheys of pure cultures of lactobacilli adjusted to the same concentration of amino acids, were very similar, whereas differences were observed when wheys were used before adjustment of the concentration of amino acids (data not shown). These results confirm those obtained with RMCs and could be due to the strains of lactobacilli present in the RMCs, which may have been selected over the years by successive culturing during the development of mixed cultures. This may explain why the characteristics of the strains presently used are very similar.

In order to study a more heterogeneous collection of lactobacilli, 36 strains, able to grow at 45 °C and only producing D-lactate, isolated from milk products manufactured outside Switzerland, were evaluated

Figure 2. Influence of the natural flora of two milks on PAB growth. Before (A) and after (B) adjustment of the concentration of amino acids. Milk 1 (■); milk 2 (□). The natural flora was enriched by incubation of the milks at 30 °C for 4 d after addition of rennet (8 $\mu\text{L}\cdot 40\text{ mL}^{-1}$) and CaCO_3 . In B, the volume of the two wheys was adjusted so that the same level of amino acids was added to the defined medium.

Figure 2. Influence de la flore naturelle de deux laits de mélange sur la croissance des BP avant (A) et après (B) ajustement de la concentration en acides aminés. Lait A (■); lait B (□). La flore naturelle a été enrichie par incubation des laits crus à 30 °C pendant 4 j, après adjonction de présure (8 $\mu\text{L}\cdot 40\text{ mL}^{-1}$) et de CaCO_3 . En B, le volume des deux lactosérums a été ajusté pour ajouter la même concentration d'acides aminés au milieu défini.

in the simulation assay. Again the results obtained showed that the amounts of liberated amino acids varied depending on the strains examined but no difference in the growth of PAB could be detected when the amino acid concentrations were adjusted to the same level (data not shown). These results confirm those obtained with RMCs and isolates from the same RMCs, suggesting that, under our experimental conditions, free amino acids are the sole factor influencing PAB growth.

3.4. Influence of *S. thermophilus* on PAB growth

A simulation assay using 3 strains of *S. thermophilus* isolated from RMCs was

performed. The amount of FAA present in the whey after 4-d incubation at 30 °C was equivalent to 3.2 mmol·leucine·L⁻¹. At this low concentration, which is almost the same as the concentration of FAA in milk, the growth of PAB was poor when compared to the growth observed on addition of peptone (1.0 g·L⁻¹). However, better growth was observed after further addition of 3 mmol of a mixture of amino acids, corresponding to the concentrations of FAA found in Emmental cheese [25] (figure 3), demonstrating that amino acids are probably the limiting factor for PAB growth in our experimental conditions. Important variations in the stimulatory effect of *S. thermophilus* have previously been described by Piveteau et al. [20] who have shown that

Figure 3. Growth of PAB in the defined medium in the presence of peptone (1 g·L⁻¹) (▲), whey after the growth of *S. thermophilus* in the simulation assay (□); further addition to the whey of 3 mmol·L⁻¹ of a mixture of pure amino acids, corresponding to the concentrations of FAA determined in Emmental cheese (■).

Figure 3. Croissance des BP dans le milieu défini en présence de peptone (1 g·L⁻¹) (▲), lactosérum obtenu après croissance de *S. thermophilus* en expérience de simulation (□) et après addition supplémentaire au lactosérum de 3 mmol·L⁻¹ d'une solution d'acides aminés purs, correspondant aux concentrations des acides aminés libres dans un fromage d'emmental (■).

stimulation was dependent on both the strain of *S. thermophilus* as well as the PAB strain used. The low production of FAA by *S. thermophilus* compared to lactobacilli may be due to the different rate of autolysis of the two bacterial species. Amino peptidases are intracellular enzymes which are liberated into the growth medium only after cell lysis [10]. Lysis of thermophilic streptococci in cheese is a slow process since it is detected only after 30 d of ripening in model cheese [18].

In contrast, FAA can be detected after 0.7 d in the simulation assay (figure 4A). The production of FAA coincided with the lysis of lactobacilli, since significant amounts of X-prolyl dipeptidyl peptidase were detected at the same time (figure 4B).

3.5. Relationship between FAA in cheese and secondary fermentation

Secondary fermentation in Emmental cheese, characterized by cracks or splits in the cheese body, is always accompanied by increased proteolysis of casein as identified by high values of the trichloroacetic acid soluble N (TCAS-N) fraction. An increase in propionate production together with a decrease in the concentration of lactic acid was also observed in cheeses with a secondary fermentation, suggesting that a stronger propionic acid fermentation occurs [25, 26]. Since the intensity of propionic acid fermentation is related to PAB growth, it is assumed that the amount of FAA in cheese is correlated with the intensity of secondary fermentation. Analyses were performed on 30 Emmental cheeses made on the same day in various cheese factories, using the two new types of PAB cultures (20 cheeses with culture A, 10 cheeses with culture B). The raw mixed cultures used varied according to the choice of cheese makers. After 24 h, the cheeses were removed to the same ripening cellar. Figure 5 shows a plot of the degree of sec-

ondary fermentation, estimated by a professional panel on an arbitrary scale of 1, corresponding to good cheeses, to 3 for pronounced secondary fermentation, against the amount of FAA, measured after 180 d of ripening. A good correlation between the two parameters was found ($r = 0.735$), which suggests that the amount of FAA plays a key role in the development of secondary fermentation. Since the TCAS-N fraction is also increased during secondary fermentation and since this fraction contains FAA as well as small peptides, one cannot exclude peptides as the cause, as has been proposed previously [2]. This assumption was tested by plotting the intensity of secondary fermentation after 180 d of ripening against the values obtained by subtracting FAA values ($\text{mmol}\cdot\text{kg}^{-1}$) from TCAS-N values ($\text{mmol}\cdot\text{kg}^{-1}$) to obtain the amount of peptides. As can be seen from figure 6, no correlation exists between the two parameters.

According to these results, PAB clearly prefer FAA to peptides for their growth in cheese, which conflicts with a previous study [2]. In our previous work, we showed that commercial casamino acids, considered as a FAA source, resulted in competitive inhibition on PAB growth, when added to peptone. However, it was discovered, after publication, that addition of the pure amino acids did not cause inhibition, suggesting that the inhibition was due to some substance in the casamino solution, such as salt, which is present at high levels in casamino acids (unpublished results).

3.6. Influence of facultatively heterofermentative lactobacilli on PAB growth

It has been shown that the addition of facultatively heterofermentative lactobacilli (FHL), such as *L. casei* or *L. rhamnosus*, together with thermophilic raw mixed cultures reduces, but does not completely suppress the risk of secondary fermentation in

Figure 4. (A) Liberation of free amino acids by a pure culture of *L. delbrueckii* ssp. *lactis* in a simulation assay as a function of incubation time. (B) Liberation of amino acids as a function of cell lysis by the same culture as in A. Cell lysis was measured by the amount of X-prolyl-dipeptidyl aminopeptidase liberated, determined by using Gly-Pro-AMC as substrate.

Figure 4. (A) Libération des acides aminés dans une expérience de simulation avec une culture pure de *L. delbrueckii* ssp. *lactis* en fonction du temps d'incubation. (B) libération des acides aminés en fonction de la lyse de la même culture que sous (A). La lyse cellulaire a été déterminée par la mesure de la libération de la X-prolyl-dipeptidyl aminopeptidase en utilisant le substrat Gly-Pro-AMC.

Figure 5. Correlation between the degree of secondary fermentation in cheese at 180 d of ripening and the amount of free amino acids. Secondary fermentation was estimated by a professional panel on an arbitrary scale of 1, corresponding to good cheeses, to 3 for pronounced secondary fermentation.

Figure 5. Corrélation entre l'intensité de la fermentation secondaire dans le fromage après 180 j de maturation et la concentration en acides aminés. La fermentation secondaire a été estimée par un jury professionnel selon une échelle arbitraire allant de 1 pour les bons fromages, à 3 pour ceux atteints de fermentation secondaire.

Emmental cheeses [23]. Consequently numerous cheese makers, in Switzerland, use FHL. In cheeses made using FHL, secondary fermentation was observed in 12 out of 20 cheeses manufactured with the new PAB culture A, whereas none was detected in 10 cheeses made with the new PAB culture B. The average values of propionate, lactate and FAA measured in the 3 groups of cheeses are given in *table II*. The propionate concentration as well as proteolysis are higher in cheeses with secondary fermentation. However, secondary fermentation, accompanied by an increase of about 20 % of propionate, was observed in spite of the presence of FHL, but only in cheeses manufactured with PAB culture A. This suggests that this mixed culture may be less sensitive to inhibition by FHL than those of culture B. To study this hypothesis, simulation experiments were performed using

L. casei and *L. rhamnosus* alone or in addition to the most used raw mixed culture in Emmental manufactory; the wheys obtained were then tested for their activity on the growth of the two new PAB cultures. No inhibition could be detected on either PAB culture A or B. On the contrary, an increase in FAA concentration was detected in some experiments, thus favouring the growth rate of PAB. According to Jimeno et al. [15], the production of diacetyl, formate and acetate by FHL could be responsible for the growth inhibition of PAB. The concentration of these metabolites producing 5 % inhibition on the growth of the 2 PAB cultures, was determined by adding increasing amounts of metabolites to the defined growth medium. From *table III*, one can see that the minimal inhibitory concentration of formate, diacetyl and acetate is at least 10 times higher than the concentrations found in

Figure 6. Correlation between the degree of secondary fermentation in cheese at 180 d of ripening and the amount of small peptides. Peptides were determined by subtracting the values of the free amino acids (FAA) from the nitrogen soluble in trichloroacetic acid (TCAS-N).

Figure 6. Corrélation entre l'intensité de la fermentation secondaire dans le fromage de 180 j de maturation et la concentration en petits peptides. Les peptides ont été déterminés par soustraction des acides aminés (FAA) de l'azote soluble dans l'acide trichloroacétique (TCAS-N).

cheese. It seems, therefore, that these compounds are not responsible for the inhibition of PAB in cheese. However, the controversial results shown in *table II* suggest that the two PAB cultures may be differentially sensitive to factors other than FHL, e.g. temperature because secondary fermentation appears only after about 120 ripening d of storage at 12–14 °C, suggesting that PAB are still growing and producing CO₂ at this low temperature. The growth of the two cultures at 24 °C and 14 °C, which correspond to the two temperatures encountered during cheese ripening, was studied. The results (*figure 7*) show that a slight difference in the rate of growth between the two cultures exists at 24 °C, whereas only culture A, which induces secondary fermentation, is able to grow at 14 °C. These results demonstrate that differences in PAB strains may be of importance in determining the qual-

ity of cheese. They also suggest that the ability of some PAB strains to grow at low temperatures should be taken into consideration when producing new PAB cultures. Similar results were published by Hettinga et al. [13], who demonstrated that the ability to grow at low temperatures was strain-dependent.

4. CONCLUSION

The growth of the two new PAB cultures used in Switzerland is directly dependent on the concentration of FAA in cheese, regardless of the lactobacilli strains contained in the RMCs. This does not exclude the possibility that PAB preferentially use only some of the FAA. Neither acetate, formate nor diacetyl inhibited PAB growth suggesting that the inhibitory effect of FHL

Table II. Concentrations of lactate, propionate and free amino acids in 30 cheeses manufactured with the two PAB cultures recently introduced, in the presence of facultatively heterofermentative lactobacilli.

Tableau II. Concentration en lactate, propionate et acides aminés de 30 fromages fabriqués avec les deux nouvelles cultures de BP récemment introduites, en présence de lactobacilles hétérofermentaires facultatifs.

	PAB culture A Secondary fermentation	PAB culture A No secondary fermentation	PAB culture B No secondary fermentation
FAA, mmol Leucine, mEq·kg ⁻¹			
3 mo ripening	94.8	75.6	85.7
6 mo ripening	129.6	97.2	102.3
Propionate, mmol·kg ⁻¹			
3 mo ripening	64.3	53.8	55.4
6 mo ripening	80.3	67.1	60.1
D + L Lactate, mmol·kg ⁻¹			
3 mo ripening	47.0	61.5	62.6
6 mo ripening	24.1	45.3	57.4

20 cheeses were manufactured with culture A, 10 with culture B. Cheeses were ripened in the same ripening cellar. Twelve cheeses manufactured with culture A showed secondary fermentation; no secondary fermentation was detected in cheeses made with culture B.

20 fromages ont été fabriqués avec la culture A et 10 avec la culture B. Les fromages ont été mûris dans la même cave. Douze fromages fabriqués avec la culture A ont présenté une fermentation secondaire ; aucune fermentation secondaire n'a été détectée dans les fromages fabriqués avec la culture B.

Table III. Concentrations of some inhibitory products in cheese and the minimum concentration necessary to inhibit of PAB growth by 5 to 10 %.

Tableau III. Concentrations de substances inhibitrices dans le fromage et concentration minimale inhibitrice de 5–10 % de la croissance des BP.

	Formate	Acetate	NaCl	Diacetyl
Concentration in cheese	5 mmol·kg ^{-1a}	43 mmol·kg ^{-1a}	150 mmol·kg ^{-1b}	7 µmol·kg ^{-1c} 17 µmol·kg ^{-1d} 200 µmol·kg ^{-1e}
Minimum inhibitory concentration	> 50 mmol·L ⁻¹	> 330 mmol·L ⁻¹	> 350 mmol·L ⁻¹	> 250 µmol·L ⁻¹

^a Samples were steam distilled, esterified and analyzed by head space gas chromatography according to a new unpublished FAM method. Les échantillons ont été distillés à la vapeur, estérifiés et analysés par chromatographie en phase gazeuse à espace de tête, selon une nouvelle méthode non publiée de la FAM.

^b Determined according to IDF Norm 88A:1988 / détermination selon la norme FIL 88 A :1998.

^c [26]. ^d [27]. ^e [15]. Using the method described in [6] / en utilisant la méthode décrite en [6].

is not due to a biochemical process. However, analyses performed in our institute have shown that the proteolytic activity in cheeses manufactured with the addition of FHL is less intensive than in cheeses made

without FHL since the TCA–S–N fraction, as well as the amounts of FAA, is lower. This explains why these cheeses are less tasty than those made without FHL. This suggests that FHL may have an inhibitory

Figure 7. Influence of temperature on the growth of PAB culture A (▲) and PAB culture B (■) at 24 °C; culture A (△) and B (□) at 14 °C.

Figure 7. Influence de la température sur la croissance de deux cultures de BP. Culture A (▲) et culture B (■) à 24 °C ; culture A (△) et culture B (□) à 14 °C.

effect on the proteolytic activity of lactobacilli thus inducing a delay in the rate of eye formation and explaining why the cheesemaker removes the cheeses made with FHL from the warm room later than the cheeses made without FHL.

Of the two PAB cultures used, one had a higher tendency to induce secondary fermentation. This culture was able to grow at 14 °C, whereas the PAB culture which never produced secondary fermentation did not grow at this low temperature.

Our results have practical implications: RMCs with high proteolytic activity, as measured under conditions simulating cheese manufacture, stimulate PAB growth and,

hence, increase the risk of secondary fermentation. Strains of PAB able to grow at low temperature (14 °C) may have a tendency to provoke secondary fermentation. These parameters could help in producing satisfactory pairs of RMCs and PAB which would produce good cheeses without a risk of secondary fermentation.

ACKNOWLEDGEMENTS

The authors would like to thank Dr M Casey for his critical reading of the manuscript and Dr J. Meyer for performing the determination of lactic acid and the isolation of lactobacilli.

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