Lait (1995) 75, 391-400

Original article

Influence of casein proteolysis by starter bacteria, rennet and plasmin on the growth of propionibacteria in Swiss-type cheese

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Summary — The low proteinase activity of propionibacteria renders their growth dependent on the primary hydrolysis of casein by starter bacteria. The purpose of this work was to develop a simple method which would allow us to measure the effect of casein hydrolysates by starter bacteria on the growth of propionibacteria in simulated cheese manufacture. The growth of lactic acid bacteria of starter was performed in UHT milk containing calcium carbonate to maintain the pH constant. After simulating the various steps (temperatures, duration) of cheese manufacture for 24 h, growth of propionibacteria was performed in a minimum medium to which casein hydrolysates produced by starter bacteria, rennet or plasmin were added. Growth of propionibacteria was followed photometrically and proteolysis of casein determined by the quantitative reaction of free amino acids with a cadmium ninhydrin reagent. The results have shown that growth of propionibacteria was very poor on medium containing milk alone, but amino acids released by the starter bacteria in milk influenced the growth of the microorganisms. Plasmin had no effect on casein on the liberation of free amino acids, but peptides produced by the enzyme were used by the propionibacteria. Rennet added to milk alone or together with plasmin in the absence of starter bacteria did not provoke the growth of propionibacteria; the proteolytic activity of rennet on casein did not modify starter bacteria growth, but produced a two- to threefold increase in free amino acids, depending on the starter used, and influenced the kinetics of the growth of propionibacteria. It was demonstrated that rennet may have a significant effect on the growth of propionibacteria in Emmental cheese, whereas it is generally admitted that after milk coagulation, the thermo denaturated enzyme no longer plays a role during the ripening of hard cheeses. Hydrolysis of α_{s1}-casein to α_{s1} I-casein by the rennet, before heating the milk to 53°C for Emmental manufacture, produces substrates which are necessary for the liberation of amino acids by peptidases. Analyses of experimental cheeses manufactured with starter bacteria of high or low proteolytic activity have shown that a too high concentration of free amino acids in cheese may inhibit growth of propionibacteria.

propionibacteria / casein / hydrolysis / plasmin / rennet

Résumé — Influence de la protéolyse de la caséine par les levains lactiques, la présure et la plasmine sur la croissance des bactéries propioniques dans l'emmental. *Une méthode simple qui permet de mesurer l'effet des hydrolysats de caséine produits par les levains lactiques, la présure et la plasmine sur la croissance des bactéries propioniques dans le fromage a été développée. La croissance* *des levains lactiques s'effectue dans du lait UHT additionné de carbonate de calcium pour maintenir un pH constant. Après simulation des étapes (durée, températures) de la fabrication d'un fromage durant 24 h, le surnageant des solutions est utilisé comme source d'azote pour la croissance des bactéries propioniques, réalisée dans un milieu de culture minimum. La croissance des bactéries propioniques est suivie par mesure photométrique. La protéolyse de la caséine est mesurée par la réaction quantitative des acides aminés libres avec un réactif cadmium-ninhydrine. Nos résultats montrent que les bactéries propioniques ne se développent pas sur le surnageant de lait UHT, non hydrolysé par les bactéries lactiques, mais la concentration en acides aminés, libérés par les peptidases des levains lactiques, influence directement la croissance des bactéries propioniques. L'action protéolytique de la plasmine sur la caséine n'a pas d'influence sur la production des acides aminés libres par les levains lactiques mais les peptides que libère l'enzyme sont utilisés par les bactéries propioniques. L'action protéolytique de la présure sur la caséine ne modifie pas la croissance des levains lactiques. En revanche, elle agit sur la production des acides aminés libérés par les levains lactiques et influence directement la cinétique de la croissance des bactéries propioniques. La présure, ajoutée seule au lait, ne provoque pas la croissance des bactéries propioniques. Ces expériences montrent que la présure exerce une influence indirecte importante sur la croissance des bactéries propioniques, alors que l'on admet généralement qu'après la coagulation du lait, cette enzyme, thermodénaturée, ne joue pas de rôle dans la maturation des fromages à pâte dure. L'hydrolyse des caséines par la présure, avant que le chauffage du lait n'atteigne les 53°C de la fabrication, produit le ou les substrats nécessaires à la libération des acides aminés libres par les peptidases des bactéries lactiques. Un essai pratique de fabrication d'emmental avec des levains lactiques à forte ou faible activité protéolytique a montré qu'une trop grande concentration d'acides aminés libres dans le fromage pouvait ralentir la croissance des bactéries propioniques dont le développement semble dépendre beaucoup plus des peptides que des acides aminés libres.*

bactérie propionique / caséine / hydrolyse / plasmine / présure

INTRODUCTION

Emmental cheese is manufactured in Switzerland in over 600 small cheese factories, each producing an average of 3 cheeses a day. The manufacturing process is well standardized since, in Switzerland, only 3 to 6 lactic starters are used. These starters are very similar in their composition and contain approximately the same amount of *S thermophilus* and *L delbrueckii* ssp *lactis*. A liquid culture of propionibacteria is also added to each milk used to manufacture Emmental cheese. Starter bacteria and propionibacteria are produced and sold exclusively throughout the country by our dairy institute.

However, in spite of this standardization, cheese defects appear, mostly during winter. The 2 most encountered faults, due to propionibacteria, are late blowing and brown spotting. Late blowing is probably caused by excessive growth or by an increase in activity of propionibacteria during cheese ripening (Steffen and Puhan, 1976); brown spotting is due to an insufficient amount of propionibacteria being added to the vat or to partial inhibition of germ growth (Baer *et al*, 1993). The difficulty in eradicating these faults from Emmental production indicates how hard it is to control the growth of propionibacteria during cheese ripening. It also indicates that the growth of these bacteria may vary, depending on the seasonal variations of milk composition, the contaminating milk flora, the starter bacteria used as well as the manufacturing procedure.

Growth requirements of propionibacteria are well known (for a review, see Hettinga and Reinbold, 1972); in the presence of a nitrogen source such as ammonium sulphate, these microorganisms are able to grow in the absence of amino acids. However, the low proteinase activity of propionibacteria, which have numerous peptidases (Flogerghagen *et al,* 1978; Sahlström *et al,* 1989; El Soda *et al,* 1992), renders their growth in cheese dependent on the primary hydrolysis of casein by starter bacteria, micrococci of the contaminating milk flora (Ritter and Schwab, 1968) or, possibly, the proteolytic activity of rennet or plasmin.

A complex method to study the synergistic effect of micrococci on the growth of propionibacteria was proposed by Ritter *et al* (1967). The purpose of this work was to develop a simple method which would allow us to determine the effect of casein hydrolysates by starter bacteria in the presence of rennet or plasmin on the growth of propionibacteria. A correlation of the results obtained in the laboratory with those observed in practice would render it possible to influence the growth of propionibacteria in cheese by choosing the appropriate starter bacteria. Preliminary results are presented in this article.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical reagent grade. Plasmin was purchased from Sigma (Buchs, Switzerland). Standard liquid calf rennet was obtained from the experimental cheese manufacture of our institute. Amino acids obtained by acid hydrolysis of casein, "Casamino", and a pancreatic digest of casein, "Trypticase peptone" were from Difco (Chemie Brunschwig, Basel, Switzerland).

Bacteria

The liquid culture of propionibacteria as well as 1 of the 3 most used starters in Emmental manufacture were obtained from our department of microbiology.

Growth of lactic acid bacteria

One ml of starter bacteria was added to 40 ml of UHT milk, containing 1.25 g of calcium carbonate to maintain the pH at a value around 5.5, similar to that of cheese. After incubation of the milk at 31°C for 30 min, 7 µl of sterile filtered rennet was added and the mixture incubated in a water bath whose temperature simulated that of Emmental cheese manufacture over the first 24 h. Since no plasmin activity remained in UHT milk, various concentrations of the sterile filtered enzyme were added to the milk at the same time as rennet. The concentrations used were 2 to 4 times higher than the average milk, estimated at 0.5 µg/ml (Richardson and Pearce, 1981).

After a 24 h incubation, followed or not by a second incubation at 31°C for 3 d, samples were heated at 85°C for 25 min to destroy any enzymatic activity. The samples were then centrifuged at 5 000 rpm for 10 min in sterile tubes and the supernatant filtered through a 0.45 µm sterile filter (Schleicher and Schuell, Feldbach, Switzerland). Supernatants were then used as the nitrogen source for the growth of propionibacteria.

Growth of propionibacteria

Propionibacteria were grown in 10 ml of a minimum culture medium (Crow, 1986), in the absence of cysteine and ammonium sulphate but containing 0.5 ml of supernatant from incubated milk, prepared as described earlier. The culture of propionibacteria was washed twice with saline and the volume of bacterial suspension to be added to 10.0 ml to obtain an absorption of 0.05 at 650 nm was determined. This volume was then added to 10.0 ml of medium containing the various supernatants. Bacterial growth was followed photometrically.

Determination of the proteolytic activity

Proteolysis of casein was determined by a method using the quantitative and specific reaction of free amino acids with a cadmium ninhydrin reagent (Folkertsma and Fox, 1992), adapted to microvolumes and measurement on microplates (Baer *et al,* 1995).

RESULTS AND DISCUSSION

Liberation of amino acids by proteolysis of casein

Table I shows the liberation of amino acids in UHT milk by various proteolytic agents, after simulation of the first 24 h of cheese manufacture and followed by incubation at 31°C for 1 to 3 d. It appears that starter bacteria were the only proteolytic agents able to produce free amino acids. Neither plasmin nor rennet hydrolyzed casein to free amino acids when added alone to milk. However, the production of free amino acids by starter bacteria was increased by the presence of rennet. Analyses of 24 various starters has put into evidence that this increase could vary from a two- to threefold, depending on the starter used (results not shown). However, the growth of the starter lactic acid bacteria was not increased in the presence of rennet, as demonstrated by the constant amount of lactate produced (table II). The increase in the production of free amino acids by starter bacteria in the presence of rennet is most probably due to the hydrolysis of peptides, produced by rennet, by the peptidases of the starter bacteria. Since the proteolytic activity of rennet is destroyed by the cooking temperature of hard cheese

Table I. Liberation of amino acids, mmol/l, from UHT milk by various proteolytic agents, after simulation of the first 24 h of cheese manufacture and followed by incubation at 31°C for 1 to 3 d.
Libération des acides aminés (mmol/l) du lait UHT par divers agents protéolytiques après simulation des 24 premières heures de fabrication du fromage suivie d'une incubation à 31°C de 1 à 3 j.

Proteolytic agents added to milk	*Time of milk incubation*			
	24 h	*2 d*	*3 d*	*4 d*
None	1.2	1.3	1.3	1.8
Rennet	1.2	1.2	1.2	1.3
Plasmin	1.5	1.4	1.8	2
Starter bacteria	2.4	5	8.9	13.3
Rennet + starter bacteria	4.6	8.6	12.7	17.3
Plasmin + starter bacteria	3	6.1	8.8	13
Plasmin + rennet + starter bacteria	4	8	12.7	17.4
Starter bacteria + denatured rennet	2.2	4.4	6.9	9.8

Table II. Production of lactate, mmol/l, by starter bacteria in UHT milk, in the presence or absence of rennet and plasmin, after simulation of the first 24 h of cheese manufacture.
Production de lactate (mmol/l) dans le lait UHT par le levain en présence ou en absence de présure et de plasmine, après simulation des 24 premières heures de fabrication du fromage.

Proteolytic agent	*L-lactate*	*D-lactate*	*L–D lactate*
Starter bacteria	51.9	65.7	117.5
Starter bacteria + plasmin	52.8	56.6	109.5
Starter bacteria + rennet	42.1	58.2	100.3
Starter bacteria + rennet + plasmin	46.2	58.2	104.4

above 50°C, it would seem that the first 60 min of cheese manufacture is sufficient for rennet to partially hydrolyze α_{s1}-casein to α_{s1} I-casein (Desmazeaud and Gripon, 1977; Exterkate, 1987; Lawrence *et al*, 1987) to release enough substrate(s) for the peptidases of lactic acid bacteria. This hypothesis was further investigated by analyzing the casein fractions of the incubated milks after 24 h, by polyacrylamide urea gel electrophoresis. The results (not presented) showed a decrease in the intensity of the band of α_{s1}-casein, accompanied by the appearance of a new anodic faint band corresponding to the position of α_{s1} I-casein. This band was visible only in milk containing rennet; it was more distinct in the presence of starter bacteria. Therefore, while considering that the new band was not definitely identified as α_{s1} I- casein, it seems that rennet could have time to partially hydrolyze α_{s1}-casein during the very first step of Emmental manufacture.Table I also shows that plasmin did not produce a substrate which could be hydrolyzed to amino acids by lactic acid bacteria.

Influence of free amino acids on the growth of propionibacteria

Growth of propionibacteria on milk hydrolysates obtained after 24 h of simulated cheese manufacture was measured. Figure 1 shows the growth of these bacteria on UHT milk supernatant, with or without rennet, in the presence or absence of starter bacteria. There was very poor growth on milk alone or with added rennet. In contrast, the presence of starter bacteria stimulated the growth of propionibacteria, which further increased when rennet was added with the starter. These results indicate that peptides, produced by rennet at the beginning of cheese manufacture, could not be used by propionibacteria and that the growth of these microorganisms depends on the presence of free amino acids or other peptides, as illustrated in figure 2, where the concentration of amino acids obtained in various experiments was plotted against the corresponding optical densities. One should also note that no increase in the production of amino acids was observed when 5 times more rennet was used and that the distribution of amino acids was very similar when determined in various hydrolysates (table III).

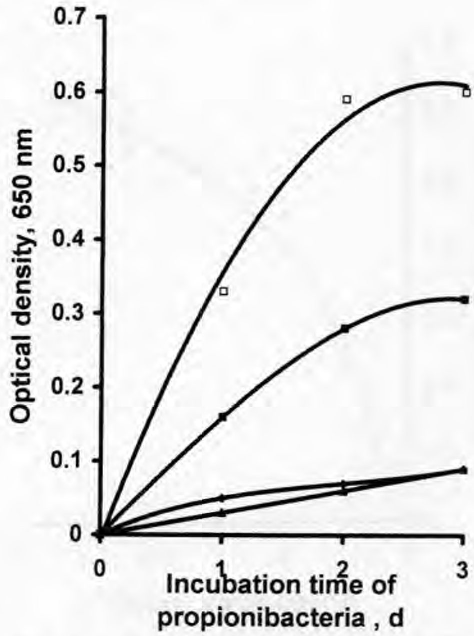

Fig 1. Growth of propionibacteria on hydrolysates of UHT milk, incubated in the presence of various proteolytic sources, according to the conditions of the first 24 h of Emmental manufacturing. Milk alone (▲), milk with added rennet (◆), starter bacteria (■), starter bacteria and rennet (❑).
Croissance des bactéries propioniques sur les hydrolysats de lait UHT, incubé avec diverses sources protéolytiques, durant les 24 premières heures de fabrication de l'emmental. Le surnageant du lait, utilisé comme source d'azote, a été ajouté à un milieu de culture minimum ; la croissance des bactéries a été suivie par mesure photométrique. Lait seul (▲), lait additionné de présure (◆), de levain lactique (■) de présure et de levain lactique (❑).

Influence of plasmin on the growth of propionibacteria

Table I shows that plasmin did not produce free amino acids and did not influence the proteolytic activity of starter bacteria. However, propionibacteria did grow on the substrates produced by plasmin, in the presence or absence of rennet or starter, after 24 h of cheese manufacture; the growth was twice as high when 4 times more plasmin was added (fig 3). Incubation of the milk, containing the normal concentration of the enzyme, for 3 d, produced growth similar to that observed with 4 times more plasmin during 24 h. These results suggest that propionibacteria may use the peptides produced by plasmin at the beginning of

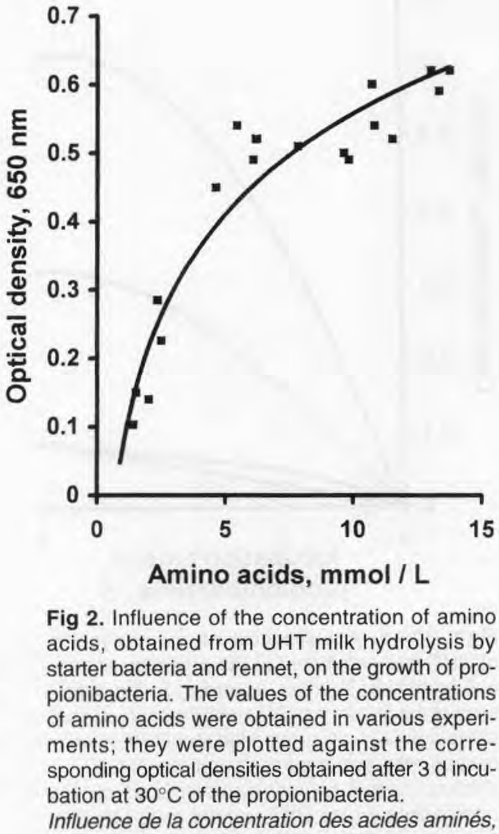

Fig 2. Influence of the concentration of amino acids, obtained from UHT milk hydrolysis by starter bacteria and rennet, on the growth of propionibacteria. The values of the concentrations of amino acids were obtained in various experiments; they were plotted against the corresponding optical densities obtained after 3 d incubation at 30°C of the propionibacteria.

Influence de la concentration des acides aminés, obtenus par hydrolyse du lait UHT par le levain lactique et la présure, sur la croissance des bactéries propioniques. Les valeurs reportées ont été obtenues lors de différentes expériences. Les densités optiques correspondantes sont celles obtenues après 3 j de croissance des bactéries propioniques à 30°C dans le milieu minimum additionné des hydrolysats.

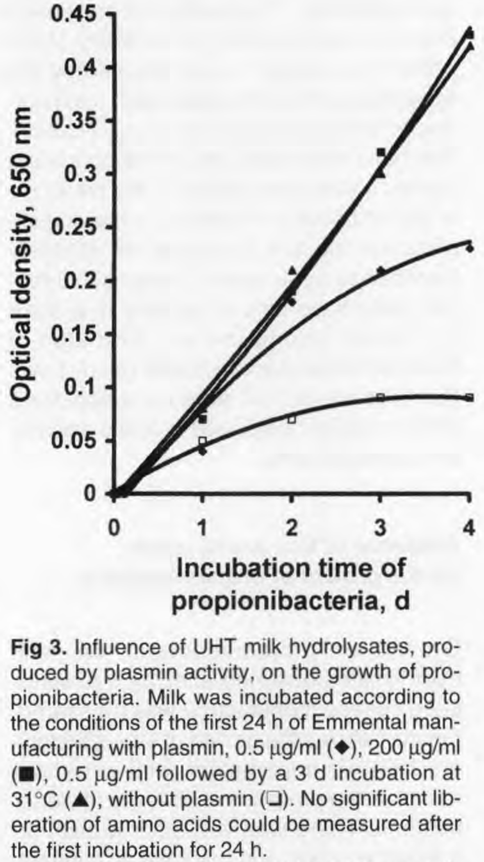

Fig 3. Influence of UHT milk hydrolysates, produced by plasmin activity, on the growth of propionibacteria. Milk was incubated according to the conditions of the first 24 h of Emmental manufacturing with plasmin, 0.5 µg/ml (◆), 200 µg/ml (■), 0.5 µg/ml followed by a 3 d incubation at 31°C (▲), without plasmin (❑). No significant liberation of amino acids could be measured after the first incubation for 24 h.

Influence des hydrolysats du lait UHT, produits par l'activité de la plasmine sur la croissance des bactéries propioniques. Le lait a été incubé durant la simulation des 24 premières heures de la fabrication de l'emmental, après addition de l'enzyme, 0,5 µg/ml (◆), 200 µg/ml (■), 0,5 µg/ml suivi d'une incubation de 3 j à 31 °C (▲), sans plasmine (❑). Aucune libération significative d'acides aminés n'a été observée après la première incubation de 24 h.

Table III. Distribution (%) of free amino acids in milk, incubated with various proteolytic agents for 2 d, after simulation of the first 24 h of cheese manufacture.
Pourcentage de distribution des acides aminés libres dans le lait incubé avec divers agents protéolytiques pendant 2 jours après simulation des 24 premières heures de fabrication du fromage.

Amino acids	*Milk + sb*	*Milk + sb + rennet*	*Milk + sb + plasmin*	*Milk + sb + rennet + plasmin*
PSE	0.0	0.2	0.0	0.0
ASP	6.8	7.2	6.9	7.8
GLU	9.2	13.0	9.5	12.0
ASN	0.8	0.8	1.0	0.9
SER	3.5	5.7	3.9	5.2
GLN	11.6	6.9	10.5	6.4
HIS	3.7	3.0	3.4	3.0
GLY	4.0	6.1	3.9	5.1
THR	1.2	1.8	1.3	1.7
CIT	1.3	1.2	1.7	1.1
ALA	1.2	2.9	1.3	2.8
ARG	1.8	2.6	2.0	3.7
GBU	1.7	1.0	1.9	1.2
TYR	2.3	1.6	2.0	1.9
ABU	0.0	0.1	0.0	0.0
VAL	7.9	9.2	7.4	9.4
MET	1.4	1.2	1.7	1.0
TRP	0.4	0.2	0.4	0.3
ILE	5.6	5.9	5.2	5.2
PHE	4.1	2.7	3.5	3.0
ORN	1.5	0.7	1.7	0.8
LEU	9.7	8.0	8.2	8.7
LYS	6.7	5.3	11.3	7.6
PRO	13.6	12.5	11.3	11.2

sb: starter bacteria

cheese ripening (Casey *et al,* 1987). One should also consider that the plasmin-induced hydrolysis of milk, before its transformation into cheese, may influence the growth of propionibacteria in Emmental cheese.

All of these experiments were repeated using raw milk; the results obtained were practically identical to those obtained with UHT milk (not shown).

Influence of the proteolytic activity of starter bacteria on the growth of propionibacteria in experimental Emmental cheeses

Two pools of starters, selected as a function of their high or low proteolytic activity, were used to manufacture 2 cheeses each. Preliminary analyses performed during the first 60 d of ripening showed that the liberation of amino acids was almost 3 times higher in cheeses manufactured with the starter bacteria of high proteolytic activity than in the other cheeses (fig 4). However, the growth of propionibacteria measured in cheeses manufactured with lactic bacteria of high proteolytic activity was about 30 times less intensive than in the other cheeses (fig 5). These results are the opposite of those expected and suggest that high concentration of free amino acids may have an inhibitory effect on the growth of propionibacteria.This hypothesis was investigated by growing the microorganisms on amino acids (casamino) and peptides (peptone) from casein. The growth measured on peptides was approximately twice as high as on amino acids, demonstrating that propionibacteria, like lactic acid bacteria generally, prefer peptides for growth. However, the addition of increasing amounts of amino acids to a constant concentration of peptides partially inhibited the growth of the bacteria (fig 6). These results may be extrapolated to the experimental cheese; they could explain the inhibition of the growth of propionibacteria in the presence of a high concentration of free amino acids.

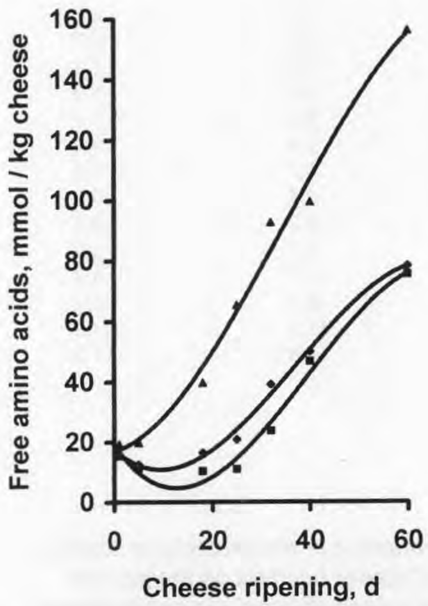

Fig 4. Liberation of amino acids in experimental Emmental cheeses, manufactured with a pool of starter bacteria of low proteolytic activity (■); high proteolytic activity (▲); controls (◆).
Libération des acides aminés dans des emmentals expérimentaux, fabriqués avec des mélanges de levains lactiques choisis en fonction de leur activité protéolytique faible (■); forte (▲); fromages de contrôle (◆).

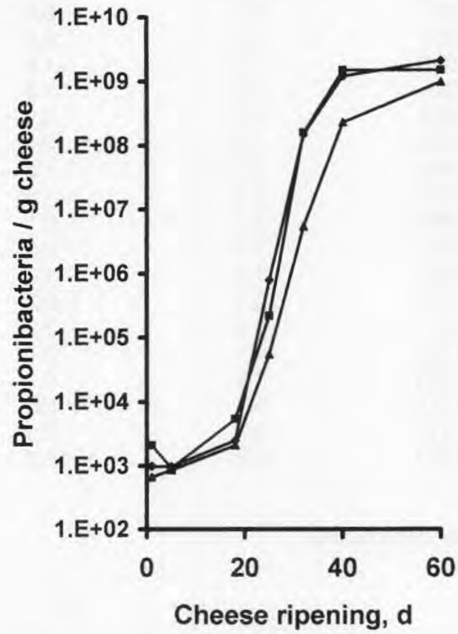

Fig 5. Growth of propionibacteria in the experimental cheeses of figure 4.Two cheeses for each pool were manufactured, using 110 l of pasteurized milk and starter bacteria of low proteolytic activity (■) and high proteolytic activity (▲). Controls (◆).
Croissance des bactéries propioniques, mesurée dans les fromages de la figure 4. Les fromages, 2 par mélange de levains, ont été fabriqués à partir de 110 l de lait pasteurisé et de levains à activité protéolytique faible (■), et forte (▲). Fromages de contrôle (◆).

CONCLUSION

Preliminary results presented here have shown that the procedure developed for studying the influence of milk hydrolysates on growth of propionibacteria may be useful to the cheese manufacturer. However, the method could be improved by simulating the brining conditions of cheese manufacture, as well as the temperature of incubation up to approximately the 25th day of ripening, which corresponds to the start of growth of propionibacteria. This further improvement should be fairly simple. The correlation between starter bacteria of high proteolytic activity and high concentration of free amino acids in experimental cheeses must be confirmed with a larger number of cheeses, since it may help in choosing adequately starter bacteria which influence the growth of propionibacteria.

The influence of rennet on the liberation of free amino acids by starter bacteria showed that the activity of the enzyme is not limited to the breakdown of κ-casein, causing milk coagulation, but may also play an important role in peptide production in Emmental cheese.

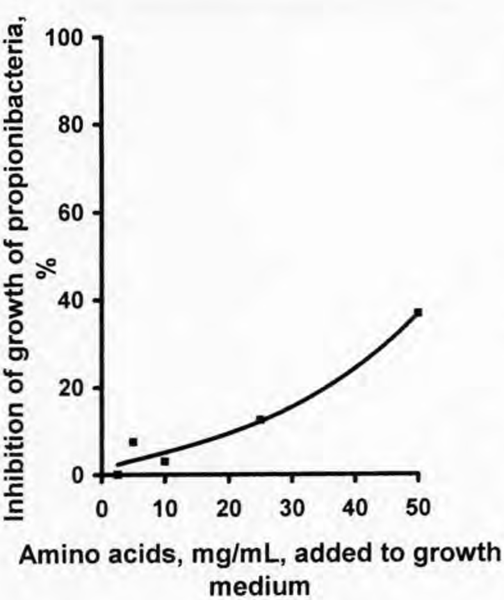

Fig 6. Inhibition of growth of propionibacteria (indicated in %) by high concentrations of amino acids, in the presence of peptides. Increasing amounts of amino acids (casamino) were added to a constant concentration of peptides (5.0 mg/ml, peptone) in minimum culture medium. Casamino and peptone are commercial products obtained by casein hydrolysis.
Inhibition, indiquée en %, de la croissance des bactéries propioniques par une grande concentration en acides aminés, en présence de peptides. Des concentrations croissantes d'acides aminés (casamino) ont été ajoutées à une concentration constante de peptides (5,0 mg/ml peptone) dans le milieu de culture minimum. Les produits casamino et peptone sont des hydrolysats commerciaux de la caséine.

ACKNOWLEDGMENTS

We thank Dr M Casey for his valuable suggestions and his critical reading of the manuscript.

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