Lait (1996) 76, 193-208

Original article

Growth of *Lactococcus lactis* in milk and rennet curd: influence of the level of inoculation

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(Received 2 March 1995; accepted 31 October 1995)

Summary — The growth of *Lactococcus lactis* subsp *lactis* CNRZ 1076 (prt^+) and CNRZ 1075 (prt^-) was followed in milk and rennet curd inoculated at levels ranging from 10^2 to 10^6 cfu/mL. Growth inhibition was observed in rennet curd inoculated with less than 10^4 cfu/mL of *L lactis* CNRZ 1076, suggesting a correlation with growth in colonies and low diffusion coefficients of nutrients and/or end-products such as H^+ and lactate. As a consequence, at low levels of inoculation acidification developed more slowly in rennet curd than in liquid milk. The growth rate of *L lactis* CNRZ 1075 (prt^-) in rennet curd was decreased in liquid and renneted milk. However, the maximum population level was higher in renneted milk than in liquid milk. This stimulation led to a more complete acid pH development in curd than in milk and was probably related to the release of peptides by chymosin activity. Microstructure studies provided evidence for colonies developing in liquid milk and rennet curd inoculated at low levels. The size of the colonies inversely depended on the level of inoculation.

level of inoculation / *Lactococcus lactis* / growth / milk / rennet curd / acidification / bacterial colonies / scanning electron microscopy

Résumé — Croissance de *Lactococcus lactis* dans le lait et le coagulum présure : influence du taux d'inoculation. *L'effet du taux d'inoculation (10^2 à 10^6 ufc/mL) sur la cinétique de croissance de* Lactococcus lactis *subsp* lactis *CNRZ 1076 (prt^+) et CNRZ 1075 (prt^-) était étudié dans le lait et dans le coagulum présure. Lorsque le taux d'inoculation était inférieur à 10^4 ufc/mL, la croissance de la souche non protéolytique CNRZ 1075 était ralentie, aussi bien dans le lait que dans le coagulum présure. Dans le coagulum présure cependant, le niveau de population atteint en fin de croissance était supérieur, ce qui conduisait à un pH final nettement inférieur à celui de la culture sur lait. Pour ces mêmes taux d'inoculation, la souche protéolytique CNRZ 1076 était également inhibée, mais uniquement dans le coagulum présure. La cinétique de croissance dans le lait était indépendante du taux d'inoculation. Ces résultats sont à relier aux observations réalisées sur les cultures en microscopie optique et en microscopie électronique à balayage qui montrent qu'en dessous d'un certain seuil d'inoculation (10^4 ufc/mL) la croissance s'effectue sous forme de colonies isolées. Il est vraisemblable que les cellules situées au cœur des colonies ne se trouvent pas dans des conditions optimales de croissance.*

taux d'inoculation / croissance/* Lactococcus lactis *** **/ *lait / coagulum présure / acidification / colonies bactériennes / microscopie électronique à balayage

INTRODUCTION

Lactic acid bacteria have been studied in-depth for nearly a century and much is known about nutrient requirements (Reiter and Oram, 1962), transport system (Konings et al, 1989), proteolytic systems (Smid et al, 1991) and to a large extent, their growth in milk. The current view is that growth of lactic acid bacteria, especially lactococci and streptococci is limited in milk because of, i) the low level of available essential nitrogenous nutrients (Mills and Thomas, 1981; Juillard and Richard, 1989); and ii) the concomitant inhibiting actions of high lactate concentration and low pH (Longsworth and Mac Innes, 1936; Bergère and Hermier, 1968; Otto et al, 1980). Lack of nitrogenous nutrients is cancelled for fast strains of starter bacteria which possess a cell wall proteinase (Hugenholtz et al, 1987). The hydrolysis of caseins supplies cells with peptides that are translocated across the membrane and used for growth to high cells densities (Juillard et al, 1994). On the other hand, the increasing lactic acid concentration causes gradual inhibition of growth (Rogers and Whittier, 1928).

During cheese making, growth of starter bacteria may occur to some extent in coagulated milk, according to the cheese type. One can wonder whether rennet action on milk affects the growth of these bacteria since: i) peptides are released from casein hydrolysis by chymosin and pepsin; ii) coagulated paracaseins as substrates for proteinase are structured in a solid network; and iii) solute diffusion may be different in rennet curd than in liquid milk.

A century ago, interesting studies showed that spotting defects seen in Swiss type cheese resulted from occurrence of colonies of bacteria (Burri, 1898; Thöni and Aleman, 1910). Brown and red spotting defects were ascribed to the genus *Propionibacterium* whereas colonies of lactic acid bacteria (rods and cocci) were likely the cause of a black spotting defect. A more recent study (Baer et al, 1992) has shown that the brown spotting defect only appeared if the population level of propionibacteria in cheese milk was low (<100 cfu/mL). Moreover, evidence for the general occurrence of small colonies of different species of bacteria in cheese has already been shown. These reports either dealt with the difficulty of counting bacteria in cheese because of their heterogeneous dispersion (Naylor and Sharpe, 1958; Dean et al, 1959) or were investigations on cheese microstructure (Hansson et al, 1966; Rousseau and Le Gallo, 1990). Though numerous data about growth of entrapped bacteria in porous matrices exist (Karel and Robertson, 1989; Yabannavar and Wang, 1991; Cachon and Diviès, 1993), the effect of milk coagulation by rennet on growth of lactic acid bacteria is still unknown. The purpose of this work was to characterize the growth of mesophilic starters in milk and rennet curd as a function of their level of inoculation.

MATERIALS AND METHODS

Milk and UF retentate culture media

Skim milk powder made from 'Bactocatch' treated raw skim milk was obtained as described by Schuck et al (1994). Milk culture medium was prepared by reconstituting 10 g powder in 90 g autoclaved distilled water at room temperature. After 5 min mixing, reconstituted milk was cooled down to 0°C in thawing ice before rennet addition and inoculation. The same batch of powder was used for all the assays.

To prepare UF retentate, bulk skim milk (Compagnie Laitière Européenne, Montauban, France) was pasteurized (75 °C, 15 s) and ultrafiltered at 50 °C up to a volume concentration ratio (VCR) of 3 or 5 using a Carbosep M1 membrane cut-off 70 000 (Techsep, Miribel, France). The UF retentate was cooled down to room temperature (20 °C) before rennet addition and inoculation.

Bacteria cultures and inoculum preparation

Lactococcus lactis CNRZ 1076 (prt^+) and CNRZ 1075 (prt^-) were from the CNRZ collection (Jouy-en-Josas, France). Stock cultures were maintained at –20 °C in M17 broth (Biokar, Beauvais, France) with 15% w/w glycerol added. For each experiment 0.2 mL of each culture was inoculated into 10 mL M17 broth and incubated overnight at 30 °C. The cultures were then centrifuged at room temperature (4000 *g*, 10 min) and cell pellets were washed with saline water (8.5 g/L NaCl).

Washing procedure was repeated once before optical density at 650 nm was adjusted to ca 0.30 with a spectrophotometer (DU 7400, Beckman Instruments, Gagny, France). This suspension was then used to inoculate reconstituted milk and UF retentate at levels ranging from 10^2 to 10^6 cfu/mL.

Milk coagulation, non-clotting milk and chymosin denaturation

Crystalline chymosin, purified according to Garnot and Mollé (1982) was a gift of D Mollé (INRA, Laboratoire de Recherches en Technologie Laitière, 65, rue de St Brieuc, 35042 Rennes cedex, France). 0.5 mg crystalline chymosin was dissolved in 1 mL sterile water (pH 6) and stocked at 4 °C. After inoculation, if necessary, 30 µL of a 500 mg/L concentrated chymosin solution was added per 100 mL of cooled reconstituted milk. Before warming it up to 30 °C in a water bath, milk was divided into 1 mL quantities in tubes for growth measurement and into 20 mL quantities in tubes for acidification measurements.

Non-clotting milk was prepared by incubating reconstituted skim milk with 5% (w/w) cation exchange resin Chelex 100, sodium form, Biotechnology Grade (Bio Rad, Ivry-sur-Seine, France) for 20 min at room temperature. pH was adjusted to 6.65 with a 1 N HCl solution.

Denatured chymosin was obtained in two ways: i) heating at 100 °C for 10 min; and ii) incubating at 43 °C and pH 9 for 24 h.

Milk acidification monitoring

pH development was followed with a multi pH-meter MPM 400 (Solomat, Évry, France) in 40 mL sterile tubes containing 20 mL milk culture medium. The temperature of the cultures was always 30 °C.

Growth rate estimation

Experiments on growth of *L lactis* CNRZ 1076 and 1075 in liquid and renneted milk inoculated with 10^2, 10^3, 10^4 and 10^6 cfu/mL were repeated three times. No significant differences were noted between repetitions of each treatment. To compare growth in liquid milk and in coagulated milk, the following method was used for all the experiments: inoculated cooled milk (with chymosin added or not) was divided among 10 separate sterile 5-mL tubes, each containing 1 mL milk. Every tube was warmed up to 30 °C in a water bath. Growth of *L lactis* CNRZ 1076 and CNRZ 1075 was static. Tubes taken at intervals were quickly cooled down to 0 °C in thawing ice. Milk or rennet curd was diluted in 9 mL sterile saline water and the mix was homogenized with an Ultra-Turrax at 20 000 rpm for 30 s at room temperature. *L lactis* enumeration was assessed by plating sample dilutions on M17 agar (Biokar, Beauvais, France) with a spiral plater (Interscience, St Nom-la-Bretèche, France). Plates were incubated for 24 h at 30 °C before reading with a colony counter by image analysis SCAN 500 (Interscience, St Nom-la-Bretèche, France). The growth rates were expressed according to Monod's equation (1958):

$$\mu = \frac{1}{\text{doubling time}} \ (h^{-1})$$

Cultures in buffered milk

To assess whether the inhibition of lactococci was related to the decrease in pH within colonies, 20 mmol/L (final concentration) phosphate buffer prepared as follows was added to milk culture medium: a stock solution consisting of 1.72 g of $CaHPO_4$, 2 H_2O (Merck, Nogent-sur-Marne, France) and 1.88 g of 85% concentrated H_3PO_4 solution (Merck, Nogent-sur-Marne, France) was adjusted to pH 6.80 with a 1 N NaOH solution (ca 40 mL) and brought to a volume of 100 mL with sterile distilled water. Reconstituted milk was prepared by diluting 10 g skim milk powder in 79 g sterile distilled water, 1 mL inoculum and 30 µL of either a 500 mg/L concentrated chymosin solution or sterile distilled water. In order to avoid phosphate precipitate decantation, 100 µL of either the phosphate stock solution or sterile distilled water was added

to culture tubes each containing 900 µL of reconstituted milk just before milk began to coagulate, ie, after 40 min incubation at 30 °C. Throughout this study, the level of inoculation of *L lactis* CNRZ 1076 was 10^2 cfu/mL.

Slide culture

In order to visualize how bacteria grow in milk and rennet curd, slide cultures were made according to a modification of the method of Postgate et al (1961) as follows. Milk was reconstituted using the method of Turner et al (1963) with an aqueous solution of 0.01% triphenyl 2,3,5 tetrazolium chloride (TTC) (AES, Combourg, France) and 0.05% arginine (Sigma, St Quentin Fallavier, France). Milk was cooled down to 0 °C, inoculated and divided in two equal parts: to one, 30 µL/100 mL chymosin (500 mg/L concentrated solution) was added, while the other was untreated. 5-µL milk samples were incubated at 30 °C between slides sealed with paraffine to avoid evaporation. Photographs were taken at intervals using a microscope Nikon Optiphot, a camera Nikon F 301 (darkfield condenser and objectives ×10 and ×40) and an Ektachrome160 ASA Tungsten film.

Scanning electron microscopy (SEM)

Five kg of freshly prepared retentate at VCR 5 was cooled down to 30 °C and divided in two equal parts of 1.8 kg before inoculation with *L lactis* CNRZ 1076. Two levels of inoculation were tested, 10^2 and 10^6 cfu/mL. 40 mL of a 520 mg/L concentrated rennet solution (Granday, Beaune, France) was added per 100 kg of retentate. Each vat was incubated at 30 °C until the pH had dropped to a stable value. Samples were regularly taken for scanning electron microscopy assay.

Prisms (2 × 5 × 10 mm) were taken in the middle of coagulated retentate and fixed with a solution of 2.5% glutaraldehyde (Sigma, St Quentin Fallavier, France) in 10 mmol/L cacodylate buffer (pH 7.2) (Merck, Nogent-sur-Marne, France), for at least 15 h according to Rousseau (1988). Glutaraldehyde was removed by washing samples in cacodylate buffer for 1 day, replacing the washing solution five times. Samples were then dehydrated by incubating in aqueous ethanol (Prolabo, Paris, France) solutions of increasing concentration (10, 25, 50, 75, 95% for 20 min each) and three times in absolute ethanol (30 min each). They were dried in carbon dioxide at critical point (Balzers CPD 030), fractured, mounted on aluminium stubs and sputter coated (20 nm) with gold (Balzers SCD 050). Scanning electron microscopy was carried out with a Philips XL 20 microscope at 8 or 12 keV.

RESULTS

Growth of L lactis CNRZ 1076 (prt⁺)

Effect of inoculation at low levels on the growth of *L lactis* CNRZ 1076 (prt⁺) in renneted milk

The growth of *L lactis* CNRZ 1076 was followed in liquid milk and milk to which 30 µL/100 mL chymosin was added. The growth was not affected by chymosin addition if milk was inoculated with more than 10^6 cfu/mL. With a level of inoculation smaller than 10^6 cfu/mL, the growth was inhibited in renneted milk compared to that in liquid milk.

Figures 1a–c illustrate that the decrease in growth rate occurred at populations of ca $3\ 10^8$, $3\ 10^7$, $3\ 10^6$ cfu/mL in renneted milk, respectively inoculated with ca 10^4, 10^3, and 10^2 cfu/mL.

As a consequence, at low levels of inoculation, acidification developed more slowly in rennet curd than in liquid milk (fig 2).

Microbial contaminants from chymosin could not account for this phenomenon since the level of population of mesophilic aerophilic flora of the chymosin was very low (less than 1 cfu/mL). Moreover, the growth of contaminants from milk powder was very slow in inoculated reconstituted milk and did not differ whether the milk was coagulated or not. Their maximum population level, ca 10^6 cfu/mL, was achieved after 15 h of the lactococcal culture. At that time, the minimum population level of *L lactis* CNRZ 1076 observed over the whole experiments was 10^8 cfu/mL (fig 1c).

The inhibition of the growth of *L lactis* CNRZ 1076 in renneted milk inoculated at a low level was likely caused by milk clot-

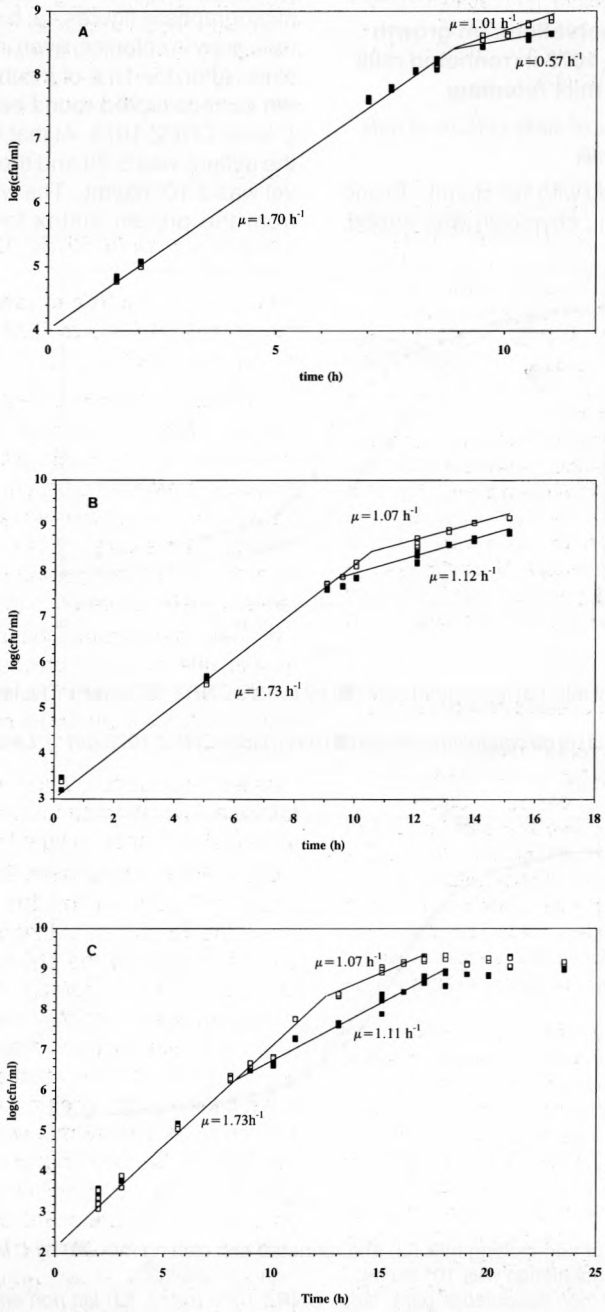

Fig 1. Growth of *L lactis* CNRZ 1076 (prt⁺) in milk (❑) and rennet curd (■). The level of inoculation was ca: **A**) 10^4 cfu/mL; **B**) 10^3 cfu/mL; **C**) 10^2 cfu/mL.
Croissance de L lactis *CNRZ 1076 (prt⁺) dans le lait* (❑) et dans le coagulum (■). Le taux d'inoculation était de : ***A)*** *10^4 ufc/mL ;* ***B)*** *10^3 ufc/mL;* ***C)*** *10^2 ufc/mL.*

ting since chymosin action in non-clotting milk had no expected inhibitory effect (fig 3).

Microscopic observations on growth of *L lactis* CNRZ 1076 in renneted milk and ultrafiltered milk retentate

Light micrographs of slide culture of milk and coagulated milk

Milk was inoculated with 10^2 cfu/mL. To one part, 30 µl/100 mL chymosin was added. Milk was prepared in thin films trapped between glass slides. Low magnification light micrographs in figures 4a, b show that bacteria grew in colonies, even in the liquid milk films. After 15–16 h of incubation, the rennet curd disrupted round each colony of - *L lactis* CNRZ 1076. At that time, the pH of the culture was 5.90 and the population level was 3 10^8 cfu/mL. The whey separated from the protein matrix forming a liquid

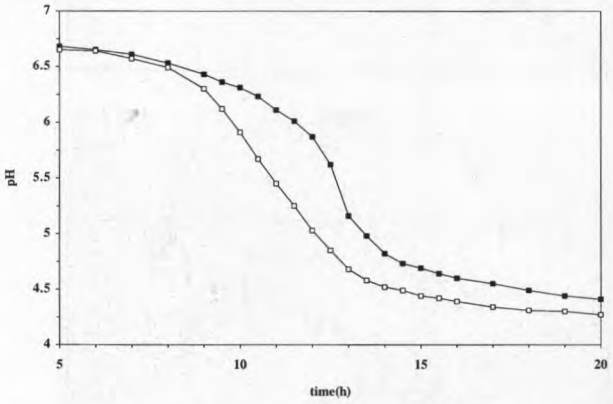

Fig 2. Acidification of milk (❑) and rennet curd (■) by *L lactis* CNRZ 1076 (prt⁺). The level of inoculation was 10^4 cfu/mL.
Acidification du lait (❑) et du coagulum présure (■) par L lactis *CNRZ 1076 (prt⁺). Le taux d'inoculation était de 10^4 ufc/mL.*

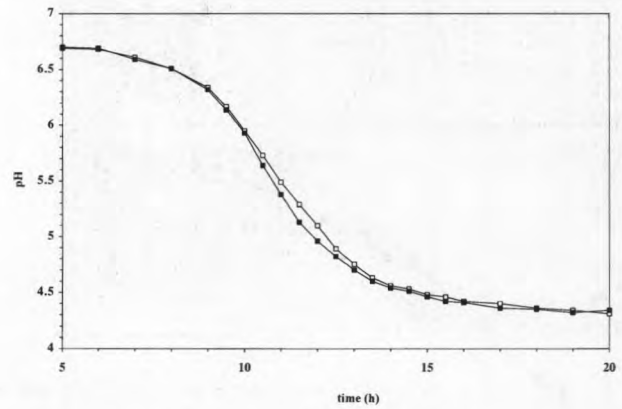

Fig 3. Acidification of non-clotting milk (❑) and renneted non clotting milk (■) by *L lactis* CNRZ 1076 (prt⁺). The level of inoculation was 10^4 cfu/mL.
Acidification de lait 'non coagulable' par L lactis *CNRZ 1076 (prt⁺). (❑) lait non emprésuré, (■) lait emprésuré. Le taux d'inoculation était de 10^4 ufc/mL.*

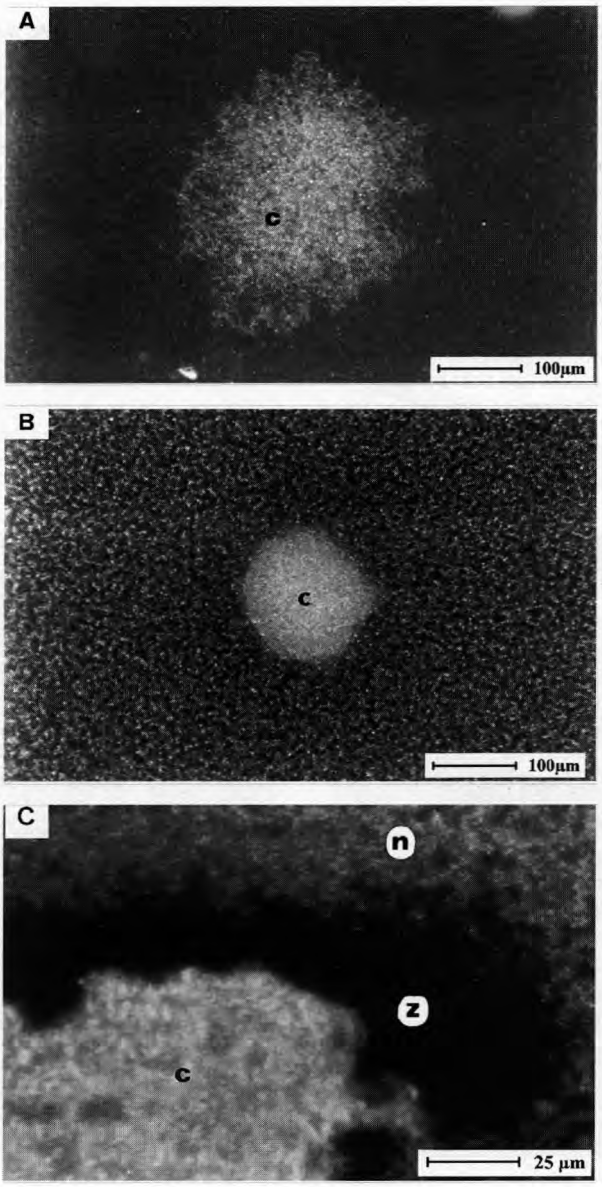

Fig 4. Colony in slide culture of *L lactis* CNRZ 1076 (prt+) in liquid milk (**A**) and renneted milk (**B**) inoculated with 10^2 cfu/mL (t = 14 h) ; (**C**) details of the colony border in renneted milk (t = 17 h ; pH 5.90 sample) ; (c) : colony ; (z) : zone of whey : (n) : casein network.
*Culture sous lame ; colonie dans une culture de L lactis CNRZ 1076 (prt+) dans du lait non emprésuré (**A**) et dans du coagulum présure (**B**). Le taux d'inoculation était de 10^2 ufc/mL (t = 14 h) ; (**C**) détail de la périphérie de la colonie (t = 17 h ; pH de l'échantillon : 5,90) ; (c) : colonie ; (z) : vacuole de sérum ; (n) : réseau de caséines.*

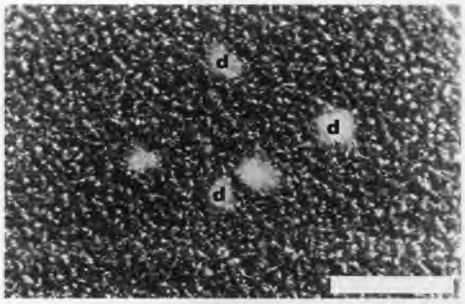

Fig 5. Points of dissemination (d) of cells of *L lactis* CNRZ 1076 in the whole curd from a slide culture inoculated with 10^6 cfu/mL; pH 5.90 sample.
Culture sous lame de L lactis *CNRZ 1076 (prt⁺) dans du coagulum présure. Le taux d'inoculation était de 10^6 ufc/mL, pH de l'échantillon : 5,90). (d) : centres de dissémination des bactéries.*

zone (fig 4c) which grew slowly at the periphery of colonies.

If milk was inoculated with 10^6 cfu/mL, bacteria invaded the whole curd from points of dissemination (fig 5).

Scanning electron microscopy of colonies in coagulated retentate

At pH 6.30, 5.70, 5.40 and 4.90 samples of UF retentate VCR 5 inoculated with 10^2 or 10^6 cfu/mL and to which 30 µL/100 mL chymosin was added were examined by SEM. The general aspect of the rennet curd of retentate was in accordance with the findings of Prokopek et al (1976) and Gavaric et al (1989).

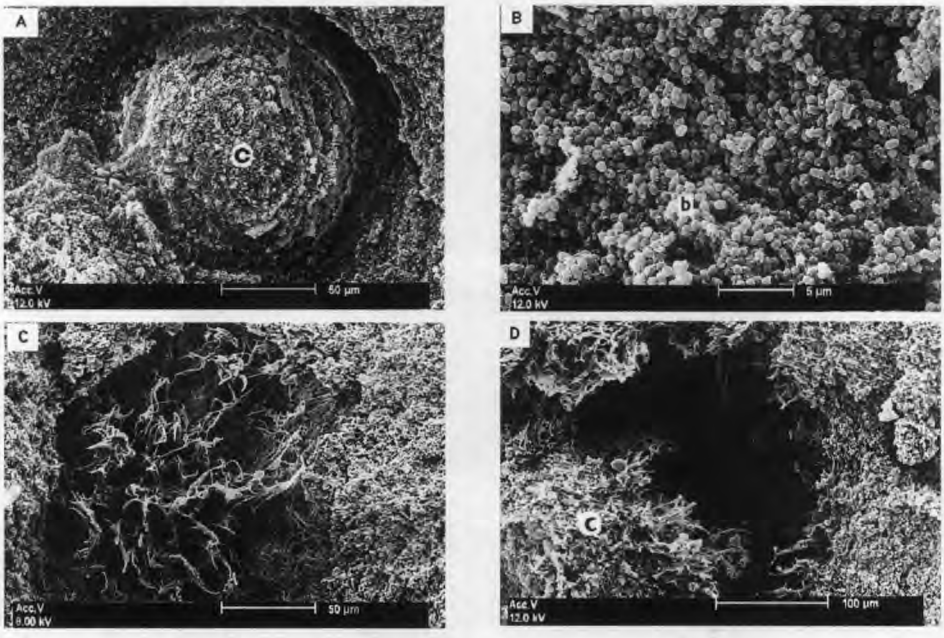

Fig 6. Culture of *L lactis* CNRZ 1076 (prt⁺) in renneted VCR5 UF retentate from skim milk; the level of inoculation was 10^2 cfu/mL; pH 6.30 sample. **A**. Colony (c). **B**. Bacteria cells in the middle of a colony (b). **C**. Details of the protein network close to a colony in the same culture; pH 5.70 sample. **D**. Disruption of the casein network close to a colony (c) in the same culture; pH 5.40 sample.
Culture de L lactis *CNRZ 1076 (prt⁺) dans du rétentat UF emprésuré (facteur de concentration volumique 5). Le taux d'inoculation était de 10^2 ufc/mL. pH de l'échantillon : 6,30. **A**. Colonie (c). **B**. Cellules bactériennes au centre d'une colonie (b). **C**. Détails du réseau de caséines à la périphérie d'une colonie dans la même culture. pH de l'échantillon 5,70. **D**. Fissure du réseau de caséines à la péripherie d'une colonie (c) dans la même culture, pH de l'échantillon : 5,40.*

The low magnification micrograph of pH 6.30 samples illustrate that bacteria actually grew in large colonies when the retentate was inoculated with 10^2 cfu/mL (fig 6a, b). In the pH 5.70 and 5.40 samples from retentate inoculated with 10^2 cfu/mL (fig 6c, d), the rearrangement of casein which appeared to contract in the vicinity of the colonies, led to the formation of large zones of whey, already observed in slide cultures (fig 4c). When the retentate was inoculated with 10^6 cfu/mL, the colonies were much smaller and closer to each other than in the 10^2 cfu/mL inoculated retentate (fig 7a, b).

Growth of *L lactis* CNRZ 1076 (prt⁺) in liquid and renneted milk supplemented with 2 mg/mL casein hydrolysate

As described above, growth and acidification in rennet curd was slower than in liquid milk when low levels of inoculation were used. A possible explanation is a slower degradation of casein by cell wall proteases when bacteria are entrapped in large colonies and casein micelles are immobilized in a network. If casein degradation is involved, addition of peptides should reduce inhibition of growth rate and of acidification development in clotted milk. Milk was supplemented with 2 mg/mL casein hydrolysate (Casitone, Difco, Detroit, USA). Acidification and growth were followed in liquid milk and milk to which 30 µL/100 mL chymosin was added. Addition of casitone to milk did not prevent the inhibitory effect of milk coagulation on acidification and growth of *L lactis* CNRZ 1076 (prt⁺): addition of Casitone similarly stimulated acidification and growth whether the milk was coagulated or not (figs 1a, 8).

Growth of *L lactis* CNRZ 1076 (prt⁺) in rennet curd from phosphated milk and from ultrafiltered milk retentate

A low pH in the vicinity of large colonies is a possible explanation of the inhibition of the growth of *L lactis* CNRZ 1076 (prt⁺). To test this possibility, bacteria were grown in media of higher buffering capacities as follows: i) rennet curd from milk to which 20 mmol/L phosphate buffer (final concentration) was added; and ii) rennet curd from ultrafiltered milk retentate VCR 3. The inoculum level was 10^2 cfu/mL.

i) Addition of phosphate buffer to a milk culture medium had no effect on growth rates of *L lactis* CNRZ 1076, whether the

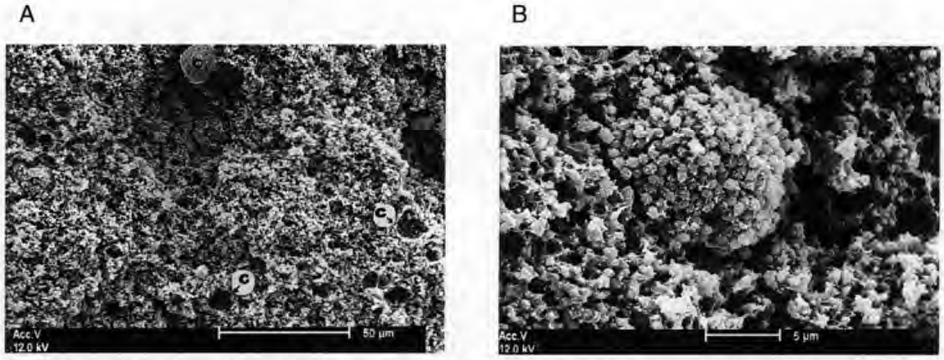

Fig 7. Culture of *L lactis* CNRZ 1076 (prt⁺) in renneted milk VCR 5 UF retentate from skim milk; the level of inoculation was 10^6 cfu/mL. pH 4.90 sample. **A**. Colony (c). **B**. Details of a colony in the same culture. *Culture de* L lactis *CNRZ 1076 (prt⁺) dans du rétentat UF emprésuré (facteur de concentration volumique 5). Le taux d'inoculation était de 10^6 ufc/mL. pH de l'échantillon : 4,90.* ***A****. Colonie (c).* ***B****. Détails d'une colonie dans la même culture.*

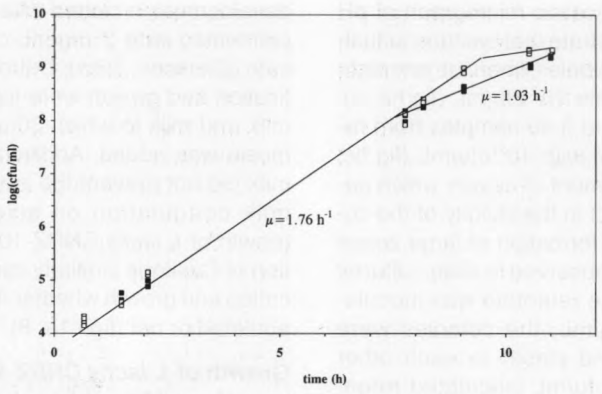

Fig 8. Growth of *L lactis* CNRZ 1076 (prt+) in milk (❑) and rennet curd (■) supplemented with 2 mg/mL casein hydrolysate (Casitone, Difco, Detroit, USA). The level of inoculation was ca 10^4 cfu/mL.
Croissance de L lactis *CNRZ 1076 (prt+) dans le lait (❑) et dans le coagulum présure (■) supplémentés avec 2 mg/mL d'hydrolysat de caséines. (Casitone, Difco, Detroit, États-Unis). Le taux d'inoculation était de 10^4 ufc/mL.*

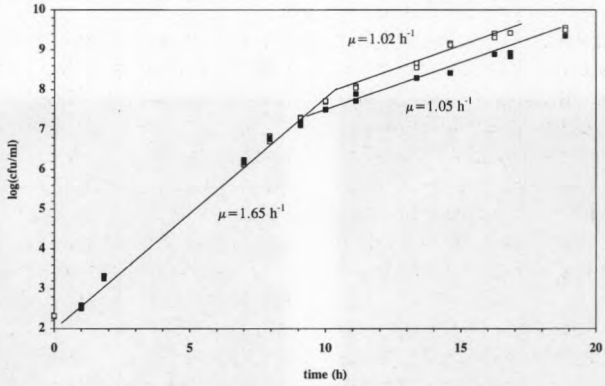

Fig 9. Growth of *L lactis* CNRZ 1076 in VCR 3 UF retentate inoculated with 10^2 cfu/mL. (❑) growth in liquid retentate, (■) growth in renneted retentate.
Croissance de L lactis *CNRZ 1076 (prt+) dans un rétentat UF (facteur de concentration volumique 3). (❑) rétentat liquide, (■) rétentat emprésuré. Le niveau d'inoculation était de 10^2 ufc/mL.*

milk was coagulated or not. The phosphate buffer, at the concentration tried, thus failed to relieve the inhibition observed in rennet curd inoculated at a low level.

ii) The media compared in further experiments were pasteurized bulk skim milk and UF retentate, VCR3, prepared from it. A comparison of figure 9 with figure 1c shows that the effect of coagulation on changes in growth rate of *L lactis* CNRZ 1076 was identical in milk and in retentate. The decrease in growth rate on coagulation occurred as soon as the population level reached ca 3 10^6 cfu/mL in rennet curd from milk (fig 1c) or ca 3 10^6 cfu/g in rennet curd from retentate (fig 9).

Growth of L lactis CNRZ 1075 (prt⁻)

Growth of *L lactis* CNRZ 1075 (prt⁻) in liquid and renneted milk

Observations of slide cultures grown from low inocula showed that *L lactis* CNRZ 1075 (prt⁻) also grew in colonies in the liquid and renneted milk films (results not shown).

Figures 10a, b show the growth of *L lactis* CNRZ 1075 (prt⁻) and the acidification patterns in milk and rennet curd. The level of inoculation was ca 10^2 cfu/mL. The maximum population level achieved was higher in rennet curd (ca 2 10^8 cfu/mL) than in liquid milk (10^8 cfu/mL) (fig 10a). Consequently, the acidification development was faster in rennet curd than in liquid milk after 20 h of incubation and led to a lower final pH, ca 4.7–4.8 (fig 10b). This possibly indicates that peptides released by chymosin activity on caseins were a significant source of nitrogen.

Acidification developed more slowly in the rennet curd inoculated with *L lactis* CNRZ 1075 (prt⁻) than in the rennet curd inoculated with *L lactis* CNRZ 1076 (prt⁺). The final pH, above the isoelectric point of casein, and the low acidification rate could explain why, in slide cultures of *L lactis* CNRZ 1075 (prt⁻), in contrast to what was observed with *L lactis* CNRZ 1076 (prt⁺), no rennet curd disruption developed at the periphery of bacterial colonies, even after 30 h of incubation (results not shown).

On the other hand, in contrast to what was observed with *L lactis* CNRZ 1076 (prt⁺), a decrease in growth rate of CNRZ 1075 (prt⁻) was noticed at a population level of ca 3 10^6 cfu/mL in liquid milk as well as in rennet curd (fig 10a).

Growth of *L lactis* CNRZ 1075 (prt⁻) in liquid and renneted milk supplemented with 2 mg/mL casein hydrolysate

The above-mentioned decrease in growth rate at a population level of ca 3 10^6 cfu/mL was similar in liquid culture to which 2 mg/mL Casitone was added and in the control culture. However, the maximum population level achieved was higher in the supplemented culture than in the control culture (fig 11).

Surprisingly, the inhibition at a population level of ca 3 10^6 cfu/mL was stronger in supplemented renneted culture than in the control renneted culture. No experiment could explain the latter phenomenon.

DISCUSSION

When the population level used to inoculate milk was less than 10^6 cfu/mL, the growth of *L lactis* CNRZ 1076 (prt⁺) significantly differed in liquid milk compared to coagulated milk. In liquid milk, the growth curve shape was in accordance with the observations of Juillard and Richard (1989), whatever the level of inoculation. The transition from the maximum specific growth rate (1.70 h^{-1}) to a lower one (1.01 h^{-1}) when the population level was ca 10^8 cfu/mL has been shown to reflect a requirement for amino acids and peptides (Juillard and Richard, 1989). When compared to growth in liquid milk, *L lactis* CNRZ 1076 was inhibited in rennet curd inoculated with less than 10^6 cfu/mL. Moreover, the data in this report clearly

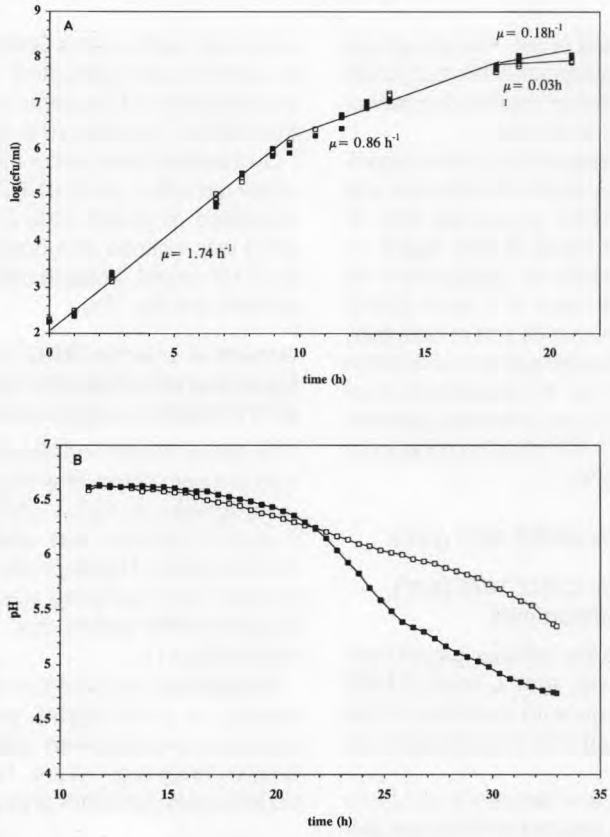

Fig 10. Growth (A) and acidifying (B) activity of L lactis CNRZ 1075 (prt⁻) in milk (❑) and in rennet curd (■) inoculated with 10^2 cfu/mL.
Croissance (**A**) et activité acidifiante (B) de L lactis CNRZ 1075 (prt⁻) dans le lait (❑) et dans le coagulum présure (■). Le taux d'inoculation était de 10^2 ufc/mL.

show that the lower the inoculum level, the sooner the occurrence of growth inhibition in rennet curd. More precisely, the decreases in growth rate always occurred as soon as the population level in culture increased by ca 10^4 cfu/mL over the initial population.

Similar inhibition was observed in renneted and also in liquid milk, inoculated with low levels of the prt⁻ *L lactis* strain CNRZ 1075.

These results can partly find their explanation in microstructural observations on the growth of *L lactis* CNRZ 1076 and CNRZ 1075 in rennet curd and in milk. Light and SEM micrographs show that, if the level of inoculation was less than 10^4 cfu/mL, the lactic acid bacteria examined grew in large colonies in milk or ultrafiltration retentate, in liquid form or coagulated by rennet. When the renneted retentate was inoculated with ca 10^6 cfu/mL, data from SEM also indicated colonies, though smaller in size (than those when a lower inoculum level was used). However, in renneted milk ino-

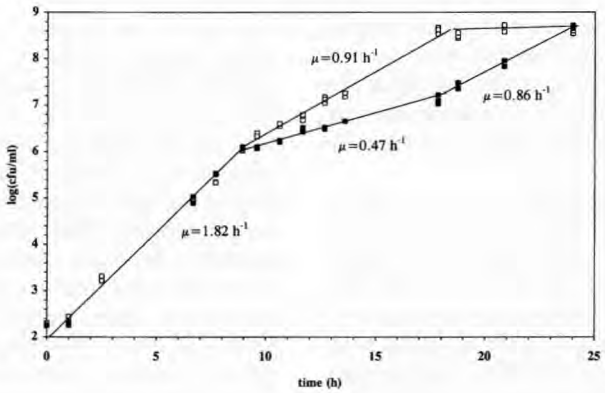

Fig 11. Growth of *L lactis* CNRZ 1075 (prt⁻) in milk (❑) and in rennet curd (■) supplemented with 2 mg/mL casein hydrolysate (Casitone, Difco, Detroit, USA). The level of inoculation was 10^2 cfu/mL. *Croissance de* L lactis *CNRZ 1075 (prt⁻) dans le lait (❑) et dans le coagulum présure (■) supplémentés avec 2 mg/mL d'hydrolysat de caséines (Casitone, Difco, Detroit, États-Unis). Le taux d'inoculation était de 10^2 ufc/mL.*

culated at this level, there was no evidence for individual colonies: bacteria were disseminated throughout the rennet curd, likely because of a lower diffusion of bacteria through the renneted retentate than through the renneted milk.

A possible explanation for the inhibition of *L lactis* culture grown from low inocula is therefore the colonial growth structure. The lower the inoculum level, the more individual and larger the colonies. The colony diameters observed in cultures grown from low inocula were ca 200–300 μm. Cachon and Diviès (1993) showed that the lactococcal cell density in gel beads in a depth of about 150 μm was ca 75% of that found at the bead periphery. From the results on inhibition in liquid and renneted curd it is reasonable to suggests that growth in large colonies was inhibited as soon as colonies achieved a critical population size (ca $3\ 10^4$ cells). The inhibition (at a population of ca $3\ 10^6$ cfu/mL) of the prt⁻ strain culture grown in liquid and renneted milk inoculated with 10^2 cfu/mL suggest that cells in the centre of the expanding microcolony are being inhibited. According to studies about lactococcal cell density in gel beads (Yabannavar and Wang, 1991) lactose is likely not the limiting factor since it is present in medium in high concentrations (ca 50 g/L) and therefore can diffuse fast enough into the centre. However, it is possible that cells in the centre of colonies did not get sufficient essential amino acids in available form (ie, small peptides), depleted by cells in the periphery. Surprisingly, data in our study indicate that amino acids and peptides from pancreatic digest of casein failed to relieve the inhibition of the prt⁻ strain cultures grown from low inocula. A more precise study concerning the effect of addition of known quantities of essential amino acids on the growth of *L lactis* CNRZ 1075 (prt⁻) in low inoculated milk could validly explain the results.

In cultures of the prt⁺ strain grown in liquid milk from low inocula, no decrease in growth rate occurred when the population level in culture increased by ca 10^4 cfu/mL over the initial population. It should be assumed that the colonial growth structure could not on its own account for the inhibition observed in low level inoculated rennet

curd. One can imagine that another condition, the decreased diffusion of solutes through the rennet curd, was necessary to inhibit cultures of the prt⁺strain grown from low inocula. Possible solutes involved are as follows:

– i) Though *L lactis* can use whey proteins (β-lactoglobulin and to a lesser extent α-lactalbumin), casein is the main nitrogen source for growth in milk (Mills and Thomas, 1981). In renneted milk, casein micelles are immobilized and one can wonder if they are still available to *L lactis* cells grown in colonies. According to figure 6a, b, this nitrogen source is located outside of the colony and seems unavailable to *L lactis* cells in its centre. However, since addition of pancreatic digest of casein failed to relieve the inhibition in rennet curd inoculated with low level, it is unlikely that immobilization of caseins micelles could play a role in the decrease in growth rate.

– ii) It has already been reported that the diffusion coefficient of salts such as NaCl is five-fold reduced in cheese moisture as compared to that in pure waters (Geurts et al, 1974). Because of the limited diffusion of protons through the rennet curd, a possible reason for the occurrence of growth inhibition of *L lactis* CNRZ 1076 when colony populations increased beyond the critical size is possibly the rapid decrease of pH near the colonies. Observations on rennet curd to which 2 mg bromocresol purple/100 mL was added presented evidence for concentration of protons in the vicinity of the colonies since this blue pH indicator turned to yellow more quickly at the periphery of colonies than in the middle of the curd (unpublished results). Yabannavar and Wang's study (1991) showed that the use of large beads in order to immobilize cells of *Lactobacillus delbrüeckii* led to significant build-up of inhibitory products, ie, proton and lactate.

The microstructure of the network also supports the assumption that the pH values were much lower in the vicinity of the colonies than in the middle of the curd. Aggregation and concentration of casein micelles actually occurred in a sample of pH 6.30, whereas these phenomena usually indicate a pH value lower than 5.20–5.10 (Heertje et al, 1985), if it is assumed that the renneting of milk does not perturb these rearrangements. The growth of lactic acid bacteria is generally affected by a decrease in the pH of the medium (Longsworth and Mac Innes, 1936; Harvey, 1965; Hugenholtz et al, 1987). The decrease in external pH finally leads to a decrease in internal pH which affects the activities of many enzymes (Kashket, 1987) and also perturbs the transport systems (Poolman et al, 1987). Nevertheless, addition of 20 mmol/L calcium phosphate buffer to renneted milk culture medium (final concentration) did not have any restoring effect on inhibited growth rate, which led us to suggest that bacteria in colonies were not inhibited by the local decrease in pH. This assumption was supported by the observation on growth inhibition in renneted VCR 3 retentate. Despite the higher buffering capacity of the medium, the inhibition ratio of *L lactis* CNRZ 1076 was unchanged compared to that found in renneted milk.

– iii) Moreover, one can suppose that the increase in solubilized micelle bound phosphate concentration on local acidification near the colonies could play a role in the growth inhibition observed in rennet curd inoculated at a low level. Indeed, previous studies (Bergère and Hermier, 1968; Ledford and Speck, 1979; Wright and Klaenhammer, 1984) have shown that some lactic acid bacteria were injured when grown in milk and culture media containing at least 100 mmol/L phosphate. However, the concentration of soluble phosphate in acidified milk is limited to an upper value of 25 mmol/L (Le Graet and Brulé, 1993). On the basis of these observations, unless phosphate is concentrated in bacterial colonies, it seems unlikely that it could inhibit the growth of *L lactis* CNRZ

1076. According to the Nernst equation, accumulation of phosphate in colonies depends on the existence of a positive electrical potential between colonies and curd. Because of high conductivity of acidified milk (> 50 10^{-4} mho, Alais, 1984) generation of a ca 150 A current by colonies would be necessary to obtain a four-fold higher concentration of phosphates in colonies as compared to their concentration in curd!

– iv) Another explanation for the growth inhibition of *L lactis* CNRZ 1076 observed in rennet curd is related to a possible increase of lactate concentration near the bacterial colonies. According to the energy recycling model (Michels et al, 1979), it is well known that a lactate gradient across the cytoplasmic membrane results in the excretion of one molecule of lactate in symport with more than one proton. The generated electrochemical proton gradient was shown to increase both the amount of energy obtained in cells per mol of lactose and of uptake of the amino acid leucine (Otto et al, 1980). With increasing external lactate concentration, the H^+/lactate stoichiometry decreased from 2 to 1, resulting in a negligible energy gain. On the basis of observations on H^+ diffusion through rennet curd, it is reasonable to suppose that rennet curd around the colonies also limits diffusion of lactate. This would lead to a high concentration close to the cells and a possible decrease in growth rate. In liquid milk, however, no inhibition of growth in colonies was observed since H^+ and possibly also lactate could rapidly diffuse. More detailed information on both lactate concentration in the vicinity of colonies and H^+/lactate stoichiometry as related to the growth rate depletion have to be obtained to support this hypothesis.

ACKNOWLEDGMENTS

The authors are indebted to J Berrier, P Boyaval, H Goudédranche, S Lortal, MN Madec, D Mollé and P Schuck for advice and help.

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