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Indirect interaction in milk between proteolytic and isogenic non-proteolytic strains of *Lactococcus lactis*. I. Effect of pre-culturing by a non-proteolytic variant

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Summary —The indirect interaction between proteolytic and isogenic non-proteolytic strains of *Lactococcus lactis* was studied by performing sequential cultures in skim milk. After pre-culturing with a non-proteolytic strain for specific periods of time, the milk was pasteurized, re-inoculated with a second strain and then re-incubated. This second strain was either the same non-proteolytic strain, or the parental proteolytic strain.

In the control milk, both proteolytic parental and non-proteolytic strains grew exponentially at the same rate. Whereas the non-proteolytic variant stopped growing, its parental strain continued to grow exponentially, but at a lower growth rate than in the first exponential growth phase.

When grown in milk pre-cultured by the non-proteolytic strain, the same strain exhibited a slower growth rate and a lower maximum population than when grown in control milk. While abundant growth of the pre-culture strain led to lower maximum populations of the second culture, the combined cell populations in the first and second cultures were constant. Furthermore, this sum was equal to the maximum population attained by the non-proteolytic strain in control milk.

When grown in pre-cultured milk, the proteolytic strain exhibited biphasic exponential growth and attained the same maximum population as when cultured in control milk. While the growth rates during the 2 exponential phases were not affected, the shift from the first to the second growth rate occurred earlier in the pre-cultured than in the control milk, with the shift in growth rates occurring earlier in milk containing higher populations of pre-culture organisms.

This apparent inhibitory effect of pre-culturing milk with a non-proteolytic variant was suppressed by adding some non-proteic nitrogen sources to the milk, thus indicating that the pre-cultured milk was depleted in non-proteic nitrogen sources. The result of this depletion was an indirect competition in milk between isogenic proteolytic and non-proteolytic strains for non-proteic nitrogen. This indirect competition was also observed using 11 other isogenic pairs of proteolytic and non-proteolytic strains belonging to the species *L lactis*.

***Lactococcus lactis* / non-proteolytic variant / pre-cultured milk / non-proteic-nitrogen depletion**

Résumé — Interaction indirecte entre souches isogéniques de *Lactococcus lactis* dans le lait. I. Effet de la pré-culture par un variant non protéolytique. *L'interaction indirecte entre souches isogéniques protéolytiques et non protéolytiques de* Lactococcus lactis *a été étudiée en réalisant des cultures séquentielles dans le même lait : celui-ci était pré-cultivé par une souche non protéolytique pendant une période de temps donnée, pasteurisé, puis réensemencé avec une seconde souche avant d'être incubé à nouveau. Cette seconde souche était soit la même que celle utilisée pour la pré-culture du lait, soit la souche parentale, protéolytique.*

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Dans le lait témoin, la souche protéolytique et la souche non protéolytique poussaient exponentiellement, avec le même taux de croissance. Lorsque le variant non protéolytique s'arrêtait de croître, sa souche parentale continuait à se développer exponentiellement, avec un taux de croissance plus lent que le premier.

Le variant non protéolytique, lorsqu'il était cultivé dans un lait pré-cultivé par la même souche, atteignait un niveau maximal de population plus faible que dans le lait témoin et ce, avec un taux de croissance moins rapide. L'inhibition de la seconde culture était d'autant plus marquée que le niveau de population atteint à l'issue de la pré-culture était important. Cependant, la somme des niveaux en fin de première et de seconde culture restait constante, et correspondait au niveau maximal atteint par cette souche non protéolytique cultivée dans le lait témoin.

La souche parentale, lorsqu'elle était cultivée dans un lait pré-cultivé par son variant non protéolytique, atteignait la même densité cellulaire maximale que dans le lait témoin. La pré-culture du lait n'affectait pas non plus les taux de croissance de la souche parentale pendant les deux phases exponentielles. En revanche, le niveau cellulaire auquel le changement de taux de croissance était observé était d'autant plus faible que le niveau cellulaire atteint à l'issue de la pré-culture était important.

Cet effet inhibiteur de la pré-culture du lait par un variant non protéolytique était supprimé en rajoutant au lait une source d'azote non protéique, ce qui démontre clairement que cette interaction était due à un appauvrissement du lait en certaines fractions azotées non protéiques, et non pas à la production d'une substance inhibitrice par la souche cultivée en premier. Cette interaction se traduisait donc par une compétition indirecte entre les deux souches pour ces fractions azotées. Cette compétition indirecte, entre souches isogéniques protéolytiques et non protéolytiques, a été constatée avec onze autres paires de souches de Lactococcus lactis. *Elle semble donc être un caractère commun aux souches de cette espèce cultivées séquentiellement dans le lait.*

Lactococcus lactis / *variant non protéolytique / pré-culture du lait / azote non protéique / épuisement*

INTRODUCTION

A preliminary study (Juillard and Richard, 1989) has shown various interactions between protease-positive (prt$^+$) strains of *Lactococcus lactis* subsp *lactis* and the corresponding protease-negative (prt$^-$) variants of the same strain when grown in milk. In sequential cultures of these pairs of isogenic prt$^+$ and prt$^-$ strains (*ie*, milk pre-cultured with one of the 2 strains for a specific period of time, followed by pasteurization, re-inoculation with the second strain and continued incubation), the growth of the second strain was always inhibited, regardless of the order of strains (Juillard and Richard, 1989).

To explain these observations, 2 hypotheses have been proposed. In the first case, it is assumed that each strain produces one or more substances that are inhibitory to the other strain. Since these substances can withstand normal pasteurization of milk, they should also be thermostable.

The second hypothesis (which is in better agreement with what is presently known about growth of these bacteria in milk) is based on their nitrogen requirements. All such organisms require non-proteic nitrogen sources such as free amino acids and peptides for growth (Reiter and Oram, 1962; Mills and Thomas, 1981; Marshall and Law, 1983). However, since the concentration of these compounds in milk is low (Mills and Thomas, 1981; Law and Kolstadt, 1983; Thomas and Pritchard, 1987; Hugenholtz *et al*, 1987), the prt$^-$ variants stop growing when these compounds have been consumed, whereas the prt$^+$ strains continue to grow as a result of their proteolytic activity (Thomas and Mills,

1981; Thomas and Pritchard, 1987). Thus pre-culturing milk with a prt⁻ strain of lactococci will induce depletion resulting in an indirect competition between the first and the second culture for non-proteic nitrogen nutrients naturally present in milk.

The present study was conducted to investigate the indirect effect of pre-culturing milk with a prt⁻ strain followed by addition of either the same strain or the parental prt⁺ strain, and to quantify this effect.

MATERIALS AND METHODS

Strains

The study was initiated using one reference pair: *Lactococcus lactis* subsp *lactis* CNRZ 1076 (prt⁺) and one of its prt⁻ variants (strain CNRZ 1075). The main results were then confirmed using 11 other pairs of prt⁺ and prt⁻ strains of *L lactis* from our collection. Seven of these strain pairs belonged to the subspecies *lactis*, and the last 4 prt⁺, prt⁻ strain pairs belonged to the subsp *cremoris* (see table III). Culture propagation and handling of these strains have been previously described (Juillard and Richard, 1989).

Culture medium

The culture medium was reconstituted skim milk (10% Nilac low heat milk powder, Nizo, Ede, the Netherlands), in sterile water. Given the high bacteriological quality of this milk (under 10^2 bacteria per ml) no further heat treatment was deemed necessary.

Partial depletion of the milk in non-proteic nitrogen (NPN) content was obtained by first culturing the milk with the prt⁻ strain. After readjusting the pH of the milk to that of the control milk (pH 6.70), the NPN-depleted milk was pasteurized for 30 min at 63 °C to give a residual flora that was consistently $< 5 \times 10^2$ cfu (colony-forming units)/ml. Previous work (Juillard and Richard, 1989) has shown that moderate acidification of the milk followed by pH re-adjustment and pasteurization do not significantly affect the behaviour of the second culture.

NPN content of the milk was also increased by adding an acid casein hydrolysate (Bacto vitamin-free casamino acids, Difco), or a mixture of the 18 usual amino acids (all at the same concentration), or a peptide source (bactotryptone or proteose-peptone No 3, Difco) to samples of both control and NPN-depleted milk.

Culture conditions

Pre-culture conditions and method of milk inoculation have been previously described (Juillard and Richard, 1989). All cultures were incubated at 30 °C, without shaking or any type of pH control system. To minimize the influence of oxygen, the dimension of the flask and the volume of the culture medium were chosen so that the ratio of surface to volume did not exceed 0.1 cm^{-1}. In addition, all milk samples were taken from the middle rather than the top or bottom of the flask.

Bacterial enumerations

Cell populations were estimated by plating samples on M17 medium (Terzaghi and Sandine, 1975), with a spiral plater (Spiral System, model DS, Interscience), after mechanically disrupting the cell chains (Ultra-Turrax, model T25, 20 000 rpm for 30 s, Hassan *et al*, 1989). The homogeniety of the prt⁺ population (possible presence of prt⁻ variants) was checked at both the beginning and end of each culture, using FSDA medium (fast–slow–differential–agar; Huggins and Sandine, 1984). The strain under study always represented more than 97% of the total population.

Measurement of pH

The pH of the milk was determined by a combination electrode (Ingold), connected to a Knick pH-meter (Portamess, model 654).

Determination of bacterial ATP

Intracellular ATP was extracted from bacteria as follows (Jakubczak and Leclerc, 1980) : 900 µl of dimethyl-sulfoxide solution (Biosys) were added to 100 µl of culture sample. After 15 s of vigorous stirring, the mixture was stored at room temperature for 1.25 min. Extracted ATP was then stabilized by adding 5 ml of morpholino-propane-sulfonic acid buffer (Biosys) (Jakubczak and Leclerc, 1980). After slow homogenization and storage for 1.5 min, 50 µl of a luciferin-luciferase reagent (Biosys) were added to 400 µl of the extract. Light emission was measured at 562 nm by using an ATP-meter (Biolumat LB9500T, Berthold).

RESULTS

Growth of prt⁺ and prt⁻ strains and pH change in milk pre-cultured with the prt⁻ variant

The prt⁺ strain (CNRZ 1076) and its prt⁻ variant (CNRZ 1075) were grown in control milk and milk pre-cultured with the prt⁻ strain. Figure 1 gives an example of the population dynamics and pH evolution for a particular level of the prt⁻ strain at the end of the pre-culture (≈ 10^8 cfu/ml).

The maximum population attained by the prt⁺ strain grown in milk pre-cultured with the prt⁻ strain did not seem to be significantly different from that observed in control milk. However, the time needed to reach a particular level was definitely longer in pre-cultured than in control milk. As previously observed in control milk (Juillard and Richard, 1989), growth of the prt⁺ strain in pre-cultured milk was biphasic; however, the growth rate changed earlier in the pre-cultured milk (after 1 rather than 2 h in control milk). As a result, the population level at which this change in growth rate was observed was lower in pre-cultured rather than control milk (2 x 10^7 cfu/ml *vs* 10^8 cfu/ml; fig 1a). However, values for growth rates during the first and second exponential phases were not affected by pre-culturing.

These modified growth patterns for the prt⁺ strain led to slower acid development with a 1-h delay noted between pH 6.5 and 5.0 to reach the same pH values as the control. However, as expected, pH values of both control and pre-cultured milk were the same following 9 h of incubation (≈ 4.5 pH unit). This is a direct result of both cultures attaining the same maximum population after 7–8 h of incubation.

The effect that pre-culturing of the prt⁻ strain had on subsequent growth of the same strain in milk was quite different (fig 1b). The maximum population was definitely lower (*ca* 0.3 log cfu/ml) in pre-cultured than in non pre-cultured milk, with a decrease in the exponential growth rate also in pre-cultured milk. The duration of the exponential growth period seemed to be slightly shortened in pre-cultured milk (0.5 h of difference between pre-cultured and control milk), althought this shortened duration was probably non significant (see next section). Furthermore, acidification was slower in pre-cultured rather than in control milk (difference of 0.2 pH unit after 6 h of incubation).

Effect of the pre-culture level on subsequent growth of prt⁺ and prt⁻ strains

Milk samples were pre-cultured with prt⁻ strains for various lengths of time to obtain different cell populations.

The effect of the pre-culturing on growth parameters of parental strain CNRZ 1076 (as a second culture) is presented in table I. This culture remained biphasic in milk,

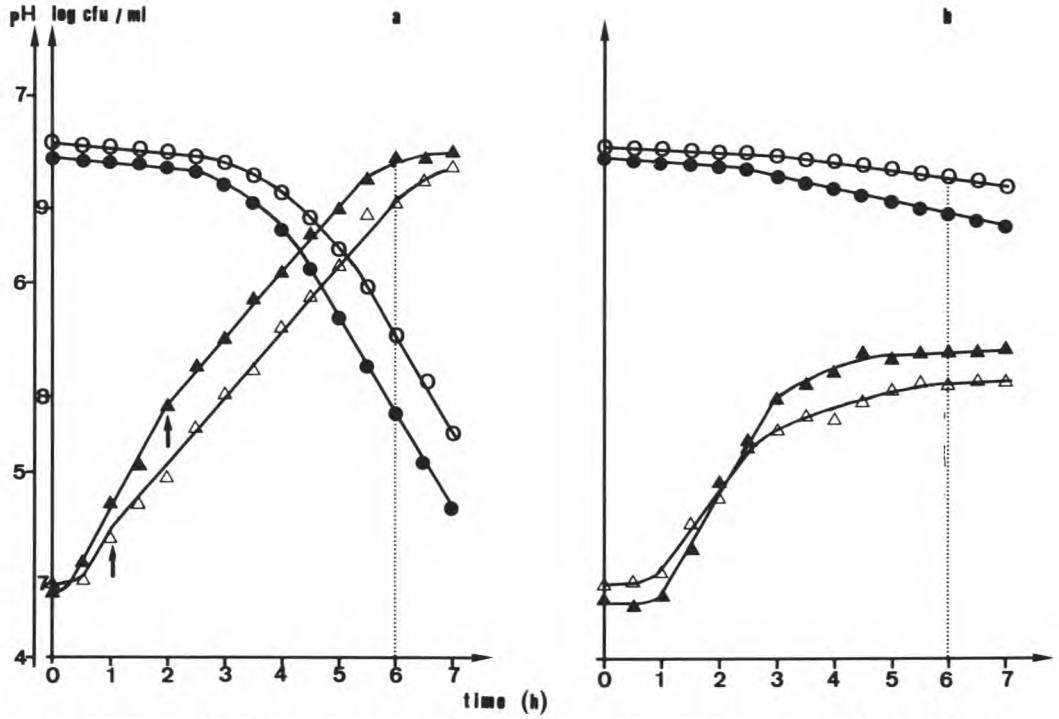

Fig 1. Growth of *Lactococcus lactis* subsp *lactis* CNRZ 1076 (prt⁺ strain) and CNRZ 1075 (prt⁻ strain) in control milk and milk pre-cultured with the prt⁻ variant. a: prt⁺ strain; b: prt⁻ strain; ●: pH; ▲: cell population; : change in exponential growth rate; closed symbols: growth in control milk, open symbols: growth in pre-cultured milk. The prt⁻ population after pre-culturing was $1.6 \cdot 10^8$ cfu/ml (*ie,* in stationary phase) in a, and $1.0 \cdot 10^8$ cfu/ml *(ie,* at the end of the exponential growth phase) in b.
Croissance de Lactococcus lactis *subsp* lactis *CNRZ 1076 (souche prt⁺) et CNRZ 1075 (variant prt⁻) dans du lait témoin et dans du lait pré-cultivé par le variant prt⁻.* a: *souche prt⁺;* b: *souche prt⁻; ●: pH; ▲: densité cellulaire; : changement de taux de croissance; symboles pleins: croissance dans le lait témoin; symboles ouverts : croissance dans le lait pré-cultivé. Le niveau cellulaire de la souche prt⁻, en fin de pré-culture, était de $1,6 \cdot 10^8$ ufc/ml (correspondant à la phase stationnaire) en* a, *et de $1,0 \cdot 10^8$ ufc/ml (correspondant à la fin de la phase exponentielle) en* b.

except when the prt⁻ pre-culture was extended to 20 h at 30 °C. The first exponential growth phase of the second culture was markedly shorter using pre-cultured samples in which the first strain had previously attained higher levels – *ie,* from 4 h in control milk, to 1 h in pre-cultured milk containing 1.55×10^8 cfu/ml (corresponding to the stationary phase). However, when this pre-culture was extended to 20 h at 30 °C, resulting in a small increase in cell population (1.6×10^8 cfu/ml), the first exponential growth phase of the second culture was no longer observed. In addition, duration of the second exponential growth phase of the prt⁺ strain increased as the cell density of the pre-culture increased (from 3.5 h in control milk to 7 h

Table I. Growth of *Lactococcus lactis* subsp *lactis* CNRZ 1076 (prt⁺ strain) in milk pre-cultured with one of its prt⁻ variants (strain CNRZ 1075).
Croissance de Lactococcus lactis *subsp* lactis *CNRZ 1076 (souche prt⁺) dans du lait pré-cultivé avec un de ses variants prt⁻ (souche CNRZ 1075).*

1st culture (prt⁻ strain)	*2nd culture (Prt⁺ strain)*					
Level at end of culture (cfu/ml x 10^8)	*1st exponential growth phase*		*2nd exponential growth phase*		*Bacterial populations (cfu/ml) when:*	
	Duration (h)	*Growth rate (log/h)*	*Duration (h)*	*Growth rate (log/h)*	*Changing exponential growth phase (x 10^7)*	*Entering stationary phase (x 10^9)*
0[a]	4.0	0.51 ± 0.04[b]	3.5	0.36 ± 0.02[b]	7.9 ± 1.3[b]	2.2 ± 0.7[b]
1.1	3.5	0.46	3.5	0.34	6.7	2.2
1.3	3.0	0.48	4.0	0.36	4.4	1.7
1.55	1.0	0.49	6.0	0.36	0.6	2.2
1.6[c]	0	NO	7.0	0.37	NO	2.5

[a, b]: Control milk (without pre-culture); average of 4 determinations. [c]: After 20 h of incubation at 30°C. NO: Not observed (absence of the first exponential phase). [a, b]: *Lait témoin (sans pré-culture); moyenne de 4 déterminations.* [c]: *Après 20 h d'incubation à 30°C. NO: Non observé (absence de première phase exponentielle).*

with maximum pre-culture). As a result, the exponential growth phase of the second culture changed sooner as the density of the prt⁻ strain in the pre-culture was higher. However, the total duration of the 2 exponential phases remained constant, at ≈ 7 h. As long as the first exponential growth phase of the prt⁺ strain was observed, its rate was apparently not affected by pre-culturing the prt⁻ variant. But as the duration of the first exponential growth phase decreased, the number of the experimental points belonging to the first exponential growth phase decreased, and it resulted in a lower accuracy of slope estimation (Hassan *et al,* 1989). Overall, the slope of the second exponential growth phase remained unchanged (in the range of 0.34–0.35 log cfu/ml per h), regardless of the level of pre-culture organisms. Moreover, the final population density of the second culture was not dependent upon the cell density of the first culture (average 2.2 ± 0.4 x 10^9 cfu/ml).

Results obtained for the prt⁻ strain CNRZ 1075 as a second culture are shown in table II. The first consequence of pre-culturing the prt⁻ strain in milk was to decrease the growth rate of the second culture of that strain, but apparently not the duration of its exponential growth phase. The higher the cell density at the end of pre-culturing, the lower the growth rate in the second culture (from 0.53 log unit per h in control milk, to 0.31 log unit per h in pre-cultured milk containing up to 1.2 x 10^8 cfu/ml). In addition, maximum populations in the second culture were lower in pre-cultured rather than control milk. When the first culture attained maximum cell density

Table II. Growth of *Lactococcus lactis* subsp *lactis* CNRZ 1075 (prt⁻ strain) in milk pre-cultured with different levels of the same strain.
Croissance de Lactococcus lactis *subsp* lactis *CNRZ 1075 (souche prt⁻) dans du lait pré-cultivé à différents niveaux cellulaires par la même souche.*

1st culture	*2nd culture*			*Sum of the levels of the 2 cultures*
Level at end of culture (cfu/ml) x 10^8)	*Exponential phase*		*Stationary phase*	
	Duration (h)	*Growth rate (log/h)*	*Cell level (cfu/ml x 10^8)*	*(cfu/ml x 10^8)*
0[a]	3.5	0.53 ± 0.06[b]	1.7 ± 0.3[b]	1.7 ± 0.3[b]
< 0.1	3.0	0.52	1.7	1.8
0.2	3.5	0.54	1.5	1.7
0.4	4.0	0.49	1.2	1.6
0.7	4.0	0.41	0.9	1.6
1.0	4.0	0.41	0.6	1.6
1.2	4.0	0.31	0.3	1.5
1.6[c]	NO	NO	0.02	1.6 (m = 1.6 ± 0.1)

[a, b]: Control milk (without pre-culture); average of 4 determinations. [c]: After 20 h of incubation at 30°C. NO: Not observed (absence of exponential phase).
[a, b]: Lait témoin (sans pré-culture); moyenne de 4 déterminations. [c]: Après 20 h d'incubation à 30°C. NO: Non observé (absence de première phase exponentielle).

(*ie*, 1.6 x 10^8 cfu/ml), it was no longer possible to detect any exponential growth in the second culture with population increases of only 10^6 cfu/ml at the time of inoculation to 2 x 10^6 cfu/ml after 6 h of incubation at 30 °C. The sum of the combined cell populations after pre-culture and secondary culture remained constant, and was not significantly different from maximum populations attained by the prt⁻ strain in control milk.

Effect on the cultures of the prt⁺ strain of addition to milk and pre-cultured milk of different nitrogen sources

To verify that the inhibitory effects just discussed were not due to production of an inhibitory substance, but rather to depletion of nutrients from milk, particulary NPN sources, different non-proteic nitrogenic compounds were added to both control milk and milk pre-cultured with the prt⁻ variant CNRZ 1075, before culturing with the parental strain.

As shown in figure 1, pH determinations on control and pre-cultured milk after 6 h of incubation at 30 °C can be used to indirectly evaluate inhibition of the second culture. Accordingly, the effect of additions was determined using this experimental procedure. Each trial was repeated 4 times. Figure 2 illustrates results of mean determinations, with 95% confidence intervals.

The inhibitory effect of pre-culturing milk with the prt⁻ variant was again confirmed:

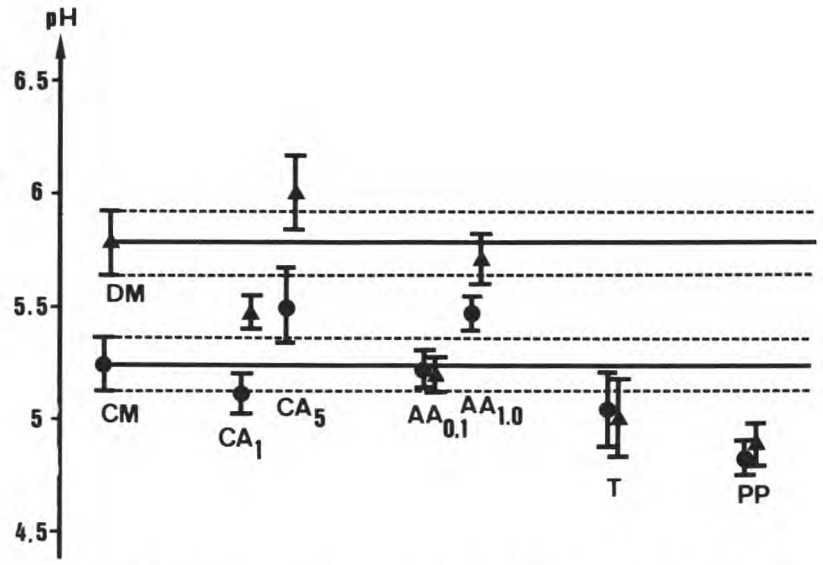

Fig 2. Effect of adding different nitrogen sources on the pH of *Lactococcus lactis* subsp *lactis* CNRZ 1076 (prt⁺ strain) culture after 6 h of incubation at 30 °C in control milk and milk pre-cultured with *L lactis* subsp *lactis* CNRZ 1075 (prt⁻ strain). ●,▲: control milk (CM) and pre-cultured (depleted) milk (DM), respectively (means of 4 repetitions with 95% confidence interval of the means). Addition of casamino acids, 1 g/l (CA_1) or 5 g/l (CA_5); a mixture of 18 amino acids, at 0.1 g/l of each ($AA_{0.1}$) or 1.0 g/l of each ($AA_{1.0}$); tryptone, 1 g/l (T); proteose–pepone, 1 g/l (PP). Milk pH and prt⁻ populations after pre-culturing were 6.30 ± 0.04 and (1.8 ± 0.1)10^8 cfu/ml respectively, corresponding to the stationary phase of the prt⁻ strain; pH of the milk was adjusted to 6.74 before re-inoculation with the prt⁺ strain at *ca* 10^7 cfu/ml for the second culture.

Effet de l'addition de diverses sources d'azote sur le pH de la culture de Lactococcus lactis *subsp lactis CNRZ 1076 (souche prt⁺), après 6 h d'incubation à 30 °C dans du lait témoin ou dans du lait pré-cultivé par L* lactis *subsp* lactis *CNRZ 1075 (souche prt⁻). ●,▲: lait témoin (CM) et lait pré-cultivé (appauvri) (DM), respectivement (moyenne de 4 répétitions avec intervalle de confiance de la moyenne à 95%). Addition de casaminoacides, 1 g/l (CA_1) ou 5 g/l (CA_5); d'un mélange de 18 acides aminés à 0,1 g/l de chaque ($AA_{0.1}$) ou à 1,0 g/l de chaque ($AA_{1.0}$); de tryptone, 1 g/l (T); de protéose-peptone, 1 g/l (PP). Le pH du lait et le niveau de population en fin de pré-culture étaient de 6,30 ± 0,04 et de (1,8 ± 0,1)•10^8 ufc/ml, respectivement, correspondant à la phase stationnaire de croissance de la souche prt⁻; le pH du lait était ajusté à 6,74 avant ré-ensemencement du lait avec la souche prt⁺ à un niveau cellulaire de l'ordre de 10^7 ufc/ml en seconde culture.*

a significant mean difference of *ca* 0.5 pH unit (5.78 ± 0.14 and 5.24 ± 0.12 respectively) was observed between depleted milk (DM) by pre-culturing the prt⁻ strain and control milk (CM).

Adding 1 g/l of casamino acids (CA_1) to both milks hastened acidification, but the decrease in pH was statistically significant only for pre-cultured milk. The beneficial effect of casamino acids disappeared when used at a concentration of 5 g/l (CA_5) : pH values of both supplemented milks were ≈ 0.25 pH unit higher than the corresponding non-supplemented milks.

Supplementing pre-cultured milk with 0.1 g/l of each of the usual 18 amino acids ($AA_{0.1}$) completely suppressed the inhibition, but failed to stimulate acidification of control milk. As already observed with the casamino acids, the beneficial effect of adding a mixture of amino acids partially disappeared when the concentration of each amino acid was raised to 1.0 g/l ($AA_{1.0}$).

Fortification of milk with 0.1% tryptone (T) also completely suppressed the inhibitory effect obtained by pre-culturing milk with the prt⁻ variant: a difference in pH was no longer observed between non-pre-cultured and pre-cultured milk. Adding tryptone to control milk slightly enhanced acid production by the prt⁺ strain; however, this difference (5.05 ± 0.15 *vs* 5.24 ± 0.12) was not statistically significant. The addition of 0.1% proteose–peptone (PP) to both milks definitely stimulated acid production by the prt⁺ strain.

Addition of proteose-peptone to milk during secondary culturing with the same non-proteolytic strain

Since addition of proteose–peptone to milk pre-cultured with the prt⁻ strain stimulated subsequent growth of the parental prt⁺ strain, such an addition should also enhance growth of the paired prt⁻ strain in second culture. Moreover, a new exponential growth phase should be observed for this second culture if proteose-peptone is added at the end of the second culture in depleted milk. To verify this hypothesis, the prt⁻ strain CNRZ 1075 was grown in control milk and milk pre-cultured with the same prt⁻ strain up to approximately 4 x 10^7 cfu/ml (*ie,* the middle of its exponential growth phase). When the second culture of the prt⁻ strain was nearing its stationary phase, 0.1% proteose–peptone was added to the milk. This moment was precisely estimated by using bioluminescence, as bacterial growth and intracellular production of ATP are coupled (Forrest, 1965; Roy and Goulet, 1985; Siro, 1985).

The inhibitory effect of culturing milk with the prt⁻ variant on subsequent growth of the same prt⁻ strain was again confirmed (fig 3): the exponential growth rate was slower in pre-cultured than in control milk (dashed line), with maximum populations also lower in the former. As expected, growth of the prt⁻ strain resumed after the milk was supplemented with proteose–peptone. The new exponential growth rate was not statistically different from that observed in control milk (0.50 log unit per h *vs* 0.53 ± 0.06 respectively). In addition, the prt⁻ strain attained maximum populations that were *ca* 10-fold higher in supplemented rather than non-supplemented control milk. This maximum level was close to that observed for the parental strain in control milk – *ca* 3 x 10^9 cfu/ml.

Extension of the study to other strains of Lactococcus lactis

In an effort to expand these results to other prt⁺, prt⁻ pairs of *Lactococcus lactis* strains, the same kind of study was repeated using 11 other prt⁺, prt⁻ strain pairs, and a simplified experimental scheme. Each prt⁺ strain was grown in control milk and milk pre-cultured with the corresponding prt⁻ variant. After pre-culturing, all prt⁻ strains attained maximum populations of *ca* 10^8 cfu/ml (*ie,* the end of the exponential growth phase). Cell densities of corresponding prt⁺ strains and the pH of both control and pre-cultured milk were taken after 4 h of incubation at 30 °C, to obtain a significant effect on both pH and population level (see fig 1). The effect of pre-culturing on the second culture was ex-

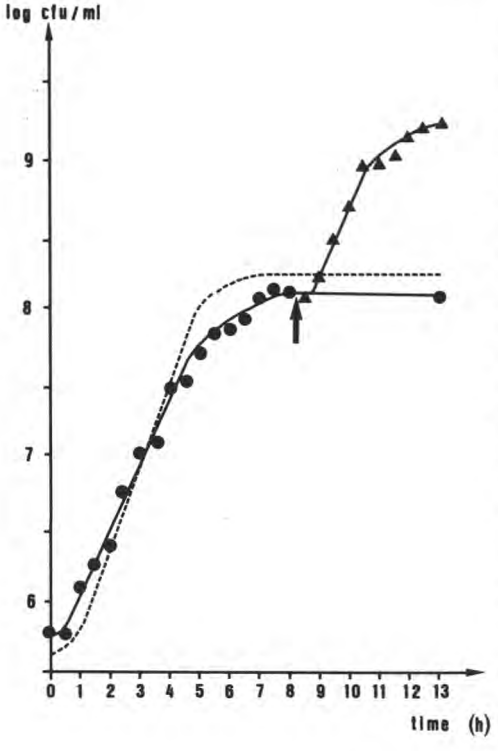

Fig 3. Effect of adding proteose–peptone to pre-cultured milk at the beginning of the stationary phase of *Lactococcus lactis* subsp *lactis* CNRZ 1075 (prt⁻ strain). ●: Growth in milk pre-cultured with the prt⁻ strain, before supplementation; : time of proteose–peptone addition (1 g/l); ↟ : growth in pre-cultured milk after supplementation. Dashed line : growth of the same strain in control milk (for clarity, experimental data are not set out in the figure). The prt⁻ population at the end of pre-culturing was $3.9 \cdot 10^7$ cfu/ml, *ie*, the pre-culture was stopped in the exponential growth phase.

Effet de l'addition de protéose–peptone au lait pré-cultivé, au début de la phase stationnaire de croissance de Lactococcus lactis *subsp* lactis *CNRZ 1075 (souche prt⁻). ● : Croissance dans le lait pré-cultivé par la souche prt⁻, avant la supplémentation; : moment de l'addition de protéose–peptone (1 g/l); ↟ : croissance dans le lait pré-cultivé, après qu'il ait été supplémenté; courbe en pointillés : croissance de la même souche dans du lait témoin (par souci de clarté, les valeurs expérimentales n'ont pas été portées sur la figure). La densité de population de la souche prt⁻ en fin de pré-culture était de $3,9 \cdot 10^7$ ufc/ml, c'est-à-dire que la pré-culture a été arrêtée pendant la phase exponentielle de croissance.*

pressed as (i) the difference in pH between pre-cultured and control milk, and (ii) the ratio of the cell densities following incubation. Thus, an inhibitory effect is characterized by a positive difference in pH (from slower acidification) and a population ratio < 1.0 (as a result of a lower population in pre-cultured rather than in control milk). On the contrary, a stimulatory effect is characterized by a negative difference in pH and a population ratio > 1.0.

In control milk, the prt⁺ strains attained a mean level of $4.9 \pm 1.6 \times 10^8$ cfu/ml following 4 h of incubation and a mean pH of 6.17 ± 0.14.

In all cases, pre-culturing milk with prt⁻ variant strains led to subsequent inhibition of the prt⁺ strains (table III). Thus, all population ratios were < 1.0 (grand mean ratio: 0.64 ± 0.09) and all pH differences were positive (grand mean value: 0.17 ± 0.04 pH unit). However, the degree of inhibition differed from one prt⁺/prt⁻ strain pair to another, with cell density ratios ranging from 0.37 (strain 187) to 0.85 (strain 385).

Addition of 1 g/l of proteose–peptone to depleted milk by pre-culturing the prt⁻ strain suppressed the inhibition of all second cultures: the population ratios ranged from 0.95 (strain CNRZ 112) to 1.90 (strain CNRZ 380), and the pH differences from + 0.06 (strain CNRZ 114) to 0.21 (strain CNRZ 257). As the mean population ratio and the mean pH difference for supplemented milk are significantly different from one and zero respectively, one can assume that the addition of proteose-peptone

Table III. Effect of proteose–peptone (1 g/l) on the growth of different prt+ strains of *Lactococcus lactis* in milk pre-cultured with the corresponding prt− variant up to cell densities of about 10^8 cfu/ml.
Effet de la protéose–peptone sur la croissance de différentes souches prt+ de Lactococcus lactis *incubées dans du lait pré-cultivé par les variants prt− correspondants jusqu'à des densités cellulaires de l'ordre de 10^8 ufc/ml.*

Strain	*Pre-cultured milk*		*Pre-cultured milk + proteose–peptone*	
	Population ratio[a]	*pH difference*[b]	*Population ratio*[a]	*pH difference*[b]
L lactis subsp *lactis*				
CNRZ 145	0.72	+ 0.18	1.13	− 0.14
CNRZ 261	0.70	+ 0.17	1.05	+ 0.03
CNRZ 377	0.67	+ 0.24	1.07	− 0.09
CNRZ 1076	0.63	+ 0.22	1.03	− 0.05
L lactis subsp *lactis* biovar *diacetylactis*				
CNRZ 125	0.40	+ 0.25	1.34	− 0.13
CNRZ 187	0.37	+ 0.14	1.23	− 0.16
CNRZ 257	0.71	+ 0.13	1.32	− 0.21
CNRZ 267	0.76	+ 0.13	1.14	− 0.02
L lactis subsp *cremoris*				
CNRZ 112	0.51	+ 0.16	0.95	+ 0.03
CNRZ 114	0.58	+ 0.15	0.96	+ 0.06
CNRZ 379	0.78	+ 0.25	1.11	+ 0.02
CNRZ 380	0.85	+ 0.03	1.90	− 0.08
Mean	0.64 ± 0.09	+ 0.17 ± 0.04	1.19 ± 0.16	− 0.06 ± 0.05

Cell density and pH were taken after 4 h of incubation at 30°C. [a]: Cell density in pre-cultured milk / cell density in control milk. [b]: pH of pre-cultured milk minus pH of control milk.
La densité cellulaire et le pH étaient estimés après 4 h d'incubation à 30°C. [a]: Densité cellulaire du lait pré-cultivé/ densité cellulaire du lait témoin. [b]: pH du lait pré-cultivé moins pH du lait témoin.

to depleted milk not only restores the growth of all second cultures, but also stimulates the growth of some of them (for example, strains CNRZ 125, 187, 257).

DISCUSSION

Pre-culturing milk with a prt− strain of *Lactococcus lactis* caused an inhibition of the same strain in a second culture. A similar inhibitory effect was also observed when the parental prt+ strain was used in a second culture. On the other hand, addition of a nitrogen source (either amino acids or peptides) to the pre-cultured milk totally suppressed the inhibition of the prt+ strain. This phenomenon of inhibition of the prt+ strain in second culture, and its suppression by adding a NPN source was observed with all prt+, prt− pairs of *L lactis* strains tested. It seems therefore to be a characteristic feature of that species when sequentially cultured in milk.

Since addition of a NPN compound stimulated the growth of the prt$^+$ strains used as second cultures, the hypothesis of the production of an inhibitory substance by the corresponding prt$^-$ variants can be definitively rejected, whereas that of the depletion of the milk in NPN content by the prt$^-$ pre-culture can be considered as valid. However, the nature and/or the concentration of the added nitrogen source is important, as exemplified by stimulation by 1 g/l of casamino acid, but inhibition by 5 g/l of this source. This inhibition may be due to the inhibitory effect of an excess of a particular amino acid, as shown with glutamate (> 2 mM) in the case of *L lactis* subsp *cremoris* Wg2 (Otto, 1981).

The conclusion that milk depletion is caused by pre-culturing a prt$^-$ strain is in agreement with the acceptance that the NPN fraction of the milk is growth-limiting for lactococci (Mills and Thomas, 1981; Law and Kolstadt, 1983; Hugenholtz *et al*, 1987; Laan *et al*, 1989). This was evidenced by the observation that prt$^-$ strains stop growing at a lower level than the corresponding parental prt$^+$ strains, and that addition of an extraneous source of nitrogen allows growth to resume (Pearce *et al*, 1974; Selby Smith *et al*, 1975; Otto, 1981; Thomas and Mills, 1981).

As a consequence of milk depletion in NPN compounds by culturing a prt$^-$ strain, one would expect a second culture of the same prt$^-$ strain in the same milk to grow poorly. More precisely, according to Monod (1949), the growth rate and the maximum population of the second culture should be lower, but the combined population of the first culture and the maximum population of the second should remain constant and equal the maximum level that this prt$^-$ strain is able to attain in control milk. This is exactly what was observed in our study using the reference strain CNRZ 1075.

The depletion in milk NPN compounds by pre-culturing a prt$^-$ strain of lactococci should have only one direct effect on the second culture of the corresponding prt$^+$ strain: an earlier change in growth rate in depleted than in control milk. More precisely, the higher the population level attained by the prt$^-$ strain during pre-culture, the more complete the depletion, and consequently the earlier the change in growth rate and the lower the population level at the time of change. Ultimately, the first growth rate is expected to completely disappear when the growth of the prt$^-$ is continued up to its maximum. This is exactly what was obtained.

The second growth phase of the prt$^+$ strain (due to the production of peptides resulting from proteolytic activity) stopped when the same population as that in control milk was attained. Since the first growth phase of a prt$^+$ strain in depleted milk is shortened, it results from the above observation that the second growth phase of that prt$^+$ strain should be longer in depleted than in control milk. This is again exactly what was observed.

In addition, the growth rate during this second phase remained constant regardless of the extent of the depletion, and was not significantly different from that in control milk. This is an indication that the peptides formed by proteolysis did not change in nature or had the same nutritional value throughout this second phase of growth. Since the second growth rate is lower than during the first exponential growth phase, one can assume that the NPN compounds produced by proteolysis are unable to sustain the same growth rate as the NPN compounds naturally present in milk, either because the products of proteolysis are less efficient than natural NPN compounds, or because the rate of proteolysis is too low to allow rapid growth of the prt$^+$ strain during its second exponential growth phase.

As a result of a complete kinetic study, a model of growth of *Lactococcus lactis* in milk depleted in NPN compounds by pre-culturing a prt^- variant has been obtained using a reference couple of prt^-, prt^+ strains. This model seems to be valid for any isogenic pairs of prt^+, prt^- strains of the species, since all pairs tested in this study responded in the same way to milk depletion and supplementation. More precisely, it was expected that depletion by pre-culturing a prt^- variant would hasten the beginning of the growth phase relying on proteolysis. It was also assumed that the resulting growth rate would be lower than in the natural NPN compounds of the milk. The results obtained prove that the latter assumption is also valid for any prt^+ strain of lactococci. It was verified more directly when studying growth in milk of other prt^+ strains of lactococci (unpublished results). The question now is to determine why the second growth rate is lower than the first.

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