Lait (1995) 75, 401-413

Original article

Antagonistic interactions between propionic acid bacteria and non-starter lactic acid bacteria

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Summary — *L rhamnosus* JCL 1211 and *L casei* JCL 1227 isolated from hard cheese facultatively heterofermentative microflora were shown to negatively interfere with the growth of *P freudenreichii* in hard Swiss-type cheese. As the observed inhibition could not be reproduced in batch cultures, the metabolism of inhibitory species was studied in cheese and in culture. Citrate appears to be the principal energy source for growth of facultatively heterofermentative microflora which metabolizes it to acetate, formate and carbonate. Small amounts of diacetyl are also produced during citrate fermentation. Diacetyl, acetate and formate can participate in the inhibitory effect observed.

***L rhamnosus* / *L casei* / *P freudenreichii* / cheese / citrate**

Résumé — Antagonisme entre bactéries propioniques et flore lactique non levain. L rhamnosus *JCL 1211 et* L casei *JCL 1227 isolés de la microflore des lactobacilles hétérofermentaires facultatifs présents dans les fromages à pâte dure contrarient la croissance de* P freudenreichii *dans le fromage. Comme l'inhibition observée dans le fromage n'est pas reproductible en culture, le métabolisme des souches inhibitrices a été étudié dans le fromage et en culture. Le citrate semble constituer la principale source d'énergie pour la croissance des hétérofermentaires facultatifs qui le transforment en acétate, formiate et carbonate. De faibles quantités de diacétyle sont aussi produites pendant la fermentation du citrate. Le diacétyle, l'acétate et le formiate peuvent contribuer à l'inhibition observée.*

L rhamnosus / L casei / P freudenreichii / *fromage* / *citrate*

INTRODUCTION

In Swiss-type hard cheese, growth of *Propionibacterium freudenreichii* is indispensable for the characteristic eye formation and flavor development in Emmentaler, whereas propionic acid fermentation must be maintained at a low level in Gruyere. Both types of cheese can be affected by a typical ripening problem: the so-called secondary fermentation where, instead of the desired eyes, the cheese develops cracks and slits. A conclusive microbiological and biochemical explanation and control of this problem are still lacking. The primary cause appears to be increased proteolysis during the ripening of the cheese as well as an intense pro-

pionic acid fermentation (Steffen *et al,* 1979; Steiger and Flückiger, 1979). Moreover, propionibacteria can provoke other defects such as the brown spotting observed when the cheese milk is inoculated with a low concentration of microorganisms (Baer *et al,* 1993).

While lactate utilization by propionibacteria is well understood, little is known about possible factors which induce an overactivity of the propionibacteria, leading to secondary fermentation with deliberately added cultures during Emmentaler production or, in the case of Gruyere production, wild strains present in the cheese milk. In particular, the interactions that occur between propionic acid bacteria and other species present in cheese are poorly understood. In fact, while organisms present in starter or in secondary cultures are well characterized, little is known about the identity or the growth of the microorganisms present in cheese " wild" microflora. Some recent studies, however, noted the necessity of characterizing the species belonging to this secondary microflora (Jordan and Cogan, 1993) and discussed the possible interactions between non-starter microorganisms and deliberately added bacteria during cheese making (Martley and Crow, 1993).

One can easily imagine that in the cheese ecosystem some species of the microflora can positively affect the growth of propionibacteria while others inhibit more or less intensively this growth. Positive interactions are treated in another article in this issue (A Baer, Influence of casein proteolysis by starter bacteria, rennet and plasmin on the growth of propionibacteria in Swiss-type cheese). Some negative interferences are mentioned in the literature, noting the lack of a metabolic explanation. We present here evidence that certain strains of *L casei* or *L rhamnosus* isolated from different types of cheese can substantially and reproducibly oppose the growth of *P freudenreichii.* In an attempt to elucidate the observed antagonistic effects of these lactobacilli on *P freudenreichii,* their metabolism in cheese was studied.

MATERIALS AND METHODS

Strains and culture conditions

P freudenreichii JCL 1868 was used for the growth and inhibition experiments. The species names, *L rhamnosus* and *L casei,* are used in accordance with the recommendations of Dellaglio *et al* (1991). The isolation of strains from different types of cheese – Parmesan, Gruyere, Raclette and Emmentaler – in accordance with Isolini *et al* (1990) and their identification by comparison with reference strains with DNA/DNA hybridization and SDS-PAGE of protein extracts will be published elsewhere. *Lactobacillus rhamnosus* JCL 1211, isolated from Parmesan, was found to be almost identical to *Lactobacillus casei* subsp *rhamnosus* ATCC 7469 on the basis of their band patterns after SDS-PAGE of protein extracts. Incubations were performed at 30°C on MRS medium without acetate and citrate (MRSAC0). Analytical measurements were performed on the medium at the end of the exponential phase. Bacterial mass was measured by absorption at 700 nm: 1 absorption unit at 700 nm corresponds to 0.46 mg of bacterial dry weight (BDW)/ml. Yield measurements were normalized by subtracting the values corresponding to residual growth observed on MRSAC0 without a supplied energy source (~ 0.1 absorption unit).

For growth determinations in cheese, experimental Gruyere and Emmentaler cheeses were prepared with 120 l of raw milk inoculated with the usual thermophilic starters and supplemented or not with *L rhamnosus* JCL 1211 or *L casei* 1227. Bacterial counts on selective media were performed after emulsifying samples in citrate solutions. Metabolite concentrations were measured after emulsifying samples and precipitating protein with Carrez solutions.

Analytical methods

Citrate, acetate, formate, ethanol, D-lactate, L-lactate and lactose were determined enzymati-

cally with kits from Boehringer. Ribose was measured according to White and Kennedy's method (Chaplin, 1986). Diacetyl was analyzed in accordance with Hill *et al* (1954). Carbon dioxide produced during fermentation was measured after stabilization of samples with 50 mmol/l NaOH. Measurements were performed by infrared photometry of gas released from the sample on the addition of 2.5 N H_2SO_4 according to Bosset *et al* (1980).

RESULTS AND DISCUSSION

Lactic acid bacteria from cheese secondary microflora have been isolated at our institute with the aim of finding strains capable of regulating the growth of propionic acid bacteria. Almost all the isolated lactobacilli belong to the group of facultatively heterofermentative type (FHL). This group was specially chosen because of its abundance in the microflora of hard cheese. Present at the limit of detection at the beginning of cheesemaking, the FHL microflora can reach concentrations comprised between 10^7 and 10^8 per gram of mature cheese, which is several orders of magnitude higher than the concentrations of other species such as enterococci or salt-tolerant microorganisms usually found in these types of cheese.

Out of hundreds of isolates from FHL microflora of Emmentaler, Gruyere and Appenzeler, all the identified strains were noted to be exclusively formed by 2 species: *L rhamnosus* and *L casei*. This is in contrast to the microflora of Cheddar cheese which contains a large proportion of *L plantarum* among the group of non-starter lactic acid bacteria (Jordan and Cogan, 1993). The relatively high temperatures used during cheese manufacture could probably explain the absence of *L plantarum* in Swiss-type varieties.

Preliminary experiments showed qualitative differences between control cheeses and cheeses prepared with cultures of *L rhamnosus* or *L casei*, mainly a strong tendency to reduce opening. *L rhamnosus* JCL 1211 and *L casei* JCL 1227 strains were particularly active. A typical inhibition of *P freudenreichii* growth observed over 20 experiments carried out with model cheeses of the Emmentaler type is illustrated in figure 1. It can be seen that the beginning of growth at about 2 weeks is strongly inhibited by both lactobacilli, with JCL 1211 tending to inhibit total growth more efficiently than JCL 1227. Extreme inhibition can reach 80% compared to the control growth but, in some cases, total propionibacteria counts after 60 d in cheese inoculated with the lactobacilli are similar to the counts found in the controls. The same type of inhibition is observed in full-size Emmentaler, as illustrated by the curves of figure 2 showing a reduction in total counts and in the production of propionic acid.

All of the attempts to reproduce this type of inhibition in cultures were unsuccessful, even when performed according to the usual methods used for bacteriocin detection. Growth activation instead of inhibition was even observed when *P freudenreichii* was cultivated in milk, with lactate added, in the presence of *L rhamnosus* or *L casei*. It is therefore clear that the observed inhibition appears to be specified by conditions that develop during the growth of FHL in cheese. In order to determine these conditions, we turned our attention to the lactobacilli metabolism, and in particular to *L rhamnosus* because of its higher inhibitory effect.

One of our first questions concerned the energy source utilized by the FHL for their growth in cheese. As FHL are capable of growing in Swiss-type cheese during the first months of ripening and are able to reach counts in the range of 10^8 cfu/g, they must obviously utilize an available energy source other than lactose for growth, since lactose, glucose and galactose disappear from cheese in a matter of 24 h. It has been claimed that part of the indigenous flora can

grow at the expense of metabolites liberated by the autolysis of starter bacteria (Thomas, 1987). We observed that our FHL strains can metabolize ribose but are unable to grow on *L lactis* or *S thermophilus* cell-free extracts or broth supplemented with ribonucleate. Lactate and citrate are certainly the most important, and possibly the only, energy source still available after fermentation of the lactose by the starter bacteria. In Swiss hard cheese, lactate is normally fermented almost exclusively by propionibacteria, added as a maturation culture or present in the indigenous microflora, or by microorganisms present in the smear (Keller and Puhan, 1985). Neither *L rhamnosus* nor *L casei* can use lactate as the sole energy source.

Citrate, present in milk at concentrations of 10 mmol/l, can be used by different types of lactic acid bacteria. Because the production of aromatic substances by lactococci at the expense of citrate often needs the presence of a fermentable sugar, some authors have reported that citrate metabolism does not support growth by lactic acid bacteria (Jonsson and Pettersson, 1977; Kempler and McKay, 1981) but can stimulate growth in the presence of carbohydrate-containing media (Hugenholtz, 1986). In fact, 4 different pathways are described for citrate utilization by lactococci and 1 of them, at least, can support growth with citrate as the sole energy source in some strains (Hugenholtz, 1993). In *Lactobacillus plantarum* and *Lactobacillus pentosus,* 2 different mechanisms for citrate utilization have been reported (Lindgren *et al,* 1990; Cselovsky *et al,* 1992; Kennes *et al,* 1993).

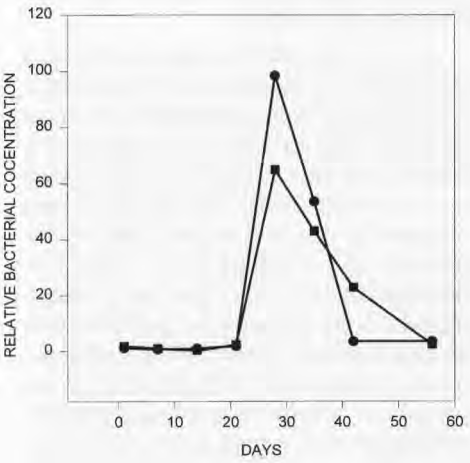

Fig 1. Growth of *P freudenreichii* in the presence of *L rhamnosus* JCL 1211(●) or *L casei* JCL 1227(■) in model Emmentaler. Bacterial concentration is expressed as the ratio of the cell counts in control cheeses to the cell counts in the presence of the lactobacilli.
Croissance de P freudenreichii *en présence de* L rhamnosus *JCL 1211 (●) ou* L casei *JCL 1227 (■) dans des fromages d'emmental expérimentaux. La concentration bactérienne est exprimée par le rapport des comptages dans les fromages témoins aux comptages dans les fromages préparés avec des lactobacilles.*

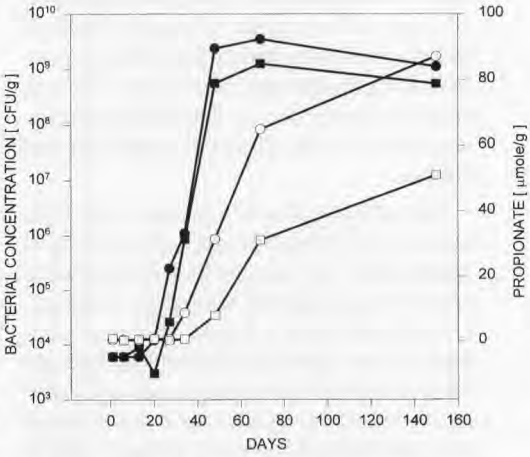

Fig 2. Growth of *P freudenreichii* in the presence of *L rhamnosus* JCL 1211 (■, ❑) and in control cheeses (●, O) in full-size Emmentaler. Closed symbols: cell counts; open symbols: propionate.
Croissance de P freudenreichii *en présence de* L rhamnosus *JCL 1211(■, ❑) et dans l'emmental témoin (●, O). Symboles pleins : comptages ; symboles vides : propionate.*

Most of the studies concerning citrate metabolism by strains belonging to the *L casei* group were performed before the revision of its taxonomy, with the ATCC 393 strain (Branen and Keenan, 1970, 1971a, b) no longer classified as *L casei* (Collins *et al,* 1989; Dellaglio *et al,* 1991) after revision or with strains insufficiently characterized (De Vries *et al,* 1970; Dirar and Collins, 1972). The work of De Vries and co-workers (1970) on the *L casei* L3 strain showed that citrate as the sole energy source supported growth of this strain with production of acetate and formate. Another study with strain NCDO 151, now well characterized as *L paracasei* subsp *paracasei* (Collins *et al,* 1989), showed only trace amounts of citrate utilized by this strain (Hickey *et al,* 1983). Some work has been done on citrate metabolism by *L rhamnosus,* but it is mainly oriented in the field of acetoin and diacetyl production (Benito de Cardenas and Oliver, 1990; Benito de Cardenas *et al,* 1991) with no mention of citrate supporting growth as the sole energy source. Moreover, no work has been reported for these species on growth in cheese or other milk products at the expense of citrate.

Table I summarizes the growth of different standard strains and isolates of *L rhamnosus, L casei* from hard-type cheese and *L plantarum* from vegetable fermentation on MRSAC0 supplemented with 30 mmol/l of sodium citrate and 10 mmol/l of $MgCl_2$. There is great variation in the ability to use citrate for growth among the different species of the FHL tested. Strain 1211 uses citrate much more efficiently as type strain *L rhamnosus* ATCC 7469, whereas the former *L casei* type strain ATCC 393 is almost unable to grow with citrate as the sole energy source. This is also the case for all the *L plantarum* tested even in the presence of lactate. Because the growth level at the end of the exponential phase is expressed as a percentage of the growth observed in the same medium supplemented with lactose (10 mmol/l) or glucose (20 mmol/l) for *L plantarum,* the differences must be considered as specific for citrate utilization and not due to some general disability in energy utilization.

Figures 3 and 4 illustrate the production of different metabolites during growth of JCL 1211 with a variable initial concentration of citrate. The main products of metabolism are acetate, formate and CO_2 produced in concentrations proportional to the initial concentration of citrate in the medium. Only minute constant quantities of ethanol and L-lactate are produced, which are presumably to be attributed to the residual carbohydrate metabolism present in the yeast extract added to the MRSAC0 medium. Small but appreciable amounts of diacetyl are produced above 10 mmol/l of added citrate. The citrate is completely consumed up to an initial concentration of 10 mmol/l and then consumption is rapidly inhibited with increasing concentrations of citrate and, concomitantly, metabolite production is also inhibited. The inhibition of metabolism is

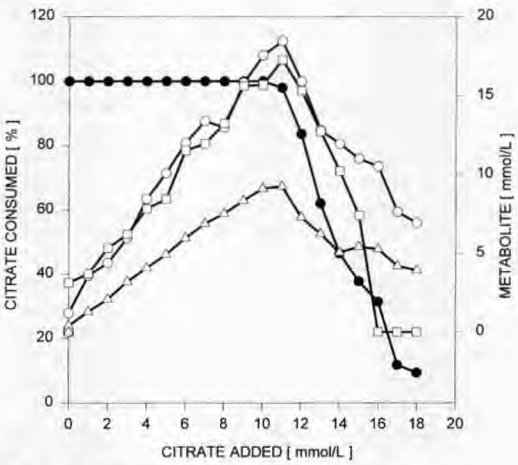

Fig 3. Citrate metabolism of *L rhamnosus* JCL 1211 in culture: citrate (●); acetate (O); carbonate (❑); formate (Δ).
Métabolisme du citrate en culture chez L rhamnosus *JCL 1211 : citrate (●) ; acétate (O) ; carbonate (❑) ; formiate (Δ).*

paralleled by growth inhibition as shown in figure 5. Growth on MRSAC0 is strongly inhibited over 10 mmol/l citrate. No inhibition is observed when the growth medium is supplemented above 10 mmol/l of $CaCl_2$ or $MgCl_2$, showing that this effect is due to the chelating power of citrate as described for the lactobacillus isolate ATCC 393 (Branen and Keenan, 1969).

Because ribose could be a potential source of energy, we also analyzed ribose utilization. The ribose metabolism illustrated in figure 6 looks more complex and varies with increasing concentration. In addition to acetate, the main product, formate is also produced together with significant quantities of L-lactate up to an initial carbohydrate concentration of 10 mmol/l. Above this concentration, formate and lactate are partially replaced by ethanol. In contrast to this type of heterofermentative growth, lactose is utilized homofermentatively, even at a low con-

Table I. Growth of facultatively heterofermentative lactobacilli with citrate as sole energy source. *Croissance de lactobacilles hétérofermentaires facultatifs avec du citrate comme seule source d'énergie.*

L rhamnosus		L casei		L plantarum L pentosus	
Strains	*Relative growth*[a]	*Strains*	*Relative growth*[a]	*Strains*	*Relative growth*[a]
JCL 1252	11	ATCC 25599	4	ATCC 11974	1
JCL 1215	19	JCL 1265	7	JCL 1280	2
JCL 1244	19	NCIMB 9709	9	JCL 1269	2
JCL 1259	21	ATCC 393 [b]	14	JCL 1284	2
JCL 1240	23	DSM 20020	22	JCL 1282	2
ATCC 7469	26	ATCC 11582	23	JCL 1268	2
JCL 1260	26	JCL 1246	28	JCL 1279	2
JCL 1258	27	JCL 1250	29	JCL 1285	2
JCL 1256	33	JCL 1249	33	JCL 1275	2
ATCC 11981	37	ATCC 27216	33	JCL 1272	3
JCL 1220	38	JCL 1247	41	JCL 1267	3
JCL 1247	41	JCL 1248	44	ATCC 8041[c]	3
JCL 1219	44	JCL 1255	44	ATCC 10012	3
JCL 1214	44	ATCC 334	47	JCL 1281	3
JCL 1218	45	JCL 1243	47	NCDO 1193	3
JCL 1217	46	JCL 1257	48	JCL 1272	3
JCL 1212	51	JCL 1253	49	ATCC 14917	4
JCL 1262	52	JCL 1254	50	ATCC 8014	4
JCL 1239	54	CNCF 242	51	JCL 1278	8
JCL 1213	57	NCFB 173	51	JCL 1263	12
JCL 1211	57	DSM 20312	55		
JCL 1216	60	JCL 1227	55		
CNRZ 185	61	CNFB 1856	62		
		ATCC 25598	72		

[a] Growth on citrate as percent of growth on lactose *(L rhamnosus, L casei)* or glucose *(L plantarum)* at the stationary phase; [b] old type strain for *L casei*. Undetermined lactobacillus; [c] *Lactobacillus pentosus*.

[a] *Croissance sur citrate exprimée en pourcentage de croissance sur lactose* (L rhamnosus, L casei) *ou glucose* (L plantarum) *en phase stationnaire.* [b] *Anciennement* L casei. *Lactobacillus non déterminé.* [c] Lactobacillus pentosus.

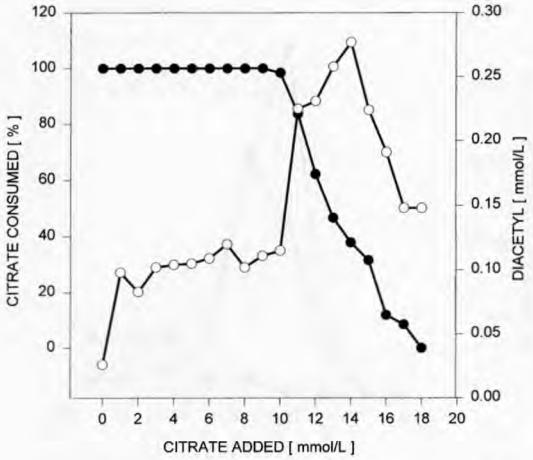

Fig 4. Production of diacetyl by *L rhamnosus* JCL 1211 cultivated with citrate as the sole energy source. Citrate consumed (●); diacetyl (O).
Production de diacétyle par L rhamnosus *JCL 1211 cultivé avec du citrate comme seule source d'énergie. Citrate consommé (●) ; diacétyle (O).*

centration (0.5 mmol/l) with quantitative production of L-lactate. With both carbohydrates, no diacetyl production was detected.

Citrate utilization of JCL 1211 in full-size Emmentaler is shown in figure 7. It can be seen that production of acetate and formate corresponds to the disappearance of citrate. This metabolism is reflected in the growth curves in figure 8. *L rhamnosus* usually grows better than *L casei,* and both considerably more than the wild FHL of the microflora. For both strains, maximal growth is reached in about 30 d, and then rapid lysis takes place. Lysis is not responsible for the inhibitory effect; as we have seen in the growth curves of figure 1, inhibition decreases precisely after 30 d.

In order to avoid complications introduced by the metabolism of *P freudenreichii,* we analyzed citrate consumption as well as acetate, formate, carbonate and cell mass

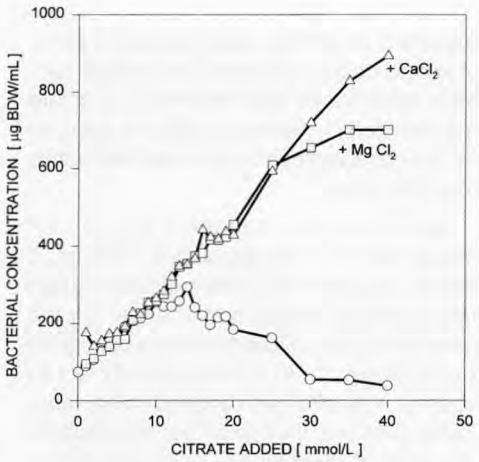

Fig 5. Growth of *L rhamnosus* JCL 1211 cultivated with citrate as the sole energy source (O); with 10 mmol/l $CaCl_2$ (Δ) or with 10 mmol/l $MgCl_2$ (❑).
Croissance de L rhamnosus *JCL 1211 cultivé avec du citrate comme seule source d'énergie (O) ; avec 10 mmol/l $CaCl_2$ (Δ) ou 10 mmol/l $MgCl_2$ (❑).*

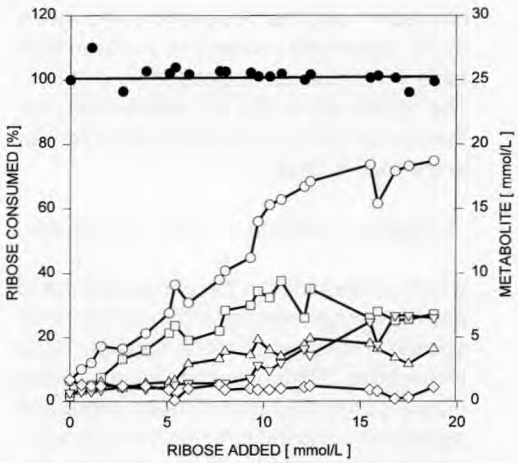

Fig 6. Ribose metabolism of *L rhamnosus* JCL 1211 in MRSAC0: ribose (●); acetate (O); formate (❑); L-lactate (∇); ethanol (Δ); carbonate (◊).
Métabolisme du ribose chez L rhamnosus *JCL 1211 dans MRSAC0 : ribose (●) ; acétate (O) ; formiate (❑) ; L-lactate (∇) ; éthanol (Δ) ; carbonate (◊).*

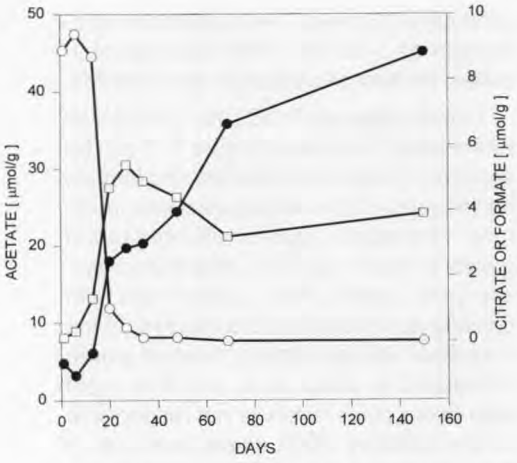

Fig 7. Citrate metabolism of *L rhamnosus* JCL 1211 in full-size Emmentaler: citrate (O); acetate (●); formate (❑).
Métabolisme du citrate chez L rhamnosus *JCL 1211 dans le fromage d'emmental : citrate (O) ; acétate (●) ; formiate (❑).*

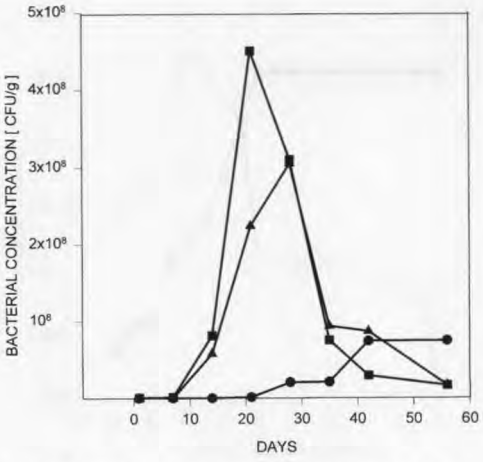

Fig 8. FHL growth in model Emmentaler: FHL microflora in control cheeses (●); *L rhamnosus* JCL 1211(■); *L casei* JCL 1227 (▲).
Croissance des lactobacilles hétérofermentaires facultatifs dans des fromages d'emmental expérimentaux : lactobacilles hétérofermentaires facultatifs dans les contrôles (●) ; L rhamnosus *JCL 1211(■) ;* L casei *JCL 1227 (▲).*

production in 3 model Gruyere cheeses between 1 and 30 d of growth. The mean of the observed values are compared in table II with the values obtained in cultures. The results show that the stoichiometry of the overall metabolism, in culture as well as in cheese, is close to:

$$1 \text{ citrate} = 2 \text{ acetate} + 1\ CO_2 + 1 \text{ formate}$$

which corresponds to the phosphoroclastic pathway proposed for different bacterial species (Hickey *et al,* 1983; Kandler, 1983; Hugenholtz, 1986). The correlation between citrate consumed and formate produced agrees very satisfactorily with such a pathway for cheese and cultures. A larger production of acetate is observed in cheese than in culture, but this could be explained by taking into account that in cheese with smear, a substantial amount of lactate is transformed to acetate (Keller and Puhan, 1985). The amount of CO_2 produced in both media is significantly larger than the expected value; this could be due to some unknown decarboxylation. The yield in bacterial mass shows that the amount of citrate metabolized in cheese can largely account for the observed cell counts reached before the lysis phase.

As citrate does not affect growth of *P freudenreichii* in lactate growth medium, a direct competition for citrate cannot explain the observed inhibition in cheese. On the other hand, the different products of citrate metabolism could synergistically act to oppose normal growth of propionibacteria. Table II shows that small but appreciable amounts of diacetyl are produced during growth of *L rhamnosus* in cheese and in culture.

In an attempt to reproduce the observed inhibition in cheese, we prepared model Emmentaler cheeses with different concentrations of diacetyl. Figure 9 summarizes the results of the growth observed.

Table II. Stoichiometry of principal metabolites produced during citrate metabolism by *L rhamnosus* JCL 1211.
Stœchiométrie des principaux métabolites produits au cours du métabolisme du citrate par L rhamnosus *JCL 1211.*

	Citrate consumed	*Acetate*	*Formate*	*Carbonate*	*Diacetyl*	*BDW*[a]
Cheese	1	2.4	0.97	1.98	0.02	17
Culture	1	1.6	0.75	1.45	0.03	23

[a] BDW: bacterial dry weight; µg/µmol.

Diacetyl inhibits the growth of *P freudenreichii* even at concentrations in the range of 0.5 mmol/l of milk. This concentration is close to the concentration observed in cheese, provided that all the diacetyl produced during FHL growth is dissolved in the water phase of cheese. Cheeses prepared with concentrations of 1 mmol/l diacetyl or higher had a uniformly brown-spotted curd, suggesting that the lower initial cell counts obtained with diacetyl cheeses is a lethal effect of this substance. We previously mentioned that brown spots in Emmentaler are found when the initial concentration of propionibacteria is low (Baer *et al,* 1993). Antibacterial properties of diacetyl have been described for other species at pH conditions near the pH values observed in cheese (Jay, 1982).

As the inhibition was not observed in batch cultures utilizing usual growth media (MRS or lactate broth), the acetate and formate produced during citrate metabolism could be inhibitory only in an environment similar to the water phase of cheese. The experiment illustrated by figure 10 is aimed to observe the effect of acetate and formate in the presence of different concentrations of copper at the concentrations usually found in Swiss-type cheese and in an ionic environment presumably comparable to the water phase of the cheese: 200 mmol/l Na^+, 60 mmol/l Ca^{++}, 10 mmol/l Mg^{++}, 2 mmol/l Zn^{++} and 300 mmol/l DL-lactate. A strong inhibition takes place in the acetate and formate media for copper concentrations which do not affect markedly the growth in the presence of the citrate alone, or without citrate.

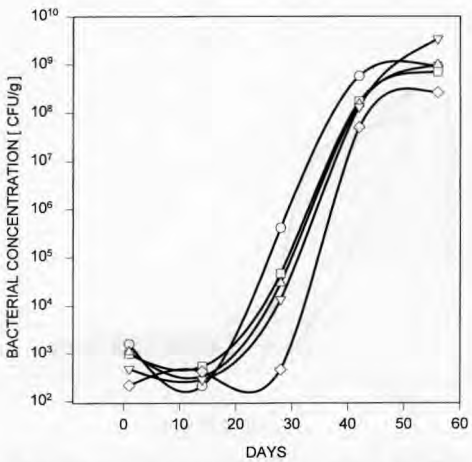

Fig 9. Growth of *P freudenreichii* in model Emmentaler with different concentrations of diacetyl added: 0.0 mmol/l (O), 0.5 mmol/l (❑), 0.75 mmol/l (Δ), 1.0 mmol/l (∇), 1.5 mmol/l (◊). *Croissance de* P freudenreichii *dans des fromages d'emmental expérimentaux préparés avec différentes concentrations de diacétyle : 0,0 mmol/l (O), 0,5 mmol/l (❑), 0,75 mmol/l (Δ), 1,0 mmol/l (∇), 1,5 mmol/l (◊).*

Since the inhibition we have described is only observed in cheese, one possibility for elucidating this phenomenon would be to compare the water phases of 1-month-old control cheeses with cheeses prepared with added FHL. Water phases can be considered as the proper growth environment of the cheese microflora and one can expect that in water phases extracted from cheeses manufactured with FHL, *P freudenreichii* will grow considerably more slowly than in water phases from control cheeses. The results illustrated by figure 11 confirm our expectations. Water phases extracted according to Morris *et al* (1988) from 1-month-old Emmentaler manufactured with *L casei* JCL 1227 considerably inhibits growth of *P freudenreichii* (A), whereas no growth is observed in juice extracted from cheese manufactured with *L rhamnosus* JCL 1211 (B).

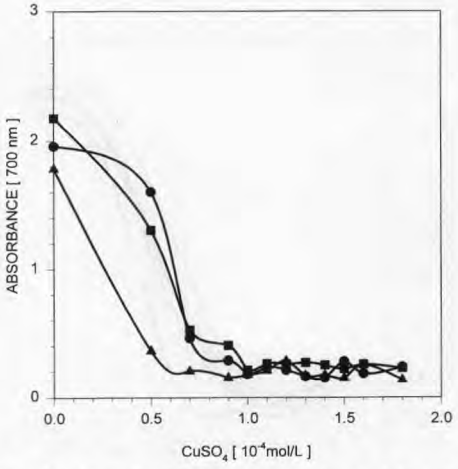

Fig 10. Growth of *P freudenreichii* with different $CuSO_4$ concentrations: control lactate broth (●); + 30 mmol/l citrate (■); + 60 mmol/l acetate and 30 mmol/l formate (▲). The salt composition of lactate broth is given in the text.
Croissance de P freudenreichii *avec différentes concentrations de $CuSO_4$: contrôle sur bouillon lactate (●), + citrate 30 mmol/l (■), + 60 mmol/l acétate et 30 mmol/l formiate (▲). La composition du bouillon lactate est donnée dans le texte.*

Table III summarizes the analysis performed on the different types of extracts. In contrast to formate and acetate, which are almost totally recovered in the cheese juice,

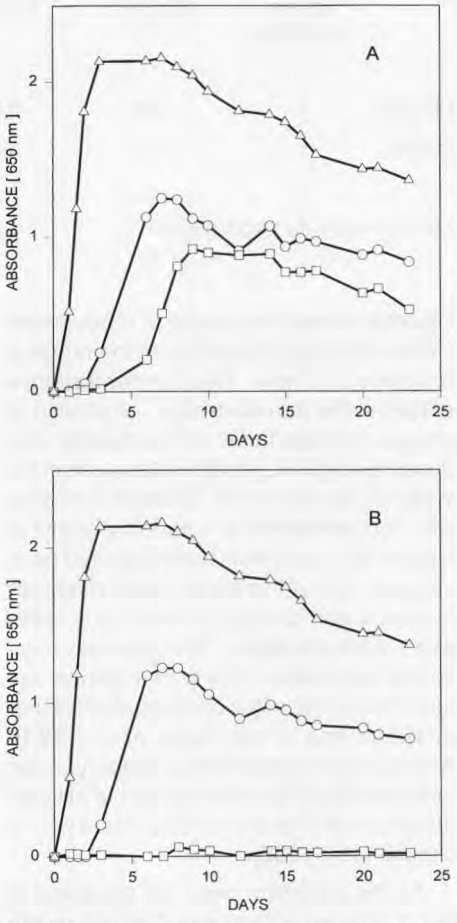

Fig 11. Growth of *P freudenreichii* in juice extracted from cheeses manufactured with *L casei* 1227 (A) or with *L rhamnosus* 1211 (B): growth in lactate broth (Δ); growth in juice from control cheese (O); growth in juice from cheese manufactured with added lactobacilli (❑).
Croissance de P freudenreichii *dans des phases aqueuses de fromages préparés avec* L casei *1227 (A) ou* L rhamnosus *1211 (B) : croissance dans le bouillon au lactate (Δ) ; croissance dans l'extrait de fromages témoins (O) ; croissance dans les extraits de fromages préparés avec les lactobacilles (❑).*

Table III. Composition of juices obtained from 1-month-old Emmentaler cheese.
Composition des jus obtenus à partir de fromage emmental d'un mois.

Compounds	*Control* [a]	L casei [b]	L rhamnosus [c]
Organic compounds (g/l)			
Dry weight	135.4	143.9	137.6
Proteins	58.1	71.4	48.8
Free amino acids	15.4	16.7	17.6
Organic acids (mmol/l)			
DL lactate	390 (388)[d]	382	320
Citrate	12.4 (25)	0.1	0.3
Acetate	0.1	49 (50)	51 (50)
Formate	0.1	20.4 (25)	21.7 (25)
Minerals (mmol/l)			
P	155 (525)	175	133
Na	340 (350)	270	330
Ca	112 (750)	136	128
K	84 (80)	93	85
Zn	0.17 (0.21)	0.33	0.09
Mg	21 (44)	24	23
Cl	141	139	166
Cu	0.058 (0.24)	0.075	0.091
Mn	0.006 (0.016)	0.003	0.003
Fe	0.393 (0.183)	0.074	0.166

[a] Juice obtained from cheeses without added *L casei* or *L rhamnosus*; [b] juice obtained from cheeses with added *L casei* JCL 1227; [c] juice obtained from cheeses with added *L rhamnosus* JCL 1211; [d] concentrations between parentheses are calculated assuming that the compounds are totally dissolved in the aqueous phase.
[a] *Jus obtenu de fromages sans addition de* L casei *et* L rhamnosus. [b] *Jus obtenu de fromages avec addition de* L casei *JCL 1227.* [c] *Jus obtenu de fromages avec addition de* L rhamnosus *JCL 1211.* [d] *Les concentrations indiquées entre parenthèses sont calculées en estimant que les composés sont totalement dissous dans la phase aqueuse.*

citrate and the majority of the analyzed cations are only partially dissolved in the aqueous phase, as previously observed by Morris *et al* (1988). Copper concentration in extracts from cheeses manufactured with FHL is higher than in extracts from control cheeses, suggesting that, as previously observed in the experiment of figure 10, the relative concentrations of citrate and copper can play an important role in the observed inhibition.

The free amino acid composition of the juices was further investigated and the results are given in figure 12. A significant difference is observed at the level of aspartate concentration: the amino acid is 3 times more concentrated in juices from cheeses prepared with FHL, pointing to the inhibition of aspartate metabolism in the propionibacteria added to the cheeses with FHL.

CONCLUSION

Our study unequivocally demonstrates that strains of *L rhamnosus* and *L casei* inter-

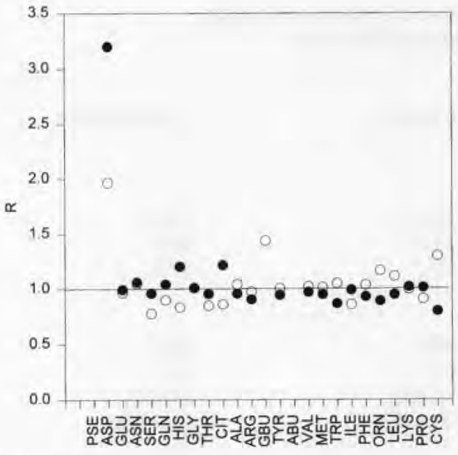

Fig 12. Amino acid composition of the aqueous phases: R is the ratio of the free amino acid concentration (mole fraction) in juices extracted from cheeses manufactured with *L casei* JCL 1227(O) or with *L rhamnosus* 1211 (●) to the concentration in juice from control cheeses.
Composition en acides aminés de la phase aqueuse : R représente le rapport de la concentration en acides aminés (fraction molaire) dans les extraits préparés avec L casei *JCL 1227(O) ou* L rhamnosus *1211 (●) à la concentration correspondante dans les extraits préparés à partir de fromages témoin.*

fere negatively with the growth of *P freudenreichii* in hard Swiss cheese. The observed inhibition of *P freudenreichii* growth in cheese cannot be reproduced in cultures with the usual media, strongly suggesting that a bacteriocin production is not responsible for the effect. All the citrate initially present in cheese is consumed during the growth of added *L rhamnosus,* with production of acetate, formate, carbonate and small but significant amounts of diacetyl. Since these metabolic products can interfere with *P freudenreichii* growth in cheese, the final observed inhibition could be the result of the interaction of several factors.

Preliminary observations show that growth of *P freudenreichii* is inhibited in juice extracted from cheeses prepared with *L casei* or *L rhamnosus* and that differences are detected between the ionic composition of juice from control cheeses and juice from cheeses prepared with FHL. Analyses in progress in our laboratory on the different types of juices, and trials currently being performed with media of similar composition are aimed at reaching a conclusive explanation of the observed inhibiting effect.

ACKNOWLEDGMENTS

We gratefully thank HP Backmann, R Badertsher, D Fuchs, B Nick, K Shafroth and A Weber for the preparation of the experimental cheeses and the analyses performed on them.

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