

Interactions between lactic and propionic acid bacteria

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Summary — In some cheeses, propionic acid bacteria (PAB) ferment lactate to propionate, acetate and CO₂, which are important in determining the flavour and texture of the cheese. Interactions between 14 strains of lactic acid bacteria (LAB) (*Lactobacillus helveticus*, *Lb acidophilus*, *Lb lactis*, *Streptococcus thermophilus* and *Lactococcus lactis*) and 4 strains of PAB (*Propionibacterium freudenreichii* and *P acidipropionici*) were studied in whey. Stimulation or inhibition was judged by the effect on growth rate and final cell mass (OD₆₀₀). No inhibition of growth was found. The growth of all 4 strains of PAB was stimulated by *Lb helveticus* and *Str thermophilus*. The degree of stimulation of the PAB by the other LAB varied. In control and *Lb helveticus* RR whey, L lactate was used preferentially over D lactate by *P freudenreichii* KM. *Lb helveticus* RR increased the levels of amino acids and peptides in the whey. All of the amino acids, except threonine and cysteine, and some of the peptides were used during subsequent growth of *P freudenreichii* KM. The addition of aspartate stimulated growth of *P freudenreichii* KM in control whey and reduced the amount of lactate converted to propionate, but not acetate. The stimulant(s) was stable to heating to 121°C for 15 min and eluted in several peaks after chromatography on Sephadex G-25. Ultrafiltration resulted in a total loss of activity.

interaction / propionic acid bacteria / lactic acid bacteria / whey / growth

Résumé — **Interactions entre bactéries lactiques et bactéries propioniques.** Dans certains types de fromage, les bactéries propioniques (PAB) fermentent le lactate en propionate, acétate et CO₂, produits importants pour la saveur et la texture de ces fromages. Les interactions entre 14 souches de bactéries lactiques (LAB) (*Lactobacillus helveticus*, *Lb acidophilus*, *Lb lactis*, *Streptococcus thermophilus* et *Lactococcus lactis*) et 4 souches de PAB (*Propionibacterium freudenreichii* et *P acidipropionici*) ont été étudiées en milieu lactosérum. La stimulation ou l'inhibition étaient jugées en fonction du taux de croissance et de la masse cellulaire (DO₆₀₀) en fin de fermentation. Aucune inhibition de la croissance n'a été observée. La croissance des 4 souches de PAB était stimulée par *Lb helveticus* et *Str thermophilus*. La stimulation des PAB par les autres LAB était variable. Dans le lactosérum produit par *Lb helveticus* RR et dans le contrôle, l'isomère L de l'acide lactique était utilisé par la souche de *P freudenreichii* KM de façon préférentielle par rapport à l'isomère D. L'ajout d'aspartate stimulait la croissance de *P freudenreichii* KM dans le lactosérum contrôle, et réduisait la proportion de lactate convertie

en propionate sans changer celle convertie en acétate. Le(s) stimulant(s) résistait au chauffage à 121°C pendant 15 min et formait plusieurs pics après chromatographie sur Sephadex G-25. L'ultrafiltration résultait en une perte d'activité.

interaction / bactérie propionique / bactérie lactique / lactosérum / croissance

INTRODUCTION

Cheese is a complex biological system which undergoes numerous biochemical changes during manufacture and ripening. In Swiss-type cheese manufacture, thermophilic lactic acid bacteria (LAB), including *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp *lactis* and *Streptococcus thermophilus*, are used as starters and degrade lactose to D and L lactate. Propionibacteria (PAB) then metabolize lactate to propionate, acetate and CO₂ during the extended ripening (Hettinga and Reinbold, 1972; Langsrud and Reinbold, 1973). The propionic acid fermentation is critical to the final quality of the cheese as CO₂ is responsible for eye formation, while propionic acid contributes to the development of the typical nutty flavour (Gautier *et al*, 1993). The quality of the cheese, therefore, depends on the extent of the lactic acid fermentation and the propionic acid fermentation. Different isomers of lactate are produced by the starter LAB. *Lactobacillus delbrueckii* subsp *lactis* produces D lactate, *Streptococcus thermophilus* produces L lactate and *Lactobacillus helveticus* produces a mixture of D and L lactate. Some strains of PAB have a preference for the L isomer (Crow, 1986a); however, the interaction between the 2 fermentations is not limited to lactate production and utilization. Interactions between LAB and PAB have been reported in co-cultures (Lee *et al*, 1976; Parker and Moon, 1982; Perez Chaia *et al*, 1987) and in sequential cultures (Hunter and Frazier, 1961; Czarnocka-Rocznikowa *et al*, 1972). The compounds involved in these interactions have not been identified. The object

of this study was to characterize the interactions between PAB and LAB more fully.

MATERIALS AND METHODS

Organisms and medium

Three strains of *Lactobacillus helveticus* (RR, 303, 3321), 1 strain of *Lactobacillus delbrueckii* subsp *lactis* (LL51), 1 strain of *Lactobacillus acidophilus* (LbA), 5 strains of *Streptococcus thermophilus* (1781, 1821, 1842, STB01 and STB02), 4 strains of *Lactococcus lactis* (C10, ML8, E8 and AM2), 1 strain of *Propionibacterium acidipropionici* (L5) and 3 strains of *Propionibacterium freudenreichii* (T, KM and H) were used. All strains were from our culture collection except STB01 and STB02 (Chr Hansen Laboratories, Cork, Ireland) and LL51 (Institut Technique du Gruyère, Rennes, France). PAB were routinely transferred weekly in sodium lactate broth (SLB) and stored at 4°C. LAB were stored at -80°C and subcultured at least twice in 10% (w/v) sterile reconstituted skim milk (RSM) before use. The composition of SLB was 1% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) KH₂PO₄ and 1% (w/v) D/L sodium lactate, pH 6.5.

Whey production

Interactions were studied by growing PAB in filter-sterilized whey obtained after growth of the LAB in RSM. Assessment of the LAB/PAB interaction was done by comparing growth rates and final OD at 600 nm in the starter whey to those in a control whey. A strain of LAB was incubated at 42°C (thermophilic strains) or 30°C (mesophilic strains) for 24 h in RSM. The coagulated milk was centrifuged (8 000 g, 10 min, 4°C); the supernatant was adjusted to pH 6.0 with NaOH (2 N)

and incubated for 30 min at 45°C to allow any precipitation to occur. This overcame subsequent precipitation of colloidal Ca salts during growth of the PAB. After a second centrifugation (8 000 g, 30 min, 4°C), the supernatant was filtered (Whatman n°1) and filter sterilized (0.45 µm). Control whey was produced by acidifying RSM with 1% (w/v) of a 50% mixture of D and L lactic acid. The coagulated milk was then processed as just described. Sterile wheys were stored at 4°C until required.

Growth conditions

The PAB were grown in SLB for 3 d at 30°C, centrifuged, washed with sterile 1/4 strength Ringer's solution and resuspended in the original volume of Ringer's solution. Whey was inoculated (1%, v/v) and dispensed in 10 ml tubes, which were incubated under static conditions at 30°C. Growth was followed by measuring OD at 600 nm. When the OD was greater than 0.5, the culture was diluted before reading to maintain linearity between OD and cell mass. Two ml of culture was centrifuged and the supernatant frozen for further analysis. Purity of cultures was checked by microscopic examination throughout growth and by plating aerobically and anaerobically, at 30°C, at the end of fermentation on sodium lactate agar (SLA).

Analytical methods

Lactose, glucose, galactose, lactate, propionate, acetate and succinate were quantified by high-performance liquid chromatography (HPLC) (column Aminex HPX 87 X at 60°C and eluted with 0.04 N H₂SO₄). D and L lactate were determined with enzymatic kits (Boehringer Mannheim). Amino acids were measured on a 120 x 4 mm cation exchange column (Na⁺ form) using a Beckman 6300 amino acids Analyser (Beckman Instruments Ltd, High Wycombe, UK). The results were processed with a PC Minichrom. Peptide profiles were determined by reverse-phase HPLC (Shimadzu HPLC system, C8 Nucleosil wide pore column; solvent gradient: A: 0.1% [w/v] trifluoroacetic acid, B: 0.1% [w/v] trifluoroacetic acid in acetonitrile).

Heat treatment

Starter whey, adjusted to pH 6.0, was pasteurized (63.5°C, 30 min) or autoclaved at 121°C for 15 min. After treatment, the whey was centrifuged (8 000 g, 10 min, 4°C) and its pH readjusted to 6.0 before filter sterilization (0.45 µm).

Ultrafiltration

Starter whey was ultrafiltered (Minitan system, Millipore) using a 10 000 cutoff polysulfone filter (10 000 NMWL plates) until a 10-fold concentration of retentate was achieved.

Perchloric acid extraction

Perchloric acid was added to the starter whey to a final concentration of 2 N. The mixture was centrifuged (5 000 g, 10 min, 4°C); the supernatant was neutralized with KHCO₃, re-centrifuged and the pH adjusted to 6.0 before filter sterilization. The original starter whey was diluted 2.5-fold by this procedure.

Gel filtration

A 100 ml Sephadex G 25 column was equilibrated for 24 h at room temperature with distilled water. Five ml of starter whey was applied and the column eluted with distilled water at a flow rate of 20 ml/h. Three ml fractions were collected and their A280 determined using the first fraction as a blank. Fractions forming a peak on the chromatogram were combined, freeze-dried, dissolved in 5 ml of distilled water, filter sterilized and assayed for growth stimulation of the PAB cultures in control whey after 72 h of incubation.

RESULTS

The influence of the 14 strains of LAB on the growth of *P freudenreichii* KM is shown in figure 1. All strains of LAB, except *Lc lactis* E8, stimulated the growth rate and final

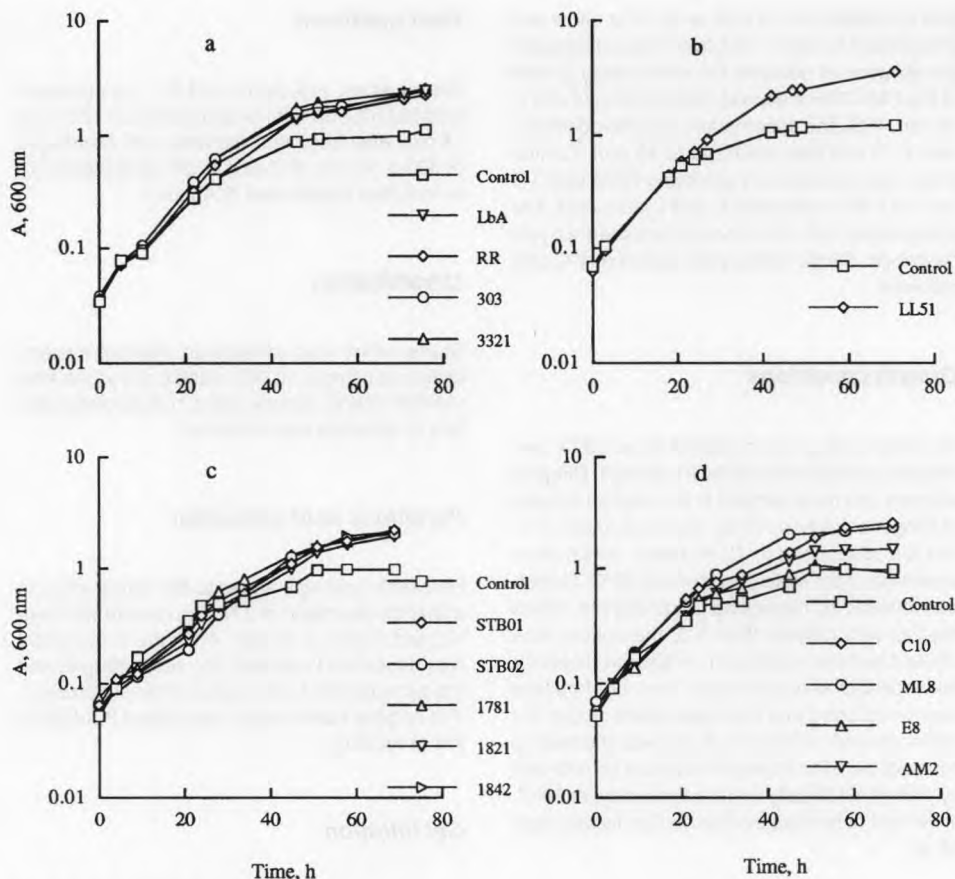


Fig 1. Influence of different strains of lactic acid bacteria on the growth of *Propionibacterium freudenreichii* KM in control whey. (a) Strains of *Lactobacillus helveticus* and *Lb acidophilus*; (b) *Lb lactis*; (c) strains of *Streptococcus thermophilus*; (d) strains of *Lactococcus lactis*.

Influence de différentes bactéries lactiques sur la croissance de Propionibacterium freudenreichii KM sur lactosérum. (a) Souches de *Lactobacillus helveticus* et *Lb acidophilus*; (b) *Lb lactis*; (c) souches de *Streptococcus thermophilus*; (d) souches de *Lactococcus lactis*.

cell mass, but to varying extents. The largest increases were observed with the *Lactobacillus* strains which increased growth rates by up to 17% and more than doubled the final cell mass. On the other hand, *Str thermophilus* strains had only a small effect on the growth rate (6% increase) but increased the cell mass significantly: a 2.2-fold increase was observed with strain STB01.

The interactions between the other strains of PAB and LAB varied (table I). Each of the 4 strains of PAB used were stimulated by *Lb helveticus* and *Str thermophilus* STB01. Some PAB/LAB pairs showed no interaction (eg PAB strain T was not affected by *Str thermophilus* 1781 and STB02 nor by the *Lactococcus lactis* strains) (table I). At the end of these preliminary experiments,

Table 1. Percentage increase in final biomass production (OD_{600}) of 4 strains of propionic acid bacteria (PAB) after growth for 72 h in lactic acid bacteria (LAB) wheys compared with control wheys.

Pourcentage d'augmentation de la production finale de biomasse (DO_{600}) de 4 souches de propionibactéries après 72 h de croissance dans le lactosérum produit par les bactéries lactiques comparé au lactosérum témoin.

LAB		Propionibacterium			
		L5	T	KM	H
<i>Lactobacillus helveticus</i>	RR	249	174	112	384
	303	173	151	128	48
	3321	247	218	144	35
<i>Lactobacillus acidophilus</i>	LbA	0	201	137	35
<i>Lactobacillus delbrueckii</i> subsp <i>lactis</i>	LL51	0	51	194	0
<i>Streptococcus thermophilus</i>	1781	77	0	116	33
	1821	0	155	98	33
	1842	0	136	117	53
	STB01	166	112	126	52
	STB02	51	0	107	34
<i>Lactococcus lactis</i>	C10	212	0	159	35
	ML8	244	0	137	60
	AM2	173	0	51	34

Lactobacillus helveticus RR and *Propionibacterium freudenreichii* KM were selected for more detailed studies, because all strains of PAB were stimulated by *Lactobacillus helveticus* RR and all strains of LAB, except *Lc lactis* E8, stimulated *P freudenreichii* KM.

Influence of milk on stimulation

Control and starter wheys were produced from 2 skim milk powders and a sample of pasteurized skim milk. The stimulation of *P freudenreichii* KM by *Lb helveticus* RR occurred regardless of the milk in which it was grown (data not shown).

Growth and fermentation in control whey

In experiments carried out in control whey, lactose was never used by *P freudenreichii* KM during the fermentation (fig 2a); at the end of fermentation, the culture reached an OD_{600} of ~0.60 and the pH decreased to 5.85. Similar results were obtained for the other 3 strains of PAB. Lactate utilization was slow during the first 24 h of growth after which L lactate was used preferentially over D lactate (fig 2a). At the end of growth, 8.3 mmol/l of L lactate and 42.1 mmol/l of D lactate remained in the whey, corresponding to an overall utilization of 82% of the L lactate and 12% of the D lactate. Propionate

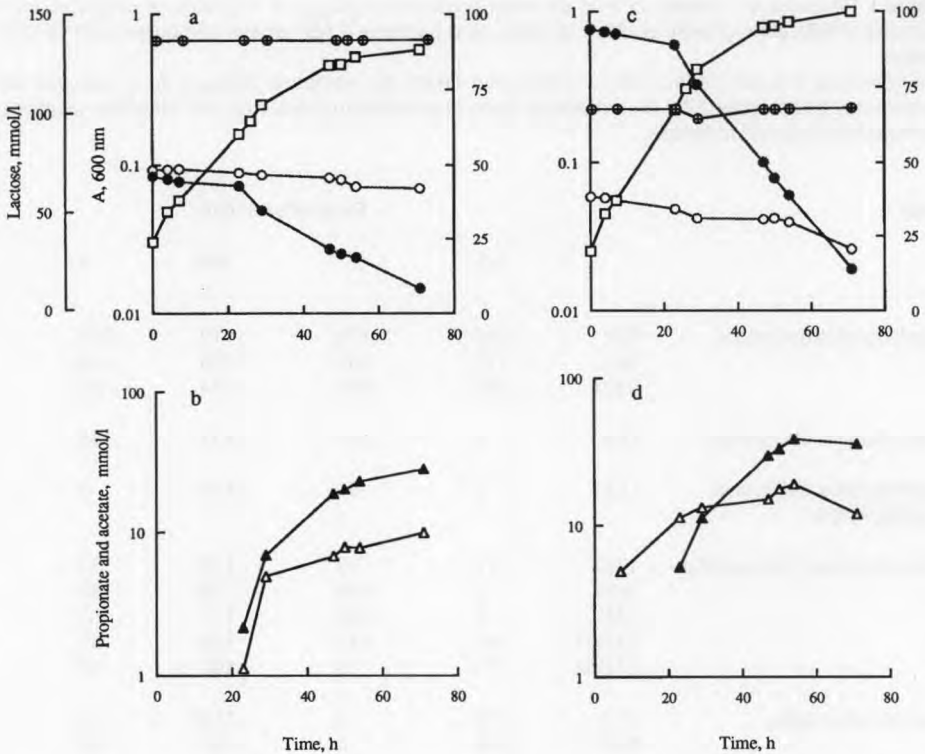


Fig 2. Growth, substrate utilization and product formation by *Propionibacterium freudenreichii* KM in (a, b) control whey and (c, d) *Lactobacillus helveticus* RR whey. (□) growth; (○) D lactate utilization; (●) L lactate utilization; (⊕) lactose utilization; (▲) propionate production; (Δ) acetate production. Croissance, utilisation des substrats et formation des produits de la fermentation par *Propionibacterium freudenreichii* KM sur (a, b) lactosérum témoin et (c, d) lactosérum produit par *Lactobacillus helveticus* RR. (□) croissance; (○) utilisation du lactate D; (●) utilisation du lactate L; (⊕) utilisation du lactose; (▲) production de propionate; (Δ) production d'acétate.

and acetate were only detected late in the fermentations, after 20 h, and the combined production of propionate and acetate was stoichiometrically related to the disappearance of lactate (fig 2b); the molar ratios of propionate:lactate and acetate:lactate were 0.70 and 0.24, respectively, and the correlation coefficients between propionate produced and lactate used and between acetate produced and lactate used were 0.99 and 0.95, respectively. Succinate was not detected.

Growth and fermentation in *Lb helveticus* RR whey

Figure 2c shows the growth of *P freudenreichii* KM in *Lb helveticus* RR whey. The culture attained a final OD of ~0.99, giving an increase in biomass of 70% compared to that in the control whey. The pH decreased from 6.0 to 5.8. As in the control whey, lactose was not utilized. Lactate utilization was slow for the first 24 h and again L lactate was used preferentially over D lactate (fig

2c). By the end of the fermentation, 85% of the L lactate and 46% of the D lactate were metabolized and 36.0 mmol/l propionate and 19.0 mmol/l acetate were produced (fig 2d). The molar ratios between propionate and lactate and acetate and lactate were 0.61 and 0.23, respectively; the correlation coefficients between propionate and lactate and acetate and lactate were 0.99 and 0.82, respectively. Succinate (13.3 mmol/l) was detected, but only after 71 h of fermentation.

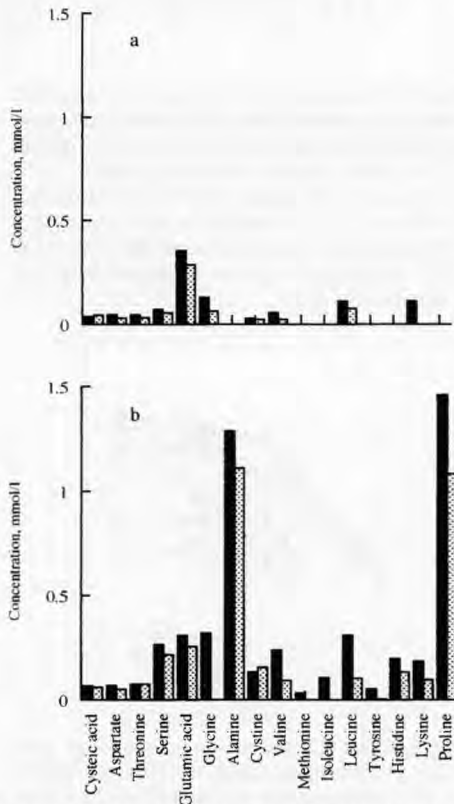


Fig 3. Amino acid composition of (a) control whey and (b) *Lactobacillus helveticus* RR whey at (■) 0 h and (▨) after 41 h of growth of *Propionibacterium freudenreichii* KM.

Composition en acides aminés du (a) lactosérum témoin et (b) lactosérum produit par *Lactobacillus helveticus* RR à (■) 0 h et (▨) après 41 h de croissance de *Propionibacterium freudenreichii* KM.

Changes in amino acid composition of whey during fermentation

The free amino acid composition of control and RR wheys are shown in figure 3. Arginine and phenylalanine were not detected in either whey. Some amino acids (eg alanine, methionine, isoleucine, tyrosine, histidine and proline), which were not found in the control whey, were present in RR whey. In addition, the concentrations of some of the other amino acids (serine, alanine, cysteine, valine and leucine) in control whey were increased by the growth of strain RR. Glutamate was not increased by the growth of strain RR. All free amino acids, except threonine and cysteine, were utilized during subsequent growth of *P freudenreichii* KM in starter whey. In order to verify if these differences in amino acid composition could explain the stimulation, growth in amino acid supplemented control whey was studied.

The addition of 0.1% and 1% vitamin free, acid-hydrolysed casein to control whey resulted in increased growth rates and cell yields of strain KM (fig 4), whereas the addition of 10% resulted in an inhibitory effect on growth rate but a stimulatory effect on cell yield. In addition to propionate and acetate (data not shown), succinate was produced during fermentation. The amount of succinate increased in direct proportion to the casein hydrolysate added and none was produced in the absence of casein hydrolysate. Several amino acids – aspartate (initial concentration 3.6 mmol/l), serine (1.4 mmol/l), glycine (1.2 mmol/l) and alanine (1.3 mmol/l) – were utilized completely during the fermentation (fig 5). The addition of 1.5 mmol/l aspartate to control whey had no influence on the growth of KM (fig 6), but higher concentrations increased both the growth rate and growth yield. The amount of lactate metabolized and propionate and acetate produced also increased with increasing aspartate addition; succinate was produced but only in the presence

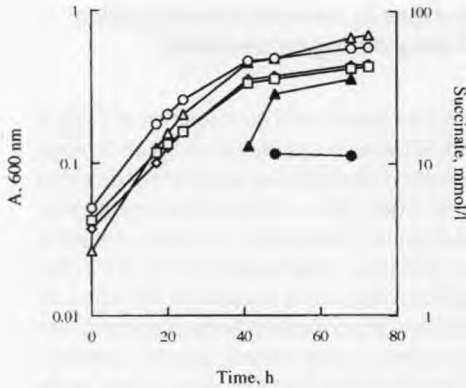


Fig 4. Effect of casein hydrolysate on growth (open symbols) and succinate production (closed symbols) of *Propionibacterium freudenreichii* KM. (□) 0.0% casein hydrolysate; (◇, ◆) 0.1% casein hydrolysate; (○, ●) 1.0% casein hydrolysate; (△, ▲) 10% casein hydrolysate. Succinate was not produced in the absence of casein hydrolysate.

Effet d'un hydrolysate de caséine sur la croissance (symboles ouverts) et sur la production de succinate (symboles pleins) de Propionibacterium freudenreichii KM. (□) 0,0% d'hydrolysate de caséine; (◇, ◆) 0,1% d'hydrolysate de caséine; (○, ●) 1,0% d'hydrolysate de caséine; (△, ▲) 10% d'hydrolysate de caséine. Le succinate n'était pas produit en absence d'hydrolysate de caséine.

of 6 and 30 mmol/l aspartate (fig 6). The molar ratios of propionate:lactate were 0.71, 0.65, 0.59 and 0.53 in the presence of 0, 1.5, 6 and 30 mmol/l aspartate, respectively. The molar ratios of acetate:lactate did not change significantly with increasing levels of aspartate. No effects were observed on addition of up to 30 mmol/l each of alanine, serine or glycine, both individually or together (data not shown).

Changes in peptide composition during fermentation

In addition to amino acids, RR whey also had higher concentrations of peptides than control whey (fig 7). Most of the peptides in

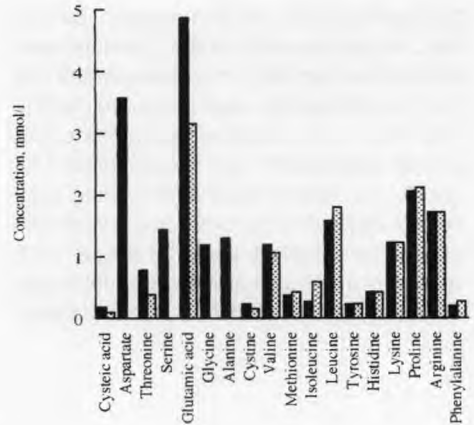


Fig 5. Free amino acid composition of control whey supplemented with 1.0% casein hydrolysate at the beginning (■) and at the end (□) of growth of *Propionibacterium freudenreichii* KM.

Composition en acides aminés libres du lactosérum témoin supplémenté avec 1,0% d'hydrolysate de caséine en début (■) et fin (71 h) (□) de fermentation par Propionibacterium freudenreichii KM.

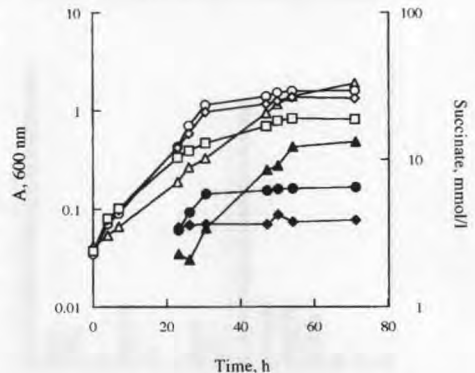


Fig 6. Effect of aspartate on growth (open symbols) and succinate production (closed symbols) of *Propionibacterium freudenreichii* KM in control whey. (□) 0.0 mmol/l aspartate; (◇) 1.5 mmol/l aspartate; (○, ●) 6.0 mmol/l aspartate; (△, ▲) 30 mmol/l aspartate.

Effet de l'aspartate sur la croissance (symboles ouverts) et sur la production de succinate (symboles pleins) de Propionibacterium freudenreichii KM sur lactosérum témoin. (□) 0,0 mmol/l d'aspartate; (◇) 1,5 mmol/l d'aspartate; (○, ●) 6,0 mmol/l d'aspartate; (△, ▲) 30 mmol/l d'aspartate.

the RR they were utilized during subsequent growth of *P freudenreichii* KM, regardless of their hydrophobicities (fig 8). Some peptides were higher in concentration after

growth of the PAB, which may be due to production of peptides by the PAB during growth or to hydrolysis of some peptides into smaller ones, which elute at different times, but which are not used or used only to a limited extent by the PAB.

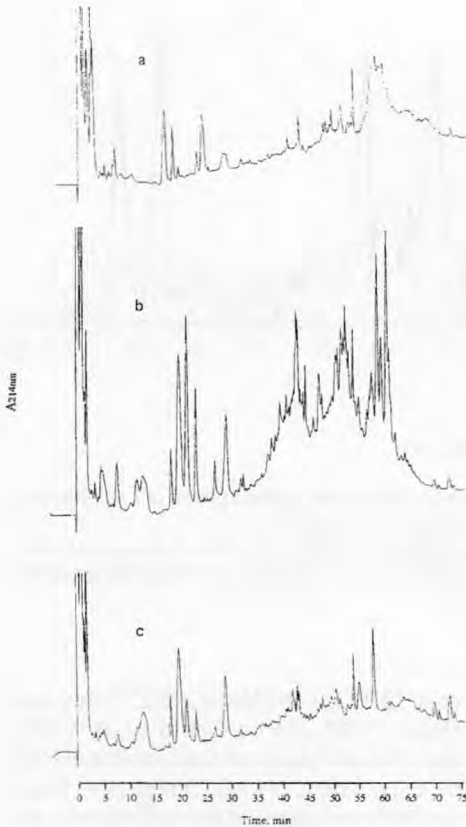


Fig 7. Peptide profiles of (a) control whey before growth of *Propionibacterium freudenreichii* KM, (b) *Lactobacillus helveticus* RR whey before growth of *Propionibacterium freudenreichii* KM and (c) *Lactobacillus helveticus* RR whey after ultrafiltration.

Profils peptidiques du (a) lactosérum témoin avant croissance de Propionibacterium freudenreichii KM, (b) lactosérum produit par Lactobacillus helveticus RR, avant croissance de Propionibacterium freudenreichii KM et (c) lactosérum de Lactobacillus helveticus RR ayant subi une ultrafiltration.

Ultrafiltration

Ultrafiltration of RR whey was used in an attempt to determine whether the stimulant(s) was a high or low molecular mass compound(s). After ultrafiltration of RR whey, KM did not grow in either the retentate, permeate or in the recombined permeate plus retentate. Ultrafiltration resulted in a dramatic reduction of the peptides of the recombined permeate and retentate (fig 7); some peptides were missing and some were present but at a much lower concentration.

Influence of heat treatment on the stimulant(s)

Pasteurization and autoclaving of RR whey resulted in a precipitate which was removed by centrifugation before filtering and filter sterilizing the supernatant. These heat treatments did not affect the rates of growth of strain KM, but a slightly reduced final cell mass was obtained in the case of autoclaved whey (data not shown).

Effect of perchloric acid extraction on the stimulant(s)

Treatment with perchloric acid precipitated the proteins from the whey. This procedure caused a 2.5-fold dilution of the whey, but stimulation was still observed in it compared with a control whey which had also been diluted 2.5-fold (data not shown).

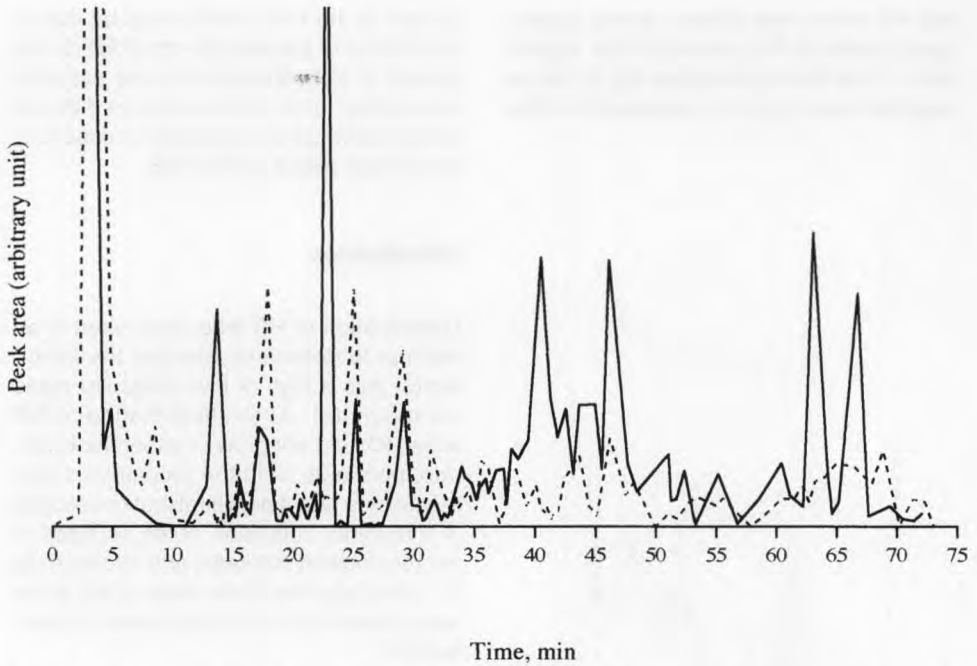


Fig 8. Effect of growth of *Propionibacterium freudenreichii* KM on the peptide profile of *Lactobacillus helveticus* RR whey. Solid line, before growth; broken line, after growth.

Modifications du profilé peptidique du lactosérum de Lactobacillus helveticus RR, induites par la croissance de Propionibacterium freudenreichii KM. Lignes pleines, avant croissance ; lignes pointillées, après croissance.

Partial isolation of the stimulant(s) by gel filtration

The gel-filtration profile of RR whey, after separation on Sephadex G25, is shown in figure 9. Eleven different peaks were obtained and peaks 1 to 4 contained most of the stimulatory activity. These results suggest that several stimulants are involved with molecular masses of less than 5 000 Da.

DISCUSSION

Earlier workers (Hunter and Frazier, 1961; Czarnocka-Roczniakowa *et al*, 1972; Lee

et al, 1976; Liu and Moon, 1982; Parker and Moon, 1982; Perez Chaia *et al*, 1987) observed stimulation of some strains of PAB by some strains of LAB. The present study confirms the ability of several starter LAB to stimulate the growth of PAB in wheys, in that 13 of 14 strains of LAB tested stimulated growth of 1 or more of 4 strains of PAB. The stimulatory activity of *Lb acidophilus* and *Lb lactis* depended on the strain of PAB used. Similar results were obtained by Liu and Moon (1982) and Parker and Moon (1982). The stimulatory activity of *Lactococcus lactis* depended, on the one hand, on the strain used (strain E8 had no effect) and on the other hand, on the PAB strain used (no stimulation by any of the 4 strains tested was observed with

PAB strain T). This variation of the stimulation according to the PAB strain used was noted by Czarnocka-Roczniakowa *et al* (1972), but the stimulation was low. The latter workers also found that *Str thermophilus* T149 stimulated the growth of 3 strains of PAB. In the present study, all 5 strains of *Str thermophilus* stimulated PAB strains KM and H. However, for PAB strains L5 and T, the stimulation depended on the strain of

Str thermophilus used. In the present work, *Lb helveticus* strains were the most consistent stimulators, in that the 3 strains used stimulated the 4 PAB strains studied. Such stimulation has also been reported previously by Czarnocka-Roczniakowa *et al* (1972) and Perez Chaia *et al* (1987).

The more detailed study of the stimulation of *P freudenreichii* KM by *Lb helveticus* RR showed that the increase in growth rate and cell yield coincided with an increased conversion of lactate to propionate and acetate (fig 2). L lactate was used faster than D lactate in both starter and control whey, in agreement with previous data of Crow (1986a). All strains of PAB used in this study metabolize lactose, but lactose was not utilized during growth in whey containing lactate which was still present when growth ceased. Marcoux *et al* (1992) observed lactose utilization by PAB in whey-based media containing lactose, lactate and yeast extract, but only when most of the lactate had been metabolized.

Amino acids are metabolized by PAB in the presence of lactate (fig 3). In a mixture of amino acids (vitamin-free, acid-hydrolysed casein), aspartate, serine, glycine, alanine and glutamic acid were mainly metabolized (fig 5). These results are in agreement with those of Brendehaug and Langsrud (1985). Crow (1986b) showed that aspartate, alanine and serine were used by PAB, but aspartate appeared to be the only one readily metabolized in a Swiss-type cheese environment. Of the 4 main free amino acids used by PAB in this study, aspartate was the only one found to stimulate strain KM (fig 6). However, the concentration of free aspartate was less than 0.1 mmol/l in starter and control wheys. In control whey, free aspartate concentrations greater than 15 times the concentration found in the starter whey were needed to stimulate the growth of KM. Succinate was detected during growth in control whey supplemented with aspartate or casein hydrolysate. This coin-

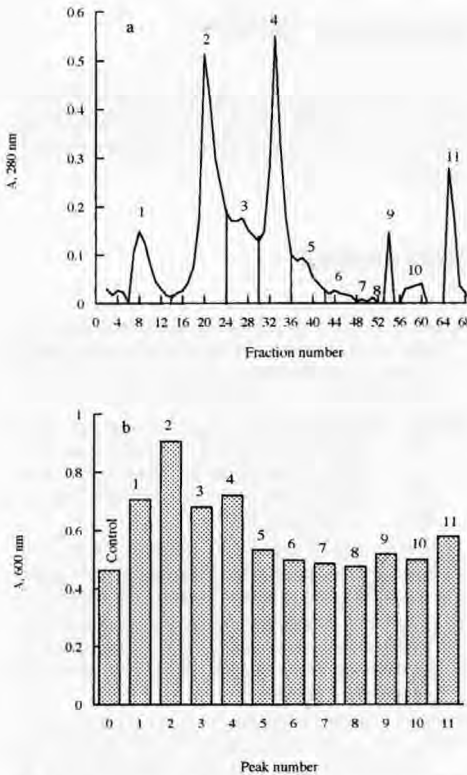


Fig 9. Separation of *Lactobacillus helveticus* RR whey on Sephadex G-25. (a) Chromatogram; (b) biomass (A_{600}) of *Propionibacterium freudenreichii* KM in control whey supplemented with material from the different fractions.

Séparation du lactosérum de *Lactobacillus helveticus* RR sur Sephadex G-25. (a) Chromatogramme; (b) biomasse (A_{600}) de *Propionibacterium freudenreichii* KM dans le lactosérum témoin et dans le lactosérum témoin supplémenté avec les différentes fractions.

cided with a decrease in the ratio of propionate:acetate formed from lactate. Crow (1986b) reported similar findings. In the present study, the amount of succinate detected at the end of fermentation was 5 mmol/l greater than the amount of aspartate used. Crow (1986b) proposed that the additional succinate was formed from pyruvate in a pathway involving CO₂ fixation and oxaloacetate production. CO₂ was not measured in the present study.

The stimulatory compound(s) was resistant to pasteurization and autoclaving and stable to precipitation with perchloric acid. These results agree with those of Hunter and Frazier (1961) but contrast with those of Czarnocka-Rocznikowa *et al* (1972), who reported a decrease of stimulatory activity after pasteurization. Attempts were made to determine the molecular mass. Ultrafiltration was useless because of absorption of the stimulant(s) on the membrane. Gel filtration with Sephadex G25 suggests that the molecular mass is less than 5 000 Da, since most of the activity eluted after the void volume. These results also suggest that several compounds are involved since stimulatory activity was present in several peaks. However, since water was used as eluent, nonspecific adsorption of one particular compound to the column may result in the compound eluting in several peaks. Such interactions can be reduced by elution with NaCl which increases the ionic strength. However, NaCl could not be used here because of the inhibitory activity of salt on the growth of PAB (Hettinga and Reinbold, 1972).

The production of high levels of amino acids and peptides by LAB in milk and cheese is not unusual (Accolas *et al*, 1980; Desmazeaud, 1983). *Lb helveticus* RR produced free amino acids and peptides, some of which were utilized during growth of strain KM. PAB have intracellular (Perez Chaia *et al*, 1990; El-Soda *et al*, 1992) and extracellular peptide hydrolase activities (Langsrud

et al, 1977). Peptide utilization has been reported during Swiss-type cheese ripening where late fermentation was linked to an increased breakdown of some peptides with molecular masses around 3 000 (Blanc *et al*, 1979). Although the concentration of free aspartate in starter whey was too low to account for the stimulation observed, it is possible that aspartate is responsible for the stimulation as a constituent of a peptide.

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