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Effects of free fatty acids on propionic acid bacteria

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Summary — The seasonal variations in milk fat composition, especially during the grazing period, often lead to poor eye formation in Swiss-type cheese. The influence of free fatty acids on the growth and metabolism of the dairy propionibacteria has been studied in this work. Linoleic ($C_{18:2}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$) and oleic acids ($C_{18:1}$) inhibited the growth and acid production of *P freudenreichii* subsp *shermanii* in the reference medium. The antibacterial activity of linoleic acid can be overcome by additions of cholesterol and soya lecithin to the medium. The four species and two subspecies of dairy *Propionibacterium* could be divided into two groups according to their high susceptibility to unsaturated fatty acids (*P freudenreichii* subsp *shermanii* and subsp *freudenreichii*) or low susceptibility (*P acidipropionici*, *P jensenii* and *P thoenii*). Nevertheless, this inhibitory action of free fatty acids was not observed in milk, retentate media or lactic curd. A total extraction of the milk lipids led to the recovery of this inhibitory effect on *Propionibacterium*. The possible modes of action of these molecules are discussed on the basis of the observed potassium effluxes and disturbances in the cell membranes.

free fatty acid / *Propionibacterium* / inhibition / linoleic acid / lauric acid

Résumé — Effet des acides gras libres sur les bactéries propioniques. *Les variations saisonnières de composition du lait peuvent avoir des répercussions importantes sur la qualité des fromages. Les laits de printemps conduisent souvent à de graves défauts d'ouverture en fabrication de fromage à pâte pressée cuite (PPC) et notamment d'emmental. Ce travail montre l'action fortement inhibitrice de faibles quantités d'acides gras libres (10 à 100 mg/l^{-1}) sur le développement, dans leur milieu de culture de référence, des bactéries propioniques, agents de l'ouverture de ces fromages. Les acides linoléique ($C_{18:2}$), laurique ($C_{12:0}$), myristique ($C_{14:0}$) et oléique ($C_{18:1}$) inhibent la croissance et la production d'acides propionique et acétique de* Propionibacterium freudenreichii *subsp* shermanii. *La présence de cholestérol ou de lécithine de soja dans le milieu de culture restaure l'activité cellulaire. Deux grands groupes de susceptibilités différentes à ces actions inhibitrices peuvent être formés parmi les souches des 4 espèces de bactéries propioniques laitières: les 2 sous-espèces de* P freudenreichii *(grande susceptibilité) d'une part et* P acidipropionici, P jensenii *et* P thoenii *(faible susceptibilité) d'autre part. Néanmoins, l'action inhibitrice de l'acide linoléique ne se retrouve ni lors de culture sur lait ou sur rétentat (facteur de concentration volumique = 5), ni dans des caillés préparés selon la technologie des fromages PPC. Une délipidation totale du lait permet à nouveau l'expression*

du caractère inhibiteur de cet acide gras libre sur les bactéries propioniques. Les modes d'action possibles de ces acides gras sont discutés au regard des résultats de pertes de potassium et des perturbations engendrées par ces acides gras libres au niveau des membranes cellulaires.

acide gras libre* / Propionibacterium / *inhibition* / *acide linoléique* / *acide laurique

INTRODUCTION

The inhibitory action of rancid milk on the growth of numerous bacteria has been shown (Tarassuk and Smith, 1940) and confirmed later (Costilow and Speck, 1951). The inhibitory effects and also the growth promoting properties of fatty acids were reviewed by Nieman (1954). The most pronounced antibacterial effects of low concentrations of fatty acids have been noted with Gram-positive bacteria.

The antibacterial activity of free fatty acids was shown at the same time, especially on lactic acid bacteria (Humfeld, 1947; Poznanski *et al*, 1968). This activity is sometimes bactericidal but, with most microorganisms, the antibacterial action of fatty acids is bacteriostatic (Nieman, 1954). Kodicek and Worden (1945) have shown that oleic and linolenic acids inhibit the growth of *Lactobacillus helveticus*. Lauric and myristic acids also proved to be inhibitory for this microorganism (Williams and Fieger, 1946). Later, Anders and Jago (1964a, b) showed that oleic acid, accumulated early during the cheese ripening process, inhibited the viability of lactic acid streptococci. This oleic acid alters the pyruvate metabolism of group N streptococci (Anders and Jago, 1970). The *cis*-form of unsaturated fatty acids exhibited a greater antibacterial activity than the corresponding *trans* isomers on *L helveticus*, *M tuberculosis* and *B subtilis* (Nieman, 1954; Galbraith and Miller, 1973; Kabara, 1979).

Milk fat mainly consists of triglycerides of fatty acids (97 to 98%) (Kurtz, 1965). Many factors determine the proportion of fatty acids in milk fat, especially the feeding, the lactation stage, the animal species or the breed of cow. The seasonal variations in the composition of milk fat influence dairy processing. This assertion is particularly true in cooked hard cheese processing, where the milk fat represents 45 to 55% of the dry matter. This fat influences the rheological properties (Creamer and Olson, 1982), the flavour (Adda *et al*, 1982) and the microbial transformation of the cheese (Chen *et al*, 1979).

As the natural lipase of milk is almost completely destroyed by the cooked hard cheese processing (Driessen, 1983), the main lipolytic activities in this type of cheese are of microbial origin: psychrotrophic bacteria (Law *et al*, 1976), lactic and propionic starters and surface flora for Comté and Beaufort.

Lactic acid bacteria exhibit a low lipolytic activity (Stadhouders and Veringa, 1973), but propionic acid bacteria, which are the main ripening agents in Swiss-type cheeses, are lipolytic (Knaut and Mazurek, 1974; Dupuis and Boyaval, 1993; Dupuis *et al*, 1993). Free fatty acid concentrations as high as 14 g kg^{-1} of hard cheese were reported after ripening (Woo *et al*, 1984). After propionic and acetic acids, which are the direct result of propionic acid bacteria activity, the major fatty acids are oleic ($C_{18:1}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$) and myristic acids ($C_{14:0}$), as in milk fat (Langler and Day, 1966; Masson *et al*, 1978; Deeth *et al*, 1983; de Jong and Badings, 1990).

The seasonal variations which change the proportions of these acids, especially in spring (Decaen and Journet, 1966; Gray,

1973; Masson *et al*, 1978), affect the Swiss-type cheese ripening. Poor eye formation and lower levels of propionic and acetic acids in the curd suggest a poor propionic acid bacteria activity. This problem has a considerable economic impact for the manufacturers who need to know the exact factors leading to these depreciated cheeses.

In this study, we have examined the influence of free fatty acids on growth, acid production (propionic and acetic acids) and lactate consumption of *Propionibacterium freudenreichii* subsp *shermanii*, the most frequently found propionic acid bacteria in Swiss-type cheese and of a type-strain of each dairy species of this genus. Moreover, potassium efflux rates, oxygen consumption and $\Delta\psi$ (transmembrane electrical potential) alterations generated by linoleic molecules on *Propionibacterium* have been measured in order to understand the nature of this influence.

MATERIALS AND METHODS

Strains and growth media

Five type-strains (Cummins and Johnson, 1986), of each species or subspecies of dairy *Propionibacterium* were used during this study: *P freudenreichii* subsp *freudenreichii* CIP 103026 (Collection de l'Institut Pasteur, Paris, France), *P freudenreichii* subsp *shermanii* CIP 103027, *P jensenii* CIP 103028, *P thoenii* CIP 103029 and *P acidipropionici* DSM 4900 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Moreover, one strain frequently used in cheese factories, *Propionibacterium freudenreichii* subsp *shermanii* LRTL 30 (INRA, Rennes, France), was also used throughout this study. Strains were propagated in a lactate-yeast extract based medium (YEL) (Malik *et al*, 1968) at 30°C without shaking. Viability was evaluated by plating 1 ml of cell suspension on YEL agar and incubated at 30°C for 4 days.

Stock cultures were maintained at –70°C in YEL medium containing 15% (v/v) glycerol (Prolabo, France). All media were sterilized by heat treatment (120°C, 15 min).

Cultures on milk were carried out with a low heat milk powder (milk 'G') recommended by Chamba and Prost (1989) for the evaluation of acidifying activity of thermophilic lactic acid bacteria.

Cheese curds were prepared according to Buisson *et al* (1987) with microfiltrated milk (Trouvé *et al*, 1991). The phase of pressing was not carried out.

Analysis

Bacterial growth was followed by optical density measurements (650 nm; Beckman spectrophotometer DU 7400, Gagny, France) correlated with dry weight measurements. Lactic, propionic and acetic acid concentrations were measured using an HPLC system (Beckman, Gagny, France) equipped with an UV detector (214 nm; Scanning detector module 167). Separation of sample of 20 µl took place in a 7.5 x 300 mm (Aminex A6, ion exchange, Biorad) stainless steel column, operated at ambient temperature with H_2SO_4 5 mmol l^{-1} (1 ml min^{-1}) as eluent.

Chemicals reagents

Acetic acid (C_2, Merck, Darmstadt, Germany); propionic acid (C_3, Sigma, St Quentin Fallavier, France); n-butyric acid (C_4, Sigma); caproic acid (C_6, Sigma); caprylic acid (C_8, Merck); capric acid (C_{10}, Merck); lauric acid (C_{12}, Prolabo, Paris, France); oleic acid ($C_{18:1}$, Prolabo) and linoleic acid ($C_{18:2}$, Prolabo) were used in this study, dissolved in ethyl alcohol of a high chemically pure form (Prolabo). Cholesterol was purchased from Prolabo, soya lecithin from Lucas-Meyer (France, 'Emulfluid E') and bovine albumin (BSA, essentially globulin free) from Sigma.

Growth and activity in the presence of free fatty acids

Cultures were carried out at 30°C in 100 ml glass bottles with 10 and 100 mg l^{-1} of each fatty acid in the YEL medium. Controls were made for each

solvent (ethanol) concentration employed. The pH was not monitored.

Oxygen consumption measurements

Oxygen consumption was measured polarographically using a Clarke-type electrode connected to a Gilson oxygraph (Model K-ITC-C, Middleton, WI, USA). Measurements were carried out at 30°C on cells incubated in phosphate buffer (50 mmol l^{-1}, pH 6.50) at a final concentration of 0.3 mg ml^{-1} (dry weight: DW).

Potassium efflux measurements

The variations in the potassium content of the cells (K^+_{in}) were determined by measuring the changes of the potassium concentration in the external medium (K^+_{out}) with a potassium-valinomycin-selective electrode (Radiometer) associated with a calomel reference electrode containing a secondary salt bridge filled with a solution of 100 mmol l^{-1} NaCl (Boulanger and Letellier, 1988). The cation electrode potential was calibrated with KCl solutions of known concentrations, which were prepared in the experimental buffer. To estimate the total potassium content of the bacteria, the cation was released by treatment of the cells with 0.4 mmol l^{-1} linoleate or 2 mmol l^{-1} laurate. The amount of released cation was then estimated with the potassium electrode. K^+_{in} was calculated from the value of K^+_{out} and expressed in nmol mg^{-1} ml^{-1} (cell DW) assuming that 4×10^9 cells correspond to 1 mg ml^{-1} (cell DW). Whatever the product nature added to the cells, the ethanol concentration was always less than 1% (v/v).

Measurement of the transmembrane electrical potential ($\Delta\psi$)

$\Delta\psi$ was determined from the accumulation of [^{14}C] TPP^+ (tetraphenylphosphonium ion) (10 µmol l^{-1}, final concentration, 2.17 GBq $mmol^{-1}$) (Ghazi *et al*, 1986). The cells were filtered on glass fibre filters (GF/F Whatman), washed with 4 ml of buffer and counted for radioactivity. TPP^+ uptake was corrected for unspecific binding by subtracting a blank obtained under identical conditions, except that the cells were pretreated with the protonophore TCS (3, 3', 4', 5 - tetrachlorosalicylanilide) (10 µmol l^{-1}, final concentration).

RESULTS

Inhibitory action of free fatty acids (FFA)

Even at very low concentrations (< 10 mg l^{-1}), the palmitic ($C_{16:0}$) and stearic acids ($C_{18:0}$) were not soluble in ethanol. They were soluble in propanol-1 and chloroform but formed a precipitate in the YEL medium. Consequently, they were not tested further in this study.

At 10 mg l^{-1}, growth of *P freudenreichii* subsp *shermanii* LRTL 30 on YEL medium was clearly inhibited by the linoleic acid and slightly by the lauric ($C_{12:0}$), myristic ($C_{14:0}$) and oleic ($C_{18:1}$) acids. These bacteriostatic effects were clearly evidenced at 100 mg l^{-1}, particularly for the lauric ($C_{12:0}$) and linoleic ($C_{18:2}$) acids (table I). A restoration of growth after 25 h was observed for the oleic acid ($C_{18:1}$). The lactate consumption was affected in the same way with no decrease for lauric ($C_{12:0}$) and linoleic acids ($C_{18:2}$) and a slight decrease for myristic ($C_{14:0}$) and oleic acids ($C_{18:1}$). The propionate and acetate productions reflected exactly the lactate evolution. Moreover, a low positive (or no) effect of the fatty acids from acetic ($C_{2:0}$) to capric acids ($C_{10:0}$) on the initial (24 h) lactate consumption and propionic and acetic acids production was noted.

The effects of caproic acid ($C_{6:0}$) and linoleic acid ($C_{18:2}$) on the type-strains of the four species and two subspecies of dairy propionibacteria are shown in table II. A slight positive effect of the caproic acid on the growth of the two *P freudenreichii* strains was observed. This effect was not significant for the three other species. Linoleic acid drastically affected the growth of

Table I. Effect of caproic acid ($C_{6:0}$) and linoleic acid ($C_{18:2}$) at 100 mg l^{-1} on the growth of the five type-strains of dairy *Propionibacterium*.
Effets des acides caproïque ($C_{6:0}$) et linoléique ($C_{18:2}$) à 100 mg l^{-1} sur la croissance des 5 souches-type de bactéries propioniques laitières.

FFA	*Strain*				
	103026	*103027*	*103028*	*103029*	*4900*
C6:0	--	-	0	0	0
C18:2	---	--	-	--	-

Inhibitory action: --- high; -- medium; - low; 0, no activity.
Action inhibitrice : --- forte; -- moyenne ; - faible ; 0 : pas d'activité.

Table II. Inhibiting action of free fatty acids on the growth, propionate production and lactate consumption of *P freudenreichii* subsp *shermanii* LRTL 30 compared to a control without fatty acid.
Action des acides gras libres sur la croissance, la production de propionate et la consommation de lactate de Propionibacterium freudenreichii *subsp* shermanii *LRTL 30 (comparée à un témoin sans addition d'acide gras libre).*

Fatty acid	*Growth*	*Propionate production*	*Lactate consumption*
C_2	0	+	+
C_3	0	+	0
C_4	0	+	0
C_6	0	+	0
C_8	0	+	-
C_{10}	-	+	-
C_{12}	---	---	---
C_{14}	--	--	--
$C_{18:1}$	--	--	--
$C_{18:2}$	---	---	---

Inhibition: ---, high; --, medium; -, low. Activation: +++, high; ++, medium; +, low; 0, no effect.
Inhibition: ---, forte ; --, moyenne ; -, faible. Activation : +++, forte ; ++ moyenne ; + faible ; 0 sans effet.

P freudenreichii subsp *freudenreichii* and, to a lesser extent, the growth of *P freudenreichii* subsp *shermanii, P theonii. P jensenii* and *P acidipropionici* growth was affected during the first 30 h, but the number of cells sharply increased afterwards to reach a level higher than the control cultures (28% more for *P jensenii* and 33% for *P acidipropionici*). Globally, these results were confirmed by the lactate consumption and the propionic and acetic acid production.

As the natural medium of *Propionibacterium* multiplication in Swiss-type cheese technology is milk, we have tried to repeat our observations on milk. The main results are summarized in figure 1. Even in a milk containing a low lipid concentration (500 mg l^{-1}), linoleic acid, at concentrations up to 5 g l^{-1}, had no effect on cell multiplication or on acid production. The inhibitory effect of linoleic acid was recovered after a previous extraction of the lipids from the milk powder with hexane before milk rehydration. Moreover, the inhibition of *Propionibacterium freudenreichii* subsp *shermanii* LRTL 30 was also not evidenced in milk retentate (volume concentration factor of 5, obtained by ultrafiltration; not shown). In order to better master the possible influence of free fatty

acids in Swiss-type cheese technology, we have examined the development of this strain in a cheese curd made from microfiltrated milk (which contained only lactic starters before addition of propionic acid bacteria). The cell multiplication, during the 340 h of the trial, was not affected by the presence of 5 g l^{-1} of linoleic acid (not shown).

Alterations of the FFA inhibition activity by addition of compounds

No growth stimulating effect of Tween 40, Tween 60, Tween 80 (at 100 mg l^{-1}) and biotin (at 10^{-5} or 10^{-4} g l^{-1}) was observed. On the other hand, Tween 80 at 30 g l^{-1} counteracted the inhibitory effect of linoleic acid for the *P jensenii* CIP 103028 culture (not shown). The inhibitory effect of linoleic acid ($C_{18:2}$) at 100 mg l^{-1} was reversed neither by the presence of 10 or 100 mg l^{-1} of caproic acid ($C_{6:0}$), nor by the presence of biotin at 10^{-5} or 10^{-4} g l^{-1}. However, soya lecithin (100 mg l^{-1}) and cholesterol (100 mg l^{-1}) greatly reduced this negative effect (fig 2).

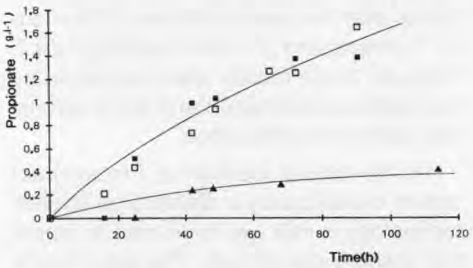

Fig 1. Effect of linoleic acid (5 g l^{-1}) on propionic acid production by *P freudenreichii* subsp *shermanii* LRTL 30 in milk G (■) and in milk G previously treated with hexane (▲). Control without addition of linoleic acid (❑). Controls are carried out with the same solvent concentrations as in trials.

Effet de l'acide linoléique (5 g l^{-1}) sur la production d'acide propionique par des cellules de P freudenreichii *subsp* shermanii *LRTL 30 cultivées sur lait G (■) et sur lait G après traitement à l'hexane (▲). (❑) représente la production d'acide propionique sans addition d'acide linoléique. Les témoins sont réalisés avec les mêmes concentrations de solvant que dans les essais.*

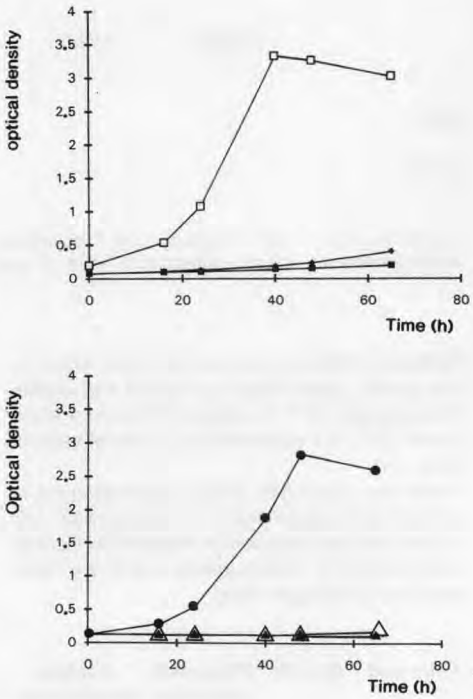

Fig 2. Effect of linoleic acid ($C_{18:2}$) (100 mg l^{-1}) (■); linoleic (100 mg l^{-1}) + cholesterol (100 mg l^{-1}) (❑); linoleic (100 mg l^{-1}) + biotin (10^{-4} g l^{-1}) (◆); linoleic (100 mg l^{-1}) + soya lecithin (100 mg l^{-1}) (●); linoleic (100 mg l^{-1}) + caproic acid ($C_{6:0}$) 10 mg l^{-1} (▲) and 100 mg l^{-1} (Δ) on the growth of *P freudenreichii* subsp *shermanii* LRTL 30. Controls are carried out with the same solvent concentrations as in trials.

Effets des acides linoléique ($C_{18:2}$)(100 mg l^{-1}) (■); linoléique (100 mg l^{-1}) + cholesterol (100 mg l^{-1}) (❑); linoléique (100 mg l^{-1}) + biotine (10^{-4} g l^{-1}) (◆); linoléique (100 mg l^{-1}) + lécithine de soja (100 mg l^{-1}) (●); linoléique (100 mg l^{-1}) + acide caproïque ($C_{6:0}$) à 10 mg.l^{-1} (▲) et à 100 mg l^{-1} (Δ) sur la croissance de P freudenreichii *subsp* shermanii *LRTL 30. Les témoins sont réalisés avec les mêmes concentrations de solvant que dans les essais.*

Alterations of the cell membrane integrity: potassium efflux

Total cellular K^+ initially present in the *Propionibacterium* cells was evaluated at 1650 nmol mg^{-1} dry weight, a value close to the 1800 nmol mg^{-1} determined by Duperray *et al* (1992) for the closely related species of *Corynebacterium glutamicum*. Figure 3 represents the effect of increasing concentrations of linoleic acid on the initial rate of K^+ efflux. The minimum concentration of linoleic acid necessary to detect an efflux of K^+ in cells incubated at pH 6.50 is 6 µmol l^{-1} (20 µmol l^{-1} for lauric acid). Addition of 125 µmol l^{-1} of linoleic acid induced a complete and rapid efflux of K^+ ions (1600 µmol min^{-1} mg^{-1} (DW)). Lauric acid also induced an efflux of cytoplasmic K^+. This efflux was maximal only for a lauric acid concentration of 900 µmol l^{-1}, more than 7 times the concentration of linoleic acid for the same rate (fig 4). Addition of 50 µmol l^{-1} of BSA to the cell suspension after addition of the free fatty acid totally counteracted the action of 25 µmol l^{-1} of linoleic acid on K^+ efflux (the same effect was observed for 200 µmol l^{-1} BSA after addition of 51 µmol l^{-1} of linoleic acid). Moreover, a 1 min incubation with 50 µmol l^{-1} BSA totally protected the internal K^+ cellular pool against the action of 51 µmol l^{-1} of linoleic acid. The addition of $MgSO_4$ at 1 mmol l^{-1} completely counteracted the K^+ efflux induced by the previous adjunction of 52 µmol l^{-1} of linoleic acid to the bacterial cells. Morever, in the presence of 20 mmol l^{-1} $MgSO_4$ in the medium, 50 µmol l^{-1} of linoleic acid have no effect on the K^+ efflux from the *P freudenreichii* subsp *shermanii* cells. A previous incubation of the *Propionibacterium* cells with caproic acid (780 µmol l^{-1}) had no protective effect on K^+ efflux induced later by linoleic acid (25 µmol l^{-1}) (not shown).

The presence of 15 mg of Tween 80 in the cell suspension had a total protective effect against linoleic acid action (on K^+

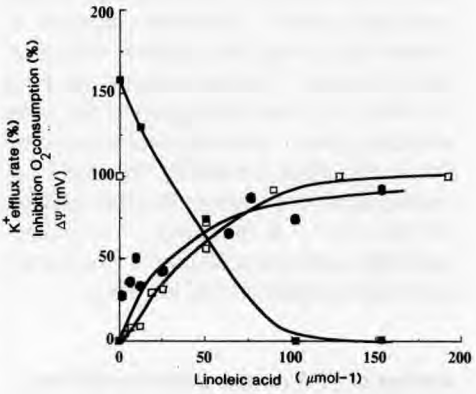

Fig 3. Effect of linoleic acid on inhibition of O_2 consumption (●), K^+ efflux rate (❑) and $\Delta\psi$ (■) in *Propionibacterium freudenreichii* subsp *shermanii* LRTL 30.
Effet de l'acide linoléique sur la consommation d'O_2 des cellules de Propionibacterium freudenreichii *subsp* shermanii *LRTL 30 (●), la vitesse de sortie du potassium intracellulaire (❑) et le $\Delta\psi$ (■).*

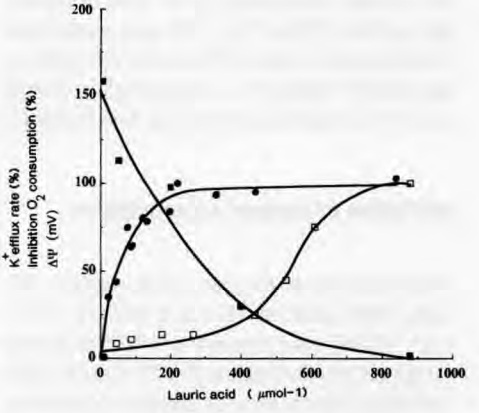

Fig 4. Effect of lauric acid on inhibition of O_2 consumption (●), K^+ efflux rate (❑) and $\Delta\psi$ (■) in *Propionibacterium freudenreichii* subsp *shermanii* LRTL 30.
Effet de l'acide laurique sur la consommation d'O_2 des cellules de Propionibacterium freudenreichii *subsp* shermanii *LRTL 30 (●), la vitesse de sortie du potassium intracellulaire (❑) et le $\Delta\psi$ (■).*

efflux and on oxygen consumption), at least up to 200 μmol l^{-1} ! Moreover, addition of Tween 80 (15 mg) after addition of linoleic acid (50 μmol l^{-1}) immediately stopped the K^+ efflux. A 2 min incubation of the cells with 200 μmol l^{-1} of cholesterol reduced by 74% the K^+ efflux induced by 128 μmol l^{-1} of linoleic acid (not shown). But the addition of 100 μmol l^{-1} or 200 μmol l^{-1} of cholesterol after addition of 50 μmol l^{-1} of linoleic acid had no effect on that K^+ efflux.

Action of FFA on the transmembrane electrical potential

At pH 6.50, the $\Delta\psi$ was 158 mV, a value close to the $\Delta\psi$ determined for other Gram-positive bacteria (162 mV for *S aureus* and 113 mV for *S lactis* (Kashket, 1981) and 190 mV for *Corynebacterium glutamicum* (Duperray *et al*, 1992)). The cell membrane also became more permeable to H^+ after addition of linoleic acid since a decrease of the $\Delta\psi$ was observed (fig 3). This decreasing $\Delta\psi$ from 158 mV to 0 mV was evidenced in the same range of linoleate concentration than K^+ efflux. At a concentration of 900 μmol l^{-1} of lauric acid, the $\Delta\psi$ was 0 (fig 4).

Inhibition of oxygen consumption

The inhibition of oxygen consumption, initially evaluated at 15 ± 2 nmol O_2 min^{-1} mg^{-1} (DW) was shown within the same range as $\Delta\psi$ decrease (fig 3). Lauric acid inhibitory effect towards oxygen consumption was roughly the same as observed for linoleic acid except that total inhibition occurred at 250 μmol l^{-1} for lauric acid and at 100 μmol l^{-1} for linoleic acid (fig 4).

Caproic acid at concentrations up to 800 μmol l^{-1} showed no action on oxygen consumption and K^+ intracellular level (not shown), confirming the above results on cell growth. Addition of 60 μmol l^{-1} of BSA had no action on the cell oxygen consumption, but protected the cells against the action of 100 μmol l^{-1} linoleic acid. But even with this protective action, 180 μmol l^{-1} of linoleic acid induced a 90% decrease in oxygen consumption. Lauric acid at 440 μmol l^{-1} achieved the same inhibition. This is the same ratio observed as without BSA protection (2.4 more lauric acid than linoleic acid).

DISCUSSION

Inhibitory action of FFA

The main effects of the fatty acids on *Propionibacterium freudenreichii* subsp *shermanii* growth on YEL medium enable a separation into three classes: class I, from $C_{2:0}$ to $C_{10:0}$ no effect on growth and a low positive effect on the fermentative capacities; class II, $C_{14:0}$ and $C_{18:1}$ negative effect on growth and acid production, intermediate effect between classes I and III; class III, $C_{18:2}$ and $C_{12:0}$ complete inhibition of the growth and metabolism of these bacteria.

To our knowledge, no information has been published on the action of free fatty acids on propionic acid bacteria. Lactobacilli, although generally not sensitive to saturated fatty acids, can be sometimes inhibited by them if the compounds have a chain length of around C_{12}, these being the most active (Hassinen *et al*, 1951). In this study, we have observed the same effects on propionic acid bacteria.

A few explanations on the exact influence of the inhibitory fatty acids on microbial cells have been proposed but not confirmed: i) they decrease the bacterial respiration (Galbraith and Miller, 1973); ii) they build an adsorption layer around the cell which changes the cell permeability, leading to an outward diffusion of vital cellular components or blocking the adsorption of essential

nutrients (Nieman, 1954); iii) they are able, in the form of soaps, to lower the surface tension of the media (Nieman, 1954) and they inhibit the active transport of amino acids (Galbraith and Miller, 1973).

As numerous works have shown that several surface active compounds do not inhibit the growth of microorganisms, the first and second hypotheses must be controlled experimentally. The fact that Gram-positive bacteria are more sensitive than Gram-negative bacteria supports the intervention of the cell membrane structures in this phenomenon. Moreover, the work of Moss *et al* (1969) on the fatty acid composition of propionibacteria leads to the separation of the species into two groups: *P freudenreichii* subsp *freudenreichii* and subsp *shermanii* on the one hand and the three other species on the other hand. This separation is in close agreement with the two groups of *Propionibacterium* species reactivity towards fatty acids found in this study.

Alterations of the FFA inhibition activity by addition of compounds

The presence of protein can counteract the inhibition of fatty acids as observed by Dubos (1947) for the serum albumin, which has high affinity binding sites for FFA (Frapin *et al*, 1993). Many other components act similarly: saponin, lecithin, charcoal, starch, cholesterol (Kodicek and Worden, 1945). The antagonistic effect of cholesterol and lecithin on an antibacterial fatty acid, as observed in this study, was already noticed by Wynne and Foster (1950), working with *Micrococcus pyogenes* var *aureus* and oleic acid. No clear interpretation of this type of results has been proposed to our knowledge.

In 1990, Somkuti and Johnson showed that *P freudenreichii* cells were able to bind the cholesterol present in the medium by polysaccharide or membrane bound proteins or by other means. These indications lead to an investigation into the role of these compounds, at the cell membrane level, with and without fatty acids in the medium.

In this study we were unable to detect any positive effect of the polyoxyethylene derivative of fatty acid monoesters of sorbitan (ester of oleic acid = Tween 80; ester of stearic acid = Tween 60 and ester of palmitic acid = Tween 40) at 100 mg l^{-1} on the growth of *P freudenreichii* subsp *shermanii* LRTL 30. This growth stimulation was frequently evidenced in the past (Dubos, 1947; Ledeoma *et al*, 1977; Cummins and Johnson, 1986). The neutralization of the antimicrobial properties of linoleic acid by Tween 80 at 30 g l^{-1} was also evidenced by Baker *et al* (1983) for lauric acid. The exact mechanism of the Tween in neutralizing the activity of this acid has not been explained in his paper.

The use of saturated fatty acids to inhibit the negative action of unsaturated fatty acids observed by Hassinen *et al* (1950) on *L bifidus* was not confirmed using our strain. If the mode of action of these acids is an interaction with the membrane layers of the cells, the affinity of the membranes for the fatty acid considered and a concentration dependence of the phenomena are probably involved. The ratios under investigation here may be out of the correct range to observe a detoxification.

Biotin is required for the growth of most strains of dairy propionibacteria (Delwich, 1949). No positive effect on growth of dairy propionibacteria by additions of biotin was observed in this study. The high level of biotin of our medium had probably hidden this potential effect. Williams *et al* (1947) supposed that biotin promotes, in some way, the formation of unsaturated fatty acids essential for the bacteria. The relationships between fatty acids and biotin in the nutrition of microorganisms is still far from being clearly defined.

Alteration of the cell membrane activity

If $\Delta\psi = 0$, at 100 μmol l^{-1} of linoleic acid, $\Delta\psi$ remained at 60% of its initial value after addition of 100 μmol l^{-1} of lauric acid. Moreover, 250 μmol l^{-1} of lauric acid induced only a very slow K^+ efflux in the cells of *P freudenreichii* subsp *shermanii*. It was then evidenced that linoleic acid is a much more active compound than lauric acid as a *Propionibacterium* cell membrane disturber.

The addition of linoleic or lauric acids in *Propionibacterium* cell suspensions results in an immediate perturbation of the permeability properties of the cytoplasmic membrane and of the bacterial energetic state: cells lose cytoplasmic potassium, they become partially or totally depolarized and their respiration is inhibited. All these changes indicate that the target of the free fatty acids is the cytoplasmic membrane.

To our knowledge, no information is available about the K^+ transport system(s) in *Propionibacterium* cells. However, we have calculated in our experiments that one cell reached by fatty acid lost potassium at a rate of 4.8 10^6 K^+ s^{-1}. This rate is many orders of magnitude higher than one expects for passive efflux through the lipid bilayer estimated at 20 ions s^{-1}/cell by Gomperts (1976) (if a permeability coefficient of 10^{-14} m s^{-1} is assumed) . This suggests that the inhibitory action of free fatty acids occurred by some global lipid bilayer disturbances.

The K^+ efflux is not the consequence of membrane depolarization since the cells incubated with the protonophore TCS at 10 μmol l^{-1}, do not show K^+ efflux (not shown). Even if the time necessary to reach the complete loss of K^+ by the cells increased when the free fatty acid concentrations decreased, the level "0" is always attained.

Free fatty acids are known to uncouple oxidative phosphorylation in mitochondria, chloroplasts and bacterial cells (Rottenberg and Hashimoto, 1986). Unsaturated long-chain free fatty acids were evidenced as far more potent uncouplers than the saturated acids (Borst *et al*, 1962). But we cannot exclude the possibility of some cofactor leakage from the cells. The protective effect of BSA against the inhibitory effect of an uncoupler, as we observed in this study, has already been observed on mitochondria since 1956 (Pullman and Racker, 1956). It has been related to the presence of an hydrophobic site in that protein, which has a great affinity for fatty acids.

As underlined by Borst *et al* (1962) the membrane disturbing activity of free fatty acids may probably be distinguished from that brought about by agents breaking up mitochondrial or bacterial envelope structure in that it is readily reversible by the subsequent addition of serum albumin. However, we have no clear interpretation of the protective action of $MgSO_4$ on *Propionibacterium* cells.

The fatty acid inhibitory effects observed on the growth and metabolism of dairy *Propionibacterium* are of great industrial importance. Indeed, the interest in propionic acid production by fermentation is increasing (Boyaval and Corre, 1987; Boyaval, 1992). This revival for biological propionic acid production is based on the highly increased productivities allowed by the technology of membrane bioreactors. As yeast extracts, which are very frequently used in that type of fermentation, contain unsaturated fatty acids (Eddy, 1958) the producer must take into account the susceptibility to these inhibitory effect in the selection of the propionic acid strains.

But the most important point is the possible involvement of these fatty acids in the inhibition of dairy *Propionibacterium* in hard cheese manufacturing. These cheeses represent more than 212 000 tons per year in France, only for Emmental (CNIEL, 1994). The highly predominant species employed in hard cheese technology is *P freudenreichii* subsp *shermanii*. If the response of most of

the strains of this species is similar to the response of the strain LRTL 30, it will probably be interesting to mix them with strains of other species in spring, when the problem of eye formation occurs. Unfortunately, we have been unable to find any information on the level of free fatty acids in the cheese curd at the end of the cold ripening, just before the development of the propionibacteria. These analyses are currently under investigation. This finding underlines once again that the lipid content of the medium has a drastic protective effect on the cells, probably by integration of the free fatty acid in the lipid globules. But scanning electron microscopic examinations of Saint Paulin cheeses suggest that the globule membrane could be disorganised by change in pH, enzymatic action and by the mechanical action of pressing (Rousseau, 1988). Membrane debris and fractured fat globule were observed. Moreover, some water was preferentially localized around the globules, leading to a kind of 'barrier' between the globules and the hydrophobic fatty acids. These observations were completed by a study on Emmental cheese where fat globules had lost their initial structure to give large masses with diverse forms (Rousseau and Le Gallo, 1990). These points underline that the fat globules, with such modifications and reduced surface have a decreased ability to trap free fatty acids in the cheese body. Our test with a curd which has not followed the complete cheese technology process (not pressed), was probably not sufficient to answer the question asked. Moreover, the alterations of fat globules, during processing and ripening of these cheeses, could overcome their trapping effect on free fatty acids as observed in milk and fresh cheese curd. Even if we have no clear biochemical data on the evolution of the lipid phase in a cheese curd from the beginning of the technology to the end of the ripening period, these results cast doubt on the major role of free fatty acids in the alteration of the eye formation during the spring. The cycle of cheese manufacturing must be completed in the initial presence of linoleic acid in order to discard this hypothesis or not.

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