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Propionibacteria in Italian hard cheeses

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Summary — Economic losses for Parmigiano Reggiano and Grana Padano, for which production is about 200 000 tons per year, can result from "late blowing" caused by propionic acid bacteria (PAB). PAB contents of 306 milk samples for Grana cheese were analyzed. The mean value was 726 cfu/ml; however, when the 8 samples with the highest PAB content (>5 000 cfu/ml) are not included, this mean decreases to 377 cfu/ml. Sixty strains of PAB were isolated, identified (65% *P freudenreichii*, 25% *P jensenii*, 5% *P thoenii* and 5% *P acidipropionici)* and tested under different incubation temperatures (30, 22, 15°C), pH of the substrates (6.0, 5.5, 5.0) and concentrations of NaCl (0.5, 1.0, 1.5, 2.0, 2.5, 3.0%). The "optimal" condition was 30°C, with considerable slowing of the growth at lower temperature. In the presence of NaCl, as the incubation temperature decreased, the number of the totally inhibited strains increased, while the total of those insensitive and those only partially inhibited remained the same up to 1.5% of NaCl and increased at higher concentrations. The strains resulted partially or strongly inhibited only at pH 5.0, while the total number of insensitive strains and those only partially inhibited increased as the temperature was lowered. An extreme adaptability of PAB even to very drastic conditions was noted: anywhere combinations such as low temperature (< 22°C), high salt concentrations (> 2%) and low pH values (<5.5) could effectively delay their development during the ripening of Grana cheese without total inhibition.

propionic acid bacteria / Grana cheese / ripening

Résumé — Bactéries propioniques dans les fromages italiens à pâte cuite. *Dans la production de Parmigiano Reggiano et de Grana Padano (environ 200 000 tonnes/an) des pertes économiques peuvent provenir du «gonflement tardif» causé par les bactéries propioniques (PAB). Trois cent six échantillons de lait destinés à la production de fromage Grana ont été analysés pour le dénombrement de PAB. La valeur moyenne était de 726 ufc/ml, mais cette valeur se réduisait à 377 ufc/ml si l'on excluait 8 échantillons avec plus de 5 000 ufc/ml. Soixante souches de PAB ont été isolées et identifiées (65%* P freudenreichii, *25%* P jensenii, *5%* P thoenii *et 5%* P acidipropionici*). On a ensuite suivi leur croissance à différentes températures d'incubation (30, 22, 15°C), pH (6,0-5,5-5,0) et en présence de concentrations croissantes de NaCl (0,5-1,0-1,5-2,0-2,5-3,0%). L'optimum de croissance était observé à 30°C, avec un considérable ralentissement aux températures plus basses. En présence de NaCl, le nombre de souches complètement inhibées augmentait lorsque la température d'incubation diminuait, tandis que l'ensemble des souches insensibles et partiellement inhibées restait stable jusqu'à 1,5% de NaCl et augmentait aux concentrations supérieures. Les souches étaient partiellement ou fortement inhibées uniquement à pH 5,0, tandis que l'ensemble des souches insen-*

sibles et partiellement inhibées augmentait lorsque la température diminuait. On a pu observer une extrême faculté d'adaptation même dans des conditions drastiques. De toutes façons les combinaisons basse température (< 22°C), concentration saline élevée (>2%) et pH bas (< 5,5) peuvent retarder la croissance des bactéries propioniques pendant l'affinage du fromage Grana, sans les inhiber complètement.

bactérie propionique / fromage Grana / affinage

INTRODUCTION

Grana Padano and Parmigiano Reggiano are 2 of the most economically important typical products of Italian agriculture, not only for the area in which they are made, but nationally.

Nearly 200 000 tons of these cheeses are produced per year (3 billion l of milk). A proportion of these are exported to almost every country in the world, but the major part are consumed by the home market. Milk is transformed into Grana cheese in about 1 000 cheesemaking dairies.

Parmigiano Reggiano and Grana Padano are hard cheeses made from raw, partially skimmed milk (2.5%). For Parmigiano Reggiano, the evening milk is poured out into small trays to rest overnight; the morning milk is used after it has stood for approximately 1 h. A portion of the naturally risen cream is skimmed off. The evening and morning milk are then poured together into a copper vat shaped like an inverted church bell. In the Grana Padano area, however, only 1 milking is used for each batch, twice a day, after it has rested for approximately 8 h; otherwise, the technology is nearly the same.

The "starter" is a natural starter, produced by fermenting the whey of the preceding batch in which the lactic flora has developed by fermentation. The rennet used is a natural extract from the stomachs of suckling calves and it is added to the milk so that the coagulation occurs within 12 to 15 min. The temperature of the curd is raised to 45°C, then the heat is increased sharply until the mass reaches a temperature of 55°C.

The "cooked" curd mass is placed in a circular wooden mold *(fascera)* for a few days. The cheese is then removed from the mold and salted by immersion in coarse salt brine for a period of 20–25 d. Ripening is natural and must continue at least until the end of the summer following the year of production. The requirements for ripened cheese may take longer than this.

Grana Padano and Parmigiano Reggiano are 2 cheeses appreciated for their pleasant organoleptic characteristics and for their high nutritional value, thus playing an important role in the dairy cheese business of Italy. The entry into the market, both Italian and foreign, of cheeses made in countries with lesser traditions of cheesemaking than Italy has made it necessary to protect the quality of its *appellation d'origine* products. It is also necessary to contain the cost of production, rationalizing the technological steps that transform the milk into cheese in order to avoid the occurrence of production defects that will affect the final cost. At present, one of the most common problems of the producers of hard cheeses is the development of what is called "late blowing," which is difficult to eliminate because it is not caused by errors in the production technology. It is related to the development of 2 gas-producing microorganisms, which are certain species of butyric acid bacteria (BAB) and propionic acid bacteria (PAB) (Politi, 1958; Bottazzi, 1959, 1963, 1966, 1983), and can cause considerable damage and notable economic losses. For this reason, several different additives have been used over the years to prevent it. Two of these additives are formalin and lysozyme, the latter being particularly effective in inhibiting the development of BAB but not PAB (Bottazzi, 1963, 1984; Bottazzi and Battistotti, 1978; Carini and Lodi, 1982; Lodi *et al,* 1983; Battistotti, 1984; Battistotti *et al,* 1984; Lodi, 1985). Formalin is a wide-spectrum bacteriostatic agent that was used for many years to inhibit all unwanted microflora in the production of Grana Padano. However, it proved to be unsatisfactory from the hygienic and health points of view (Resmini *et al,* 1980a, b; Resmini, 1987) and it became necessary to find an alternative. When formalin could no longer be used, another way to control the numbers of PAB without eliminating them was needed, since they must be present in limited numbers (Bottazzi, 1959) for the cheese to have its typical organoleptic characteristics (Cantoni *et al,* 1966; Carini *et al,* 1971).

Lack of knowledge about both the presence and the role of PAB in milk for cheese production made it necessary to quantify them in milk and to determine to which degree to vary the incubation temperature, the sodium chloride in the culture medium and the pH affecting the growth of PAB isolated from milk samples obtained from different sources. These are all factors that might be modified in standard cheesemaking technology to control the number of these microflora.

MATERIALS AND METHODS

Sampling of the milk

We analyzed 306 samples of milk obtained directly at the farm from different milkings for production of Grana in different cheese factories of the Po River plain (the Padania). The samples of milk arrived refrigerated at the laboratory not more than 12 h after milking.

Microbiological analyses

Standard bacterial count (SBC)

On Petrifilm SM (3 M), at 30°C for 72 h.

Propionic acid bacteria

On P2 agar (Carini and Casadei, 1970) at 30°C for 7 d in Gas-Pak. The stock cultures were set up in P2 broth (pH 6.7) and incubated at 30°C for 48 h.

Anaerobic sporeformer bacteria

The analyses were done by the Weinzirl method (Weinzirl, 1916) as modified by Annibaldi (1969) and the number of spores was expressed as MPN (Most Probable Number) according to Hoskins (1933).

Identification of propionic acid bacteria

Sixty isolated strains, after verification of the morphological characteristics and confirmation by the catalase test, were identified by the API SYSTEM 20A (bio-Mérieux, Marcy-l'Étoile, France) at 30°C for 48 h, under anaerobic conditions. This identification was combined with the table of sugar fermentation in *Bergey's Manual of Systematic Bacteriology* (1984).

Growth tests

The 60 identified strains were used to set up the growth tests, with inoculation of 10^5 cfu/ml PAB in P2 broth and prepared for the several tests carried out as follows:

Incubation temperature: The strains inoculated in P2 broth were incubated at 30, 22 or 15°C.

Sodium chloride concentration: Increasing amounts of NaCl were added to the P2 broth (0.5, 1.0, 1.5, 2.0, 2.5 or 3.0%) and the strains were then incubated at 30, 22 or 15°C.

Differences in pH: The strains in broth were adjusted to 6.0, 5.5 or 5.0 with lactic acid and then incubated at 30, 22 or 15°C.

For all these tests, the growth of PAB was evaluated as the absorbance at 600 nm in a UVIDEC-310/320 spectrophotometer (Tokyo, Japan Spectroscopic Co, Ltd), setting a value of 1.0 as maximal growth.

RESULTS

Propionic acid bacteria in milk

A total of 306 samples of milk for Grana cheese were analyzed between December 1991 and December 1994. The numbers of PAB ranged from a minimum of a few cfu/ml to a maximum of 27 000 cfu/ml, with a mean of 726 cfu/ml (fig 1). Only 8 samples, corresponding to 3% of the total, had more than 5 000 cfu/ml and when these were not included in the calculation of the mean, it fell sharply to 377 cfu/ml (fig 2). Samples with PAB from 1 000 to 5 000 cfu/ml made up 8% of the total; those with PAB from 500 to 1 000 cfu/ml were 10%. Thus, 79% of the samples had PAB below 500 cfu/ml (table I). The samples with the highest cfu (6 000 to 27 000) all came from the same dairy farm. These samples had SBC of about 100 000 cfu/ml, which is similar to the mean SBC in other samples with much lower numbers of PAB; therefore, there is no direct relationship between good microbiological quality and low numbers of propionic acid bacteria. In addition, determination of the numbers of clostridia spores showed that samples with large numbers of PAB contained fewer spores and *vice versa* (fig 3). Fifty-one percent of the samples had values for the 2 microbial groups at the same time lower or higher than the values considered "risky," which are 200 spores/l for BAB (Bergère *et al*, 1968) and about 400 cfu/ml for PAB (unpublished data). Forty-nine percent of the samples had 1 of the 2 groups in greater number and the other in lesser number than considered advisable. Nevertheless, there was more often too high a number of BAB (77%) than of PAB, even though the spore counts were decidely high (>10 000) in only one-tenth of the samples.

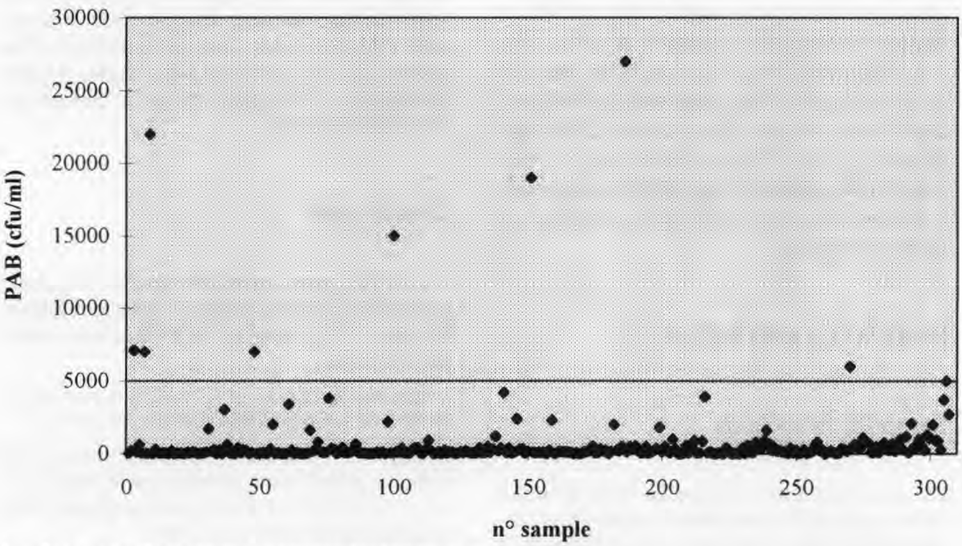

Fig 1. PAB contents in milk samples for Grana cheesemaking.
Nombre de bactéries propioniques dans les échantillons de lait destinés à la fabrication de fromage Grana.

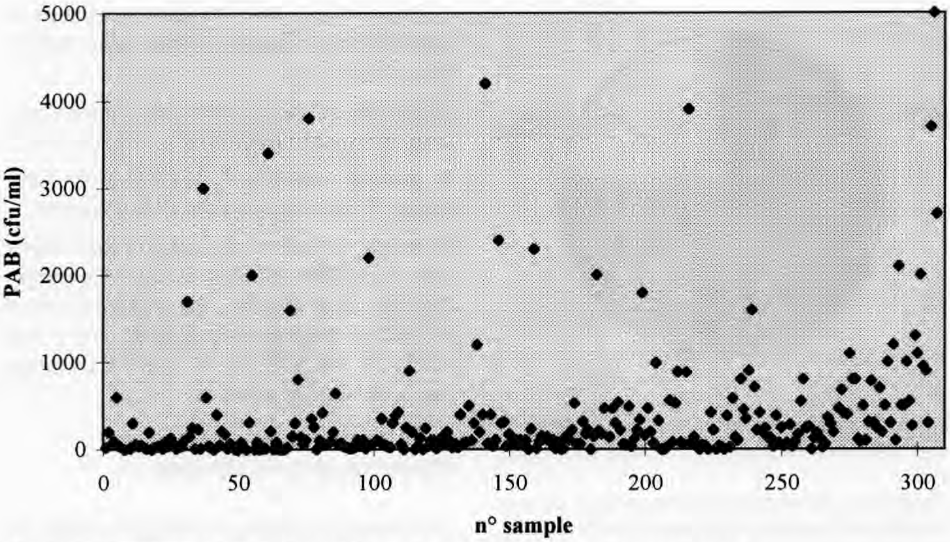

Fig 2. PAB contents (cfu/ml < 5 000) in milk samples for Grana cheesemaking.
Nombre de bactéries propioniques (ufc/ml < 5 000) dans les échantillons de lait destinés à la fabrication de fromage Grana.

Identification of the strains

A total of 60 strains were identified. They were of the following species: 34 *P freudenreichii*; 17 *P jensenii;* 6 *P thoenii;* and 3 *P acidipropionici.*

Table I. Distribution into 4 arbitrary classes of milk samples according to their PAB contents. Values expressed in %.
Distribution dans 4 classes arbitraires des échantillons de lait en fonction de leur nombre de bactéries propioniques. Valeurs exprimées en %.

PAB (cfu/ml)	*Samples (%)*
> 5 000	3
5 000–1 000	8
1 000–500	10
< 500	79

Studies of the sensitivity of the propionic acid bacteria to different factors

Having found PAB in raw milk for production of Grana cheese, we decided to study the inhibitory effects on these microorganisms of incubation temperature, concentration of sodium chloride in the medium and pH of the medium. Different combinations of these factors could be applied in cheesemaking to reduce the development of these microflora, which cannot be eliminated completely because they contribute to the development of the organoleptic characteristics of the cheese.

Influence of incubation temperature

The 60 PAB strains were incubated at 3 different temperatures: 30°C, the temperature at which their growth is optimal; 22°C, the mean temperature of the ripening room; and 15°C, the temperature in the production room, especially during the brine salting.

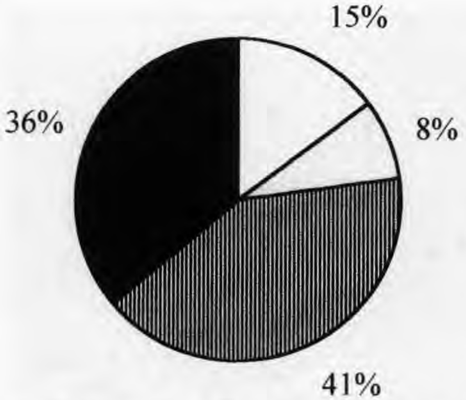

Fig 3. Distribution of milk samples according to their BAB and PAB contents. Values expressed in %.
Distribution des échantillons en fonction de leur nombre de bactéries butyriques et propioniques. Valeurs exprimées en %.
□ BAB ≤ 200 spores/l ; PAB ≤ 400 cfu/ml ; ▨ BAB ≤ 200 spores/l ; PAB > 400 cfu/ml ; ▥ BAB > 200 spores/l ; PAB ≤ 400 cfu/ml ; ■ BAB > 200 spores/l ; PAB > 400 cfu/ml.

The results confirmed that 30°C is the optimal temperature for growth, which was markedly decreased by lowering the temperature. The mean development of equivalent inocula were the same after 2–3 d at 30°C, 5–7 d at 22°C and 10–14 d at 15°C. These data show the importance of temperature in regulating the development of these microorganisms.

Inhibition tests

The inhibitory effects were evaluated as delay of the growth of the strains as compared with that of control. If we assign for each temperature the number of days needed for the control to reach growth equivalent to an absorbance value of 1.0 (considered as above the top of the logarithmic phase of growth), we can group the behaviors of the strains into the following 4 classes:

1) *insensitive to the inhibitory factor:* development time equal to or less than that of the control

2) *partially inhibited*: development time less than or equal to twice that for the control

3) *strongly inhibited*: development time longer than twice the time of the control

4) *totally inhibited*: the strains that never gave a positive reading at any time during the test: 10 d at 30°C, 30 d at 22°C, 60 d at 15°C in the presence of NaCl and 7 d at 30°C, 15 d at 22°C or 30 d at 15°C during the trials with different pH

Effects of sodium chloride

The inhibitory effects of sodium chloride on growth of 34 strains of PAB, divided into the 4 classes (fig 4), were tested. At 30°C (table II) and 1.5% NaCl in the culture medium, 44% of the strains were considerably inhibited. At 2% NaCl, 3% of the strains were totally inhibited and at 3% NaCl, 44% were totally inhibited. Some strains were insensitive to salt, but as the concentration of salt increased, 6% became partially inhibited and 47% strongly inhibited at 3% NaCl. With decreasing incubation temperature, some insensitive strains always remained so (except at 22°C and 3% NaCl) (table II), but the percentages of these were lower at 22°C and 15°C. At temperatures below 30°C, 3% became totally inhibited at 22°C and 1.5% NaCl, and also 3% became totally inhibited at 15°C and 0.5% NaCl.

It was of interest that many insensitive strains grew faster than the controls at 30°C (27%) and at 22°C (35%) when the NaCl content of the medium was 0.5%. Although there were no substantial differences in the behaviors of the 4 species of PAB as a whole, there were differences in the effects of culture conditions on the 2 species present in the highest percentages (*P freudenreichii*, 64% and *P jensenii*, 21%) (table III). As the incubation temperature was

Table II. Inhibiting effect of NaCl on PAB growth; strains repartition into 4 arbitrary classes. Values expressed in %.
Effet inhibiteur du NaCl sur la croissance des bactéries propioniques ; répartition des souches en 4 classes arbitraires. Valeurs exprimées en %.

	NaCl %	*Class*			
		1	*2*	*3*	*4*
30°C	0.5	92	8	0	0
	1.0	80	17	3	0
	1.5	56	33	11	0
	2.0	28	41	28	3
	2.5	3	47	33	17
	3.0	3	6	47	44
22°C	0.5	86	14	0	0
	1.0	69	28	3	0
	1.5	47	44	6	3
	2.0	33	47	9	11
	2.5	17	36	22	25
	3.0	0	39	28	33
15°C	0.5	86	11	0	3
	1.0	61	28	3	8
	1.5	50	39	0	11
	2.0	33	47	3	17
	2.5	17	50	8	25
	3.0	11	34	11	44

decreased and the concentration of NaCl increased, *P freudenreichii* grew even more slowly. There were 27% totally inhibited strains at 30°C and 3% salt and these increased as the temperature decreased slowly until they were inhibited at 15°C and all the salt concentration. *P jensenii* had more normal behavior, with some strains becoming totally inhibited at all 3 temperatures only when the NaCl concentration was 1.5% or higher. When compared under equivalent conditions, the sums of classes 1 and 2 and those of classes 3 and 4 for the 2 species indicated that *P freudenreichii* adapts more easily than *P jensenii* to unfavorable environmental conditions (table III).

Effects of pH

These tests were set up with 54 strains and the inhibition due to the differences in the pH of the medium at the 3 different temperatures was determined as in the initial inhibition tests. There was almost no slowing of growth of PAB at pH 6.0 and 22°C (fig 5, table IV) (96% of the strains were insensitive *versus* 85% at 30°C), which would indicate that temperatures lower than those reported in the literature might be more favorable for the growth of our isolated strains, but it must be kept in mind that at 0.5% NaCl, 35% of our strains in the first class grew more quickly than the controls. At all 3 temperatures, strains were totally inhibited only at

Table III. Inhibiting effect of NaCl on *P freudenreichii* (A) and *P jensenii* (B) growth; strains repartition into 4 arbitrary classes. Values expressed in %.
Effet inhibiteur du NaCl sur la croissance de P freudenreichii *(A) et* P jensenii *(B); répartition des souches en 4 classes arbitraires. Valeurs exprimées en %.*

	NaCl %	*Class*			
		1	*2*	*3*	*4*
A.					
30°C	0.5	91	9	0	0
	1.0	82	14	4	0
	1.5	73	23	4	0
	2.0	41	41	18	0
	2.5	4	64	23	9
	3.0	5	9	59	27
22°C	0.5	86	14	0	0
	1.0	77	23	0	0
	1.5	59	36	5	0
	2.0	50	41	5	4
	2.5	23	45	18	14
	3.0	0	55	23	22
15°C	0.5	86	9	0	5
	1.0	59	23	4	14
	1.5	50	36	0	14
	2.0	41	36	5	18
	2.5	18	55	4	23
	3.0	14	45	5	36
B.					
30°C	0.5	86	14	0	0
	1.0	100	0	0	0
	1.5	43	43	14	0
	2.0	14	43	29	14
	2.5	0	14	72	14
	3.0	0	0	29	71
22°C	0.5	86	14	0	0
	1.0	71	29	0	0
	1.5	43	43	0	14
	2.0	14	57	0	29
	2.5	14	43	14	29
	3.0	1	14	43	43
15°C	0.5	86	14	0	0
	1.0	71	29	0	0
	1.5	57	43	0	0
	2.0	29	57	0	14
	2.5	29	57	0	14
	3.0	0	14	29	57

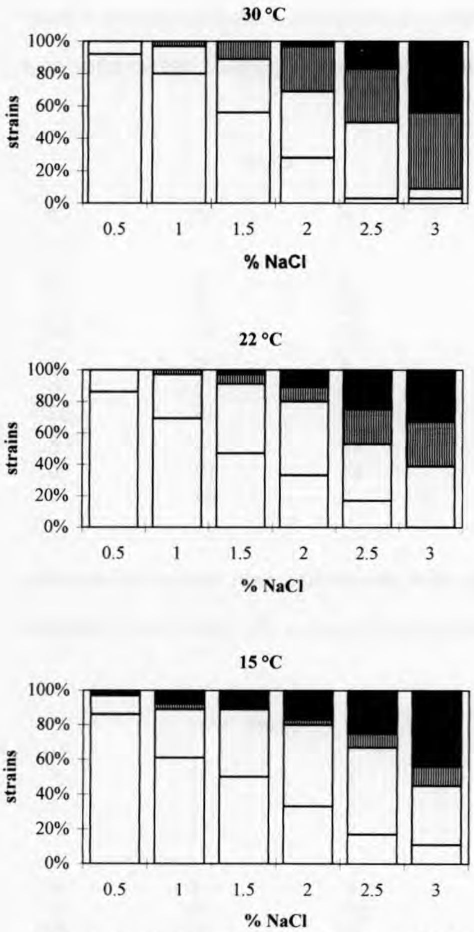

Fig 4. Inhibiting effect of NaCl on growth of PAB. Values expressed in %.

Effet inhibiteur du NaCl sur la croissance des bactéries propioniques. Valeurs exprimées en %.

□ *class 1* ; ▨ *class 2* ; ▥ *class 3* ; ■ *class 4.*

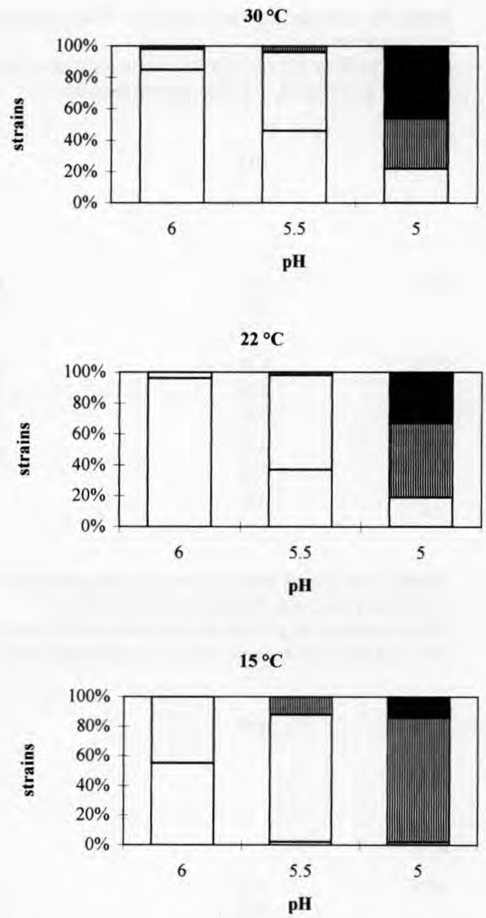

Fig 5. Inhibiting effect of pH on growth of PAB. Values expressed in %.

Effet inhibiteur du pH sur la croissance des bactéries propioniques. Valeurs exprimées en %.

□ *class 1* ; ▨ *class 2* ; ▥ *class 3* ; ■ *class 4.*

pH 5.0, and as the temperature was lowered, the percentages of these decreased from 46% at 30°C to 33% at 22°C and then 14% at 15°C. The number of strains that were strongly inhibited increased in parallel with this, so that there was an almost constant total of very disturbed strains (classes 3 + 4) (table IV), which indicates the strong adaptability of these microorganisms to unfavorable environmental conditions. Of the 2 species most represented (*P freudenreichii,* 62% and *P jensenii,* 28%), it was of interest that the first of these adapted more easily at lower temperatures to unfavorable conditions (table V), passing from 62% totally inhibited at pH 5.0 and 30°C to 17% at 15°C, while *P jenseii* was halved (from 27 to 14%). The preponderance of *P freudenreichii* strains might be due to this marked adaptability.

Table IV. Inhibiting effect of pH on PAB growth; strains repartition into 4 arbitrary classes. Values expressed in %.
Effet inhibiteur du pH sur la croissance des bactéries propioniques ; répartition des souches en 4 classes arbitraires. Valeurs exprimées en %.

	pH	*Class*			
		1	*2*	*3*	*4*
30°C	6.0	85	13	2	0
	5.5	46	50	4	0
	5.0	0	22	32	46
22°C	6.0	96	4	0	0
	5.5	37	61	2	0
	5.0	0	19	48	33
15°C	6.0	55	45	0	0
	5.5	2	86	12	0
	5.0	0	2	84	14

Table V. Inhibiting effect of pH on *P freudenreichii* (A) and *P jensenii* (B) growth; strains repartition into 4 arbitrary classes. Values expressed in %.
Effet inhibiteur du pH sur la croissance de P freudenreichii *(A) et* P jensenii *(B) ; répartition des souches en 4 classes arbitraires. Valeurs exprimées en %.*

	pH	*Class*			
		1	*2*	*3*	*4*
A.					
30°C	6.0	91	6	3	0
	5.5	62	32	6	0
	5.0	0	18	20	62
22°C	6.0	97	3	0	0
	5.5	44	56	0	0
	5.0	0	9	41	50
15°C	6.0	67	33	0	0
	5.5	3	80	17	0
	5.0	0	3	80	17
B.					
30°C	6.0	73	27	0	0
	5.5	13	87	0	0
	5.0	0	20	53	27
22°C	6.0	93	7	0	0
	5.5	27	73	0	0
	5.0	0	33	67	0
15°C	6.0	36	64	0	0
	5.5	0	93	7	0
	5.0	0	0	86	14

DISCUSSION

Our study of the propionic acid bacteria in milk for production of Grana cheese showed that their numbers vary noticeably in milk from different dairy farms. The origins of these microorganisms and possible sources of contamination must still be investigated, keeping in mind that we do not yet know whether there is a relationship between the SBC, the spore content and the propionic acid bacteria. If it is true that these last 2 bacterial groups come from the same contamination source and have similar nutritional requirements, then it is difficult to explain why no such correlations have ever been found. In addition, it is not yet clear whether there is a PAB content to consider "risky," as there is for the clostridia. As an alternative for formalin, which, until recently, had been used legally as the best antiblowing agent in the production of Grana Padano, we must now consider only technological parameters that might indicate the key points for cheesemaking: the pH of the coagulation, and the therefore more or less rapid acidification by inoculation of natural starter, the temperature of working in the vat, with the control of this and also of the concentration of salt during the brine salting and during ripening. These are the most suitable points in cheesemaking technology for intervention against the development of propionic acid bacteria, which must be held within limits. It was found that reducing the incubation temperature by 10° or so below that for optimal growth slowed down the development of PAB. In this lengthening of time for reaching the same number of cells, it is probable that the sensitivity to inhibitory factors, such as those we studied, might be reduced, or at least not increased. This observation raises the question of whether it is really essential to completely inhibit these microflora with bactericidal agents or whether it is enough to slow down their development. In Grana cheese, a not too excessive number of these bacteria produces the organoleptic characteristics that make it typical. The combination of a low temperature (22°C), a high concentration of NaCl (>2%) and a pH of the substrate of less than 5.5 effectively slowed down the development of these bacteria in the first 30–60 d of ripening, which would allow the curd to become hard enough to resist the pressure of carbon dioxide better than a soft curd.

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