Lait 80 (2000) 337–346
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**Original article**

# Indirect conductimetry in the study of propionibacteria inhibition

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(Received 22 December 1997; accepted 3 December 1999)

**Abstract** — Temperature (T), pH and NaCl concentration are the parameters that control the rate of propionic acid bacteria (PAB) growth. The indirect conductimetric technique was employed and a medium containing yeast extract, Na acetate, L (+)-cysteine chloride and Na lactate was formulated to amplify the electric signal. Sixteen PAB strains isolated from milk for Grana cheesemaking were tested in different combinations of 4 parameters (temperature, pH, NaCl and species); data were expressed as percentage of growth delay compared to standard conditions (pH 6.1, 30 °C, no NaCl added). Decreasing temperature caused the most significant growth delay (131% at 22 °C and 438% at 15 °C), while PAB growth with decreasing pH and increasing NaCl concentration was less affected in the most restrictive conditions (236% at pH 5.2 and 222% at 2.5% NaCl respectively). A slight stimulating effect was observed at 30 °C and low NaCl content (from 193% at 0% down to 187% at 0.5%). The double combinations T × pH and T × NaCl further increased delay values up to 489% (T × pH) and to 482% (T × NaCl) in the most restrictive conditions, and temperature was always the most important factor; the stimulating effect due to NaCl was amplified at 30 °C for all concentrations and at 22 °C at 0.5%. A significant difference was found in the behaviour of the 4 tested species: *P. thoeni* was the most inhibited, while *P. freudenreichii* and *P. acidipropionici* underwent the lowest growth reduction. Strictly controlled temperature (under 22 °C), curd acidification (under pH 5.4) and brine salt concentration are the cheesemaking steps identified as the critical points for containing PAB growth; the reliability of the proposed method suggests further individualization of the most suitable factor levels to contain the late blowing defect.

***Propionibacterium* / inhibition / indirect conductimetry**

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**Résumé — Application de la conductimétrie indirecte à l'étude de l'inhibition des bactéries propioniques.** La température (T), le pH et la teneur en NaCl sont 3 facteurs qui peuvent contrôler la croissance des bactéries propioniques (PAB). La conductimétrie indirecte a été utilisée pour évaluer par un plan d'expérience l'effet de ces 3 facteurs pour 16 souches de PAB représentant 4 espèces laitières. Un milieu permettant d'amplifier le signal électrique a été mis au point, contenant du lait, de l'extrait de levure, de l'acétate de sodium, du chlorhydrate de cystéine et du lactate de sodium. Le temps de détection, reflétant la vitesse de croissance, a été exprimé en pourcentage de retard par rapport à des conditions de référence (30 °C, pH 6,1, 0 % NaCl). La diminution de la température causait le plus grand retard de croissance (131 % à 22 °C et 438 % à 15 °C), alors que la diminution du pH et l'augmentation de la teneur en NaCl avaient un effet moins marqué (respectivement 236 % à pH 5,2 et 222 % à 2,5 % de NaCl). Une légère stimulation de la croissance était observée pour 0,5 % de NaCl à 30 °C (de 193 % à 0 % NaCl jusqu'à 187 % à 0,5 % NaCl). Les doubles combinaisons T × pH et T × NaCl augmentaient les retards jusqu'à 489 % (T × pH) et 482 % (T × NaCl) dans les conditions les plus restrictives, et la température était toujours le facteur le plus important. L'effet de stimulation du sel était amplifié à 30 °C pour toutes les teneurs et à 22 °C pour 0.5 %. Les 4 espèces testées montraient des différences significatives : *P. thoeni* étant la plus inhibée et *P. freudenreichii* et *P. acidipropionici* les moins sensibles. Une température contrôlée (au-dessous de 22 °C), une acidification du caillé (au-dessous du pH 5,4) et la concentration de la saumure sont les points considérés comme critiques pour limiter la croissance des PAB. La conductimétrie indirecte est une méthode utile pour améliorer les connaissances sur les conditions susceptibles de réduire le 'gonflement tardif' des fromages.

***Propionibacterium* / inhibition / conductimétrie indirecte**

## 1. INTRODUCTION

The defects due to anomalous fermentations in hard cheeses constitute a very serious problem with considerable economic effects; one of the most important defects is the 'late blowing' caused by the production of $CO_2$ by anaerobic micro-organisms such as butyric acid bacteria and propionic acid bacteria (PAB) [4, 6, 19]. Some authors have taken an interest in PAB, so that their presence has been investigated in milk and dairy products, particularly in Grana Padano and Parmigiano Reggiano cheese, during the different steps of production and ripening; these typical Italian products annually undergo serious losses because of this defect, which was kept under control for several years through the addition of formalin to the milk during the first steps of cheesemaking [2, 3, 7]. Currently this additive can no longer be used, so the problem of 'late blowing' is very topical and it is necessary to look for a rapid short-term solution. Lysozyme is considered as a suitable agent against butyric but not against propionic blowing [3, 12, 15]. However, if PAB are present in low numbers, they bring out some agreeable organoleptic characteristics of the cheese [9, 13]; so the factor(s) capable of reducing propionic blowing should not completely inhibit PAB growth.

Many factors can influence PAB growth in a medium, such as temperature, pH and NaCl concentration: a temperature lower than 20 °C, a pH near 5.0 and a concentration of NaCl around 3% (w/v**)** are conditions which may contain their growth [5]; so a number of important technological steps, such as the acidification of the curd, salt brine concentration and temperature in the storerooms have been identified as suitable for preventing 'late blowing' [8, 14].

Tests performed in our laboratory with OD measurements have demonstrated that a low temperature, high concentration of salt (> 2%) and a pH of the medium lower than

5.5 cause a remarkable slowdown of PAB development [10, 21]. However, these tests are time- and work-consuming, so the use of the conductimetric technique, already employed in the study of lactic acid bacteria [17], has recently been taken into consideration for the study of PAB.

This method, which is very sensitive to the variations in conductance of the culture medium and which is capable of furnishing data continuously for a prolonged period, requires a suitable medium to obtain amplified electric signals [22]**.** The inhibitory effect of salt and formalin determined by this technique was compared with the data resulting from OD measurements in a yeast extract–lactate medium [11]**:** the two methods allowed comparable results for formalin to be obtained, but this was not the case for NaCl, which was responsible for disturbances in the electric signal [21].

This problem was resolved using the indirect conductimetric technique, which employs the production of carbon dioxide in the medium as an index of the metabolic activity of the micro-organisms [18]; tests performed using OD measurements and indirect conductimetry provided a good correlation of data, also in the presence of different concentrations of NaCl [21].

The overall aim of this work was to find a useful medium for the indirect conductimetric study of PAB, to establish parameters and protocols for its use, and to establish whether this technique might be useful in the study of the inhibitory factors capable of reducing PAB growth.

## 2. MATERIALS AND METHODS

### 2.1. Media

#### *2.1.1. P2 broth*

P2 broth [11] consisted of the following: nutrient broth 8 g, yeast extract 5 g, Na lactate 8.4 g, distilled water 1 000 mL, pH 7.1 after sterilization.

#### *2.1.2. Lactate solution (A-solution)*

Lactate solution (A-solution) [1] was modified according to a previous study [16]: yeast extract and Na acetate 5 g, Na lactate 8.4 g, L (+)-cysteine chloride 1 g, distilled water 100 mL.

#### *2.1.3. Skim milk*

Skim milk powder was reconstituted at 10%.

### 2.2. Growth tests

#### *2.2.1. Differences in pH*

The pH of the sterilized medium was adjusted at 5.2 or 5.4 with sterile lactic acid solution.

#### *2.2.2. Sodium chloride concentration*

Increasing amounts of NaCl (0.5, 1.5, 2.5% w/v) were added to the medium before its sterilization. Trials were carried out testing 4 factors: temperature (30, 22, 15 °C); pH (6.1, 5.4, 5.2); NaCl (0, 0.5, 1.5, 2.5%) and 4 PAB species using a multifactorial experimental design.

### 2.3. Strains

Four different species of PAB for a total of 16 strains were employed: 5 *P. freudenreichii*, 4 *P. jensenii*, 4 *P. thoeni*, 3 *P. acidipropionici.* These strains, supplied by CNR Centro Studi Latte-Milano, were isolated from milk for Grana production. They were maintained by weekly transfers in P2 broth.

### 2.4. Preparation of inocula

The PAB were grown for 48 h in 10 mL of P2 broth at 30 °C. The decimal dilutions of cultures were prepared in 9 mL of sterile $^1/_4$-strength Ringer's solution, and all tests

were inoculated to obtain a final concentration of $10^5$ cfu·mL$^{-1}$, in order to reduce the test length in the less favourable conditions for PAB development.

### 2.5. $CO_2$ absorbing solution

The 0.1 N KOH, prepared in distilled water, was stored in the dark in tightly-capped bottles. Before using, the solution was degassed by 5 min vigorous boiling and allowed to cool at room temperature.

### 2.6. Preparation of indirect conductance cells

In all tests, the cells were assembled with the empty plastic insert and autoclaved at 121 °C for 15 min; 6 mL of medium were aseptically dispensed into each cell inoculated with 1% of the appropriate dilution of culture suspension, and then 500 µL of the 0.1 N KOH were carefully added to each plastic insert. The electrode caps were then closed tightly.

### 2.7. Equipment

All experiments were performed on a Malthus 2000 analyzer (Malthus Inc., Crawley, Sussex, UK). The cells containing the inoculated media were placed into a Malthus water bath incubator set at the required temperature.

The computer programme automatically supplied a recording of DT (detection time: the time necessary to reach the logarithmic phase of the microbial growth which causes a quick change in the KOH conductance); at times, in the most restrictive conditions for PAB growth, it was necessary to recalculate the DT from the graphs. The data were expressed as growth delay, determined as percentage increase compared to the control (pH 6.1, 0.0% NaCl incubated at 30 °C).

### 2.8. Statistical analysis of data

The dependent variable (growth delay increase) was analyzed with the JMP® 3.22 programme (SAS Institute Inc., Cary, NC, 1997) according to the following model with all the possible third-order interactions:

$$\begin{aligned} y_{ijklm} = {} & m + S_i + T_j + pH_k + NaCl_l \\ & + (S\cdot T)_{ij} + (S\cdot pH)_{ik} + (S\cdot NaCl)_{il} \\ & + (T\cdot pH)_{jk} + (T\cdot NaCl)_{jl} \\ & + (pH\cdot NaCl)_{kl} + (S\cdot T\cdot pH)_{ijk} \\ & + (S\cdot T\cdot NaCl)_{ijl} + (S\cdot pH\cdot NaCl)_{ikl} \\ & + (T\cdot pH\cdot NaCl)_{jkl} + e_{ijklm} \end{aligned}$$

where $y_{ijklm}$ = growth delay increase, m = overall mean, $S_i$ = fixed effect of species (i = 1 [*P. acidipropionici*], 2 [*P. freudenreichii*], 3 [*P. jensenii*], 4 [*P. thoenii*]), $T_j$ = fixed effect of temperature (j = 1 [15 °C], 2 [22 °C], 3 [30 °C]), $pH_k$ = fixed effect of pH (k = 1 [5.2], 2 [5.4], 3 [6.1]), $NaCl_l$ = fixed effect of NaCl concentration (l = 1 [0%], 2 [0.5%], 3 [1.5%], 4 [2.5%]), and $(S\cdot T)_{ij} + (S\cdot pH)_{ik} + (S\cdot NaCl)_{il} + (T\cdot pH)_{jk} + (T\cdot NaCl)_{jl} + (pH\cdot NaCl)_{kl}$ = second-order interactions; $(S\cdot T\cdot pH)_{ijk}$, $(S\cdot T\cdot NaCl)_{ijl}$, $(S\cdot pH\cdot NaCl)_{ikl}$, and $(T\cdot pH\cdot NaCl)_{jkl}$ = third-order interactions; $e_{ijklm}$ = residuals (m = 4, number of tested strains for a single species).

The significance of the levels for each factor (temperature, T, pH and NaCl) versus standard conditions (temperature = 30 °C, pH = 6.1, NaCl = 0.0%) was tested with the Dunnet test ($\alpha = 0.05$), while for species, where no controls exist, the Tukey-Kramer test was employed ($\alpha = 0.05$) which allows a simultaneous comparison of means.

## 3. RESULTS

### 3.1. Formulation of the medium

P2 broth could be used for the application of indirect conductimetry to the study of PAB growth (Fig. 1); however, we worked in order to set up a medium similar to milk,

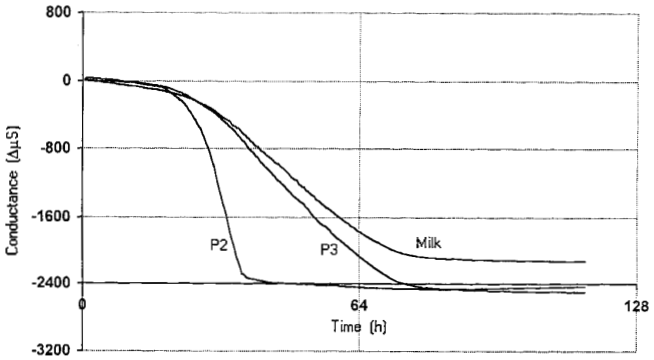


**Figure 1.** Conductimetric curves for *P. freudenreichii* in P2 broth (control), P3 broth and milk.

**Figure 1.** Courbes conductimétriques de *P. freudenreichii* sur bouillon P2 (témoin), bouillon P3 et lait.

the habitat from which the tested PAB were isolated. Skim milk in 10% solution and P2 broth enriched with 10% milk powder (P3 broth) were not as suitable media as the P2 broth used for reference (Fig. 1), so different ingredients were tested in order to obtain quicker variations in conductance.

In our laboratory, *Clostridia* are usually tested in milk to which has been added 5% (v/v) lactate solution (A-solution) with 0.4% (w/v) final concentration of Na lactate in the medium; A-solution and Na lactate were separately added to the milk at the same final concentration of Na lactate (0.4% w/v) in the medium; the milk plus A-solution formulation gave a curve comparable to that of the P2 broth, even with a delay in the DT. Adding the 4 ingredients of the A-solution to the milk one by one, only the yeast extract produced a rapid decrease in conductance; of the different combinations used, only the yeast extract tested together with cysteine chloride or Na acetate increased the response, which was further amplified by the addition of Na lactate (cyl and lya; see Fig. 2) showing a synergic interaction between all ingredients. In order to obtain a DT as similar as possible to P2 broth, increasing amounts of A-solution were added to the milk and the formulation of a

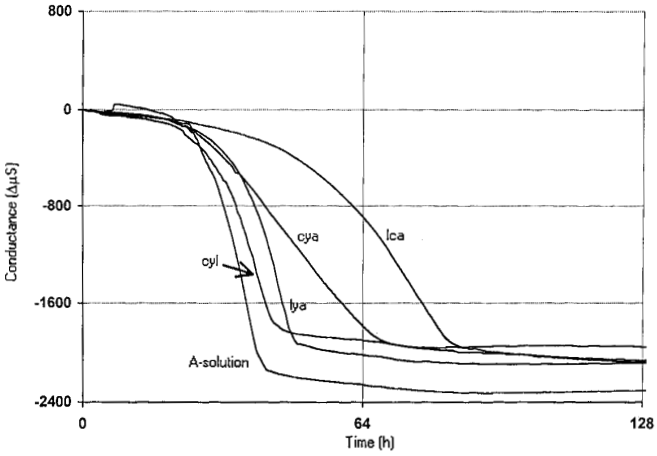


**Figure 2.** Conductimetric curves for *P. freudenreichii* in milk with A-solution added (control) and with the separate ingredients of A-solution added one by one: **y**: yeast extract, 0.25%; **l**: Na lactate, 0.4%; **c**: L (+) cysteine chloride, 0.05%; **a**: Na acetate, 0.25%.

**Figure 2.** Courbes conductimétriques de *P. freudenreichii* sur lait supplémenté avec la solution A (témoin) et avec les différents ingrédients de la solution A : **y** : extrait de levure, 0,25 % ; **l** : lactate de sodium, 0,4 % ; **c** : chlorhydrate de cystéine, 0,05 % ; **a** : acétate de sodium, 0,25 %.

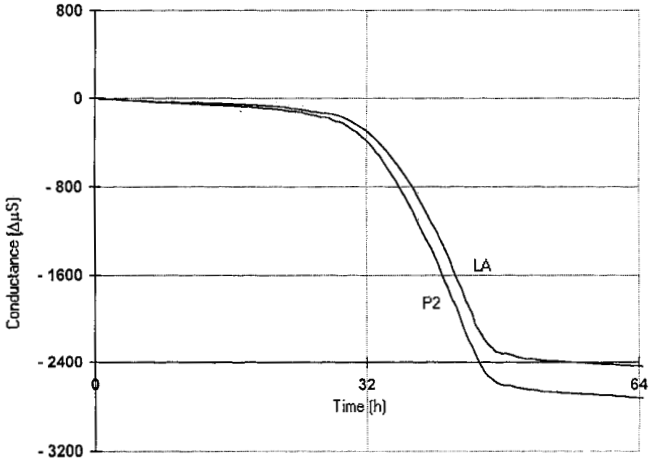


**Figure 3.** Conductimetric curves for *P. freudenreichii* in P2 broth (control) and in LA broth.

**Figure 3.** Courbes conductimétriques de *P. freudenreichii* sur bouillon P2 (témoin) et sur bouillon LA.

new medium (LA broth) obtained by adding 10% of A-solution to milk was defined (Fig. 3).

The repeatability of the method was tested by singly inoculating in LA broth 1 strain for each species chosen among those selected for the trial; for each strain 4 blocks of repetitions with a replication number within each block ranging from 4 to 8, with a total number of 95 observations were prepared at standard conditions (pH = 6.1, NaCl concentration = 0.0%, 30 °C). The coefficient of variation was 1.54%. No significant difference was found between the blocks of each strain, while the 4 species showed a significantly different behaviour ($P < 0.001$) (Tab. I). The Tukey-Kramer test indicated that the behaviour of *P. freudenreichii* and *P. jensenii* strains was significantly different from the strains of other species that had the lowest DT.

Once the formulation of the medium was determined, the work aimed at assessing through the conductimetric technique the growth of PAB in the presence of different inhibitory factors such as the pH of the medium and the presence of NaCl at different temperatures; the choice of the parameters was made with the aim of simulating cheese ripening conditions in the first months, when the NaCl concentration in the core of the cheese does not exceed 1.5–2.0% (w/w) in dry matter [20].

Data were first assessed according to the complete model on the dependent variable growth delay increase, and then all non-significant interactions were gradually

**Table I.** Repeatability of detection time evaluated in standard conditions (30 °C, pH 6.1, 0% NaCl) for 1 strain of each species. Letters a, b, c indicate the values which were found to be significantly different by the Tukey-Kramer test ($\alpha = 0.05$).

**Tableau I.** Répétabilité des valeurs du temps de détection dans les conditions de référence (30 °C, pH 6,1, NaCl 0 %) pour une souche de chaque espèce de bactérie propionique. Les lettres a, b, c indiquent les valeurs moyennes significativement différentes par le test de Tukey-Kramer.

| Species | Observation No. | Growth delay (h) Mean ± SE |
|---|---|---|
| All species | 95 | 25.30 ± 0.39 |
| *P. acidipropionici* | 29 | 22.41 ± 0.27[a] |
| *P. freudenreichii* | 27 | 30.44 ± 0.21[b] |
| *P. jensenii* | 23 | 25.25 ± 0.38[c] |
| *P. thoenii* | 16 | 21.95 ± 0.35[a] |

removed. The retained model ($R^2$ = 0.849) indicated a significant effect for all the single factors (temperature, pH, NaCl concentration and species) and of the double interactions temperature × pH, temperature × NaCl and temperature × species. The least-square means ± SE and significativeness of the effect of the single factors are reported in Table II together with the significativeness of the double interactions, while the least-square means of the latter are reported in Figure 4.

**Table II.** Least-square means (± SE), significantness of growth delay due to the single factors and significantness of the double interactions. Different letters for the levels of each factor indicate the significant differences for $\alpha = 0.05$ by the Dunnet test (small letters) and by the Tukey-Kramer test (capitals).

**Tableau II.** Moyennes ajustées (± écart-type) et signification des facteurs et des interactions sur le retard de la croissance. Les différentes lettres pour les niveaux de chaque facteur indiquent les différences significatives pour $\alpha = 0{,}05$ par le test de Dunnet (minuscule) et par le test de Tukey-Kramer (majuscule).

| Studied factors | Growth delay (h) (LSM ± SE) |
|---|---|
| Temperature (°C) | *** |
| 30 (control) | 35 ± 6.1$^{a}$ |
| 22 | 131 ± 5.0$^{b}$ |
| 15 | 438 ± 5.7$^{b}$ |
| pH | *** |
| 6.1 (control) | 172 ± 6.0$^{a}$ |
| 5.4 | 196 ± 5.6$^{a}$ |
| 5.2 | 236 ± 5.6$^{b}$ |
| NaCl concentration (%) | *** |
| 0.0 (control) | 193 ± 7.1$^{a}$ |
| 0.5 | 187 ± 6.5$^{a}$ |
| 1.5 | 204 ± 6.5$^{a}$ |
| 2.5 | 222 ± 6.5$^{a}$ |
| Species | *** |
| *P. acidipropionici* | 172 ±7.6$^{A}$ |
| *P. freudenreichii* | 178 ±5.9$^{A}$ |
| *P. jensenii* | 211 ± 6.6$^{AB}$ |
| *P. thoenii* | 243 ± 6.6$^{B}$ |
| Temperature × pH | * |
| Temperature × NaCl | ** |
| Temperature × species | *** |

Significance of the factors: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

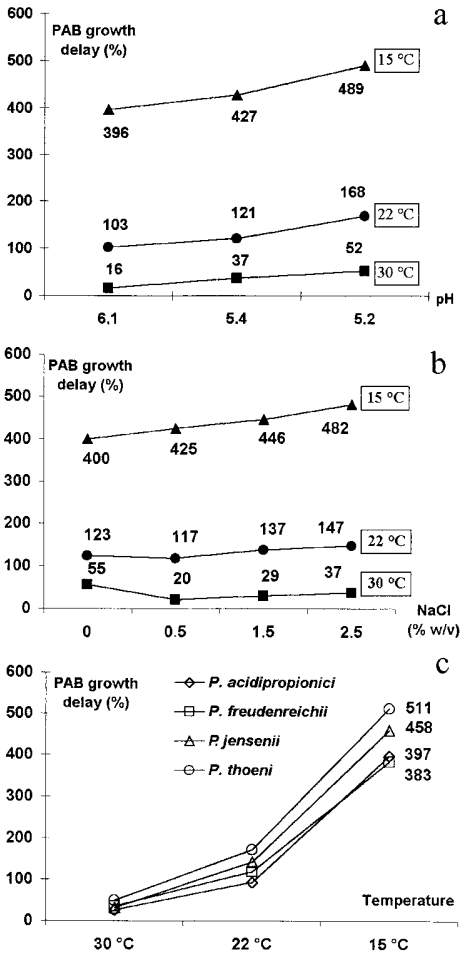


**Figure 4.** Growth delay as a function of the double interactions: **a**: temperature × pH; **b**: temperature × NaCl; **c**: temperature × species. Each value is the mean of data obtained for the 16 strains at the tested levels for each factor.

**Figure 4.** Retard de la croissance en fonction des doubles interactions : **a** : température × pH, **b** : température × NaCl ; **c** : température × espèce. Chaque valeur représente la moyenne des données des 16 souches testées aux différents niveaux de chaque facteur.

### 3.2. Effect of the single factors

#### *3.2.1. Temperature*

The analysis of variance (ANOVA) indicated that PAB growth was significantly ($P < 0.001$) affected by temperature decrease; the Dunnet test identified a significant slowdown of PAB when the temperature decreased from 30 °C (control condition) to both the lower tested temperatures (22 and 15 °C) (Tab. II).

#### *3.2.2. pH*

From the ANOVA, decreasing pH was found to cause a significant ($P < 0.001$) increase in PAB growth inhibition; the Dunnet test showed as significant only the growth difference between the lowest pH ( 5.2) and that of the control (Tab. II).

#### *3.2.3. NaCl*

Increasing NaCl concentration from 0% (control level) to 2.5% caused, as indicated by the ANOVA, a significant ($P = 0.0011$) reduction in PAB growth with a delay increase of 15% at the highest NaCl concentration. No significant growth difference was shown by the Dunnet test between the control and the other NaCl concentrations, because the lowest delay value was recorded at 0.5% (Tab. II).

#### *3.2.4. Species*

Statistical data analysis showed a significant effect ($P < 0.001$) of species, confirmed by the Tukey-Kramer test, that indicated as significantly different the behaviour of *P. thoeni*, the most inhibited species, compared to *P. freudenreichii* and *P. acidipropionici*, the least inhibited species (Tab. II).

### 3.3. Effect of the double interactions

Statistical data analysis showed a significant inhibitory effect on PAB growth for the double interactions T × pH, T × NaCl and T × species (Tab. II).

#### *3.3.1. T × pH*

The effect of the pH decrease from 6.1 to 5.4 was similar whatever the temperature, but a further decrease to pH 5.2 resulted in a more pronounced delay of growth at the lowest temperatures (15 or 22 °C) than at 30 °C (Fig. 4a). Lowering the temperature at each pH caused a higher growth reduction than for pH: when the temperature decreased from 30 to 15 °C, a growth delay increase 24, 12 and 9 times higher was recorded respectively at pH 6.1, 5.4 and 5.2, while when the pH decreased from 6.1 to 5.2, a delay only 1.2, 1.6 and 3.2 times higher was recorded respectively at 15, 22 and 30 °C.

#### *3.3.2. T × NaCl*

The effect of NaCl concentration increase from 0 to 2.5% caused a growth delay increase at 15 and 22 °C except for 0.5% at 22 °C, where a slight reduction of the delay was observed (Fig. 4b); at 30 °C, on the contrary, the highest delay value was reported at 0% with a delay decrease of 33% from the control value to the highest NaCl concentration. Lowering the temperature at each NaCl concentration caused a growth delay increase 7, 21, 15 and 13 times higher respectively at 0, 0.5, 1.5 and 2.5%, noticeably more important than that recorded at 15 and 22 °C when the NaCl concentration increased.

#### *3.3.3. T × S*

The growth of the 4 different species of PAB was significantly affected by the temperature decrease and underwent the highest delay value at 15 °C (Fig. 4c). *P. thoeni* was the most inhibited at all the temperatures, and at 15 °C showed the highest growth reduction recorded in all conditions.

## 4. DISCUSSION

The late blowing in hard cheeses due to an abnormal presence of PAB, as determined in previous research, can be limited when in the cheesemaking process, temperature, pH and NaCl concentration interact under controlled conditions. Temperature is the most important parameter and, as experienced in dairy practice, if strictly controlled under 22 °C as in the salting and ripening rooms, can strongly contain PAB growth. $CO_2$ production from PAB may damage the first steps of cheese ripening when the curd is very soft and can be reduced if the pH decrease, due to the acidifying property of the starter, reaches pH values lower than 5.4 over a time range no longer than 10 h. The confirmed inhibitory effect of NaCl, even if no discriminating concentration from the control was identified, the slight stimulatory action of 0.5% NaCl at temperatures higher than 22 °C, and the slow diffusion of salt in the curd indicate the salting step as a very crucial phase of this technology. The adaptability of *P. freudenreichii* and *P. acidipropionici* to the most inhibitory conditions and their predominant presence in dairy products (respectively 53 and 30% of the PAB) must be considered as a further weak step in this technology. The data confirmed what is experienced daily in cheesemaking, but the lack of significance of some levels and double interactions suggest the necessity for further investigation. The composition of the medium, which is so different from that of the cheese, and the lack of absolute anaerobic conditions in the tests represent the limits of the proposed method; however, its reliability and the quantity of data that can be recorded help to better define the effects of the 3 factors in reaching a solution to the topical problem of late blowing.

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