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MODELING THE GROWTH LIMIT OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* CRA7152 IN APPLE JUICE: EFFECT OF PH, BRIX, TEMPERATURE AND NISIN CONCENTRATION

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ABSTRACT

The logistic regression model was used to describe the temperature effect (25 to 50C), pH (3.5 to 5.5), soluble solids concentration (11 to 19) and nisin concentration (0 to 70 IU/mL) on the growth probability of Alicyclobacillus acidoterrestris CRA 7152 in apple juice. The model concordance was 97.3%, indicating a good fit of the observed data. The results showed that with pH 3.7 the growth probabilities were small ($<10^{-5}$) for nisin concentrations above 50 IU/mL. At 0.05 growth probability, minimum values of pH were established between 3.8 and 4.6 to inhibit the growth with the nisin synergistic action in concentration of 0 and 20 IU/mL, respectively, when the juice is incubated at 30C. The logistic model obtained can provide data to be used in quality control and at processes development.

PRACTICAL APPLICATIONS

This work applied predictive microbiology to develop a logistic model that can be used to reduce the recontamination of apple juice. The model obtained can be immediately used to food quality control, quality management, risk assessment and management, hazard analysis by control critical point, good manufacturing practices and cost reducing by allowing the prediction. The results also show that nisin plays an important role in the control of apple juice contamination by *Alicyclobacillus acidoterrestris* and it can be used in combination with pH, soluble solids concentration and temperature.

INTRODUCTION

The first report on contamination caused by an acidophilic sporeformer microorganism occurred in apple juice aseptically packed in Germany in 1982 (Cerny *et al.* 1984). That microorganism was a strict aerobic, heat resistant bacterium, able to survive to thermal treatments normally applied to fruit juice processing. From this time on, the concept of contamination in acid fruit juices has been changing and the destruction of that bacterium has become the target for the preservation of the product. Microbiological contamination is a very important factor and must be adequately controlled, including also vegetables. To ensure microbiological stability and safety in minimally processed salad vegetables, it is essential to consider the microbiology of this produce during cultivation and harvesting, and the potential changes to this microbiology through preparation, distribution and storage (Nicholl *et al.* 2004).

Alicyclobacillus acidoterrestris is a thermoacidophilic, nonpathogenic, spore forming bacterium, which was isolated and identified from forest soils as well as from several pasteurized and contaminated fruit juices, such as orange ones as shown by Parish and Goodrich (2005), apple and passion fruit (Pettipher *et al.* 1997; Splittstoesser *et al.* 1998; Walls and Chuyate 1998; Mc Knight 2003). *Alicyclobacillus* causes "off-flavor" in fruit juices. The responsible chemical compounds for this flavor were guaiacol (Splittstoesser *et al.* 1998) and phenolic compounds (Jensen and Whitfield 2003). That microorganism

grows at temperatures between 25 to 60C (Previdi *et al.* 1995.), with pHs varying from 3.0 to 5.5 (Pinhatti *et al.* 1997). Current studies on thermal inactivation have been developed by different authors (Murakami *et al.* 1998; Eiroa *et al.* 1999; Mc Knight 2003), showing that a conventional thermal pasteurization treatment, applied to fruit juices, might not be sufficient to inactivate *Alicyclobacillus* spores. Komitopoulou *et al.* (1999) and Yamazaki *et al.* (2000) studied the use of nisin (minimum inhibitory concentration method) in controlling the spore germination of *A. acidoterrestris* spores, indicating that inhibitory effect on spores depends on the temperature and pH.

Evaluating the microorganisms' responses for the medium factors become difficult when more than one variable is studied, and predictive models can assist the microbiological design of food quality and safety (Buchanan 1993). In this context, probabilistic models can be able to describe the probability that an event can happen, depending or not of the time (Elliott 1996), and to establish critical limits for the variables involved in the process. Probabilistic models based on logistics regression are useful to analyze the description of the growth/nongrowth interface, with the possibility of exploring the effects of medium conditions on microbial responses (Ratkowsky and Ross 1995; Lopez-Malo and Palou 2000). Logistics regression is a powerful tool for probabilistic microbial modeling, which having enough information about the product characteristics and storage conditions, can estimate the growth probability of microorganisms (Lopez-Malo and Palou 2000).

The logistic regression model has been used to describe the growth probability of *E. coli* in function of temperature, pH and water activity (Presser *et al.* 1998; Salter *et al.* 2000); growth probability and toxin production of *Clostridium botulinum* in fish filet, stored under modified atmospheres (Ikawa and Genigeorgis 1987), and also to determine the growth probability depending on time of incubation (shelf life) (Lopez-Malo and Palou 2000; Peña *et al.* 2004).

The purposes of this study were: (1) evaluate the growth responses of *Alicyclobacillus acidoterrestris* in apple juice with different pH values, concentrations of soluble solids and nisin subjected to different incubation temperatures; (2) model the growth/nongrowth interface using polynomial logistic regression model; and (3) predict critical values of pH, temperature and nisin concentration required to inhibit bacterial growth.

MATERIALS AND METHODS

Bacterial Strain and Culture Media

The *Alicyclobacillus acidoterrestris* CRA 7152 strain used in this research was kindly provided by Danisco Cultor.

Sporulation medium: *Alicyclobacillus acidocaldarius* medium (AAM) (Murakami *et al.* 1998): 0.05% $MnCl_24H_2O$; 1.5% agar; 1.0 g yeast extract (Oxoid, Basingstoke, UK); 0.2 g $(NH_4)_2SO_4$; 0.5 g $MgSO_47H_2O$; 0.25 g $CaCl_22H_2O$; 0.60 g KH_2PO_4, 1.0 g glucose (Oxoid) and 1000 mL water. pH was adjusted to 4.0 (Murakami *et al.* 1998).

K quantification medium: peptone 5 g (Oxoid); glucose 1 g (Oxoid); Tween-80 1 g (Synth, Diadema, Brazil); yeast extract 2.5 g (Oxoid); agar 15 g (Difco Laboratories, Detroit, Ml); 1000 mL distilled water. Medium was sterilized at 121C for 15 min and pH adjusted to 3.7 with malic acid (Vetec, São Paulo, Brazil) at 25% sterilized by filtration (Walls and Chuyate 1998).

Preparation of Spores' Suspension

Initially, the growth of viable *Alicyclobacillus acidoterrestris* cells was produced in four slant tubes containing potato dextrose agar (PDA), pH 5.6 (Oxoid), incubated at 44C for 3 days. After that, the growth result was collected from the tubes by scraping the bottle with sterile glass rods using 5 mL sterile distilled water per tube. The suspension produced was transferred to a sterile screw caped tube (25 × 200 mm), and activated at 80C for 10 min, followed by a fast cooling in ice bath until reaching room temperature. A portion (0.1 mL) of activated suspension was inoculated on each one of the 100 glass bottles (290 mL) containing 60.0 mL of solidified and slanted medium (AAM) (Yamazaki *et al.* 1996). Those 100 inoculated bottles were incubated during 9 days at 45.0C. After the 90% of field sporulation had been confirmed by microscopic observation of spore stain, spore collection was carried out (Murakami *et al.* 1998). Collected spores were washed and resuspended in sterile distilled water after three centrifugations (12,310 g/15 min at 4C), followed by alternated washing. Lysozyme at 0.3 mg/mL suspension was added after the first washing, after pH adjustment to 11 for disruption of vegetative cells (Stumbo 1973). Spore suspension was stored at 4C in sterile distilled water until its use. Spores count was performed in K medium after thermal activation for 10 min at 80C, followed by pour plating. The inverted plates were incubated at 43C for 5 days. Concentration of spores suspension was 8×10^8 spores/mL.

Determination of the Maximum Nisin Concentration

Initially, a determination test of the maximum concentration of nisin (IU/mL) to be used in the experiment was carried out. The 500, 250, 100, 80, 70, 50, 10, 5 concentrations and 0 IU nisin/mL juice were tested with pH 4, 11.5°Brix and incubation temperature at 43C in optimum bacterium growth conditions for 10 days (Komitopoulou *et al.* 1999). Plating was carried out in K medium (pH 3.7) to verify the inhibiting effect of the bacteriocin on bacteria. The inoculums concentration was approximately 2×10^5 spores/mL juice, activated at 80C for 10 min.

Experimental Design

Once the maximum nisin concentration to be used in the experiment had been determined, the effect of four factors was studied: pH (3.5, 4.0, 5.0 and 5.5), considering the minimum pH of the juice and the highest pH growth of the bacterium; temperature (25, 35, 43 and 50C), considering the minimum growth temperature up to 5C above the optimum range; nisin concentration (0, 30, 50 and 70 IU/mL), considering the result of maximum nisin concentration to be used; and soluble solids – °Brix (11, 13, 15 and 19), considering the °Brix of the simple juice (11°Brix) as an inferior limit and the 19°Brix as a superior one, as indicated by Splittstoesser *et al.* (1994), since above this value there is no growth of *A. acidoterrestris*. The SAS v.8.0 Proc Factex and Proc Optex procedures (SAS/QC Manual "user guide", SAS Institute, Cary, NC) were used to implement a consistent fractional design of 37 assays (Table 1) to be experimentally tested in duplicate. The apple juice pH was adjusted with NaOH 5N and malic acid 25% (p/v) and measured with a potentiometer (DMPH-2-Digimed). The different concentrations of the juice soluble solids were adjusted with different dilutions of sterile distilled water, added in the concentrated apple juice and measured with an ATAGO refractometer HSRO500. Before inoculation, all samples had been thermally treated at 105C for 10 min to eliminate the possible presence of competitors (Massaguer *et al.* 2002). Nisin™ was provided by Danisco Cultor and used after preparing a stock solution containing 10^4 IU/mL in 0.02 N HCl and sterilized at 121C for 15 min (Scott and Taylor 1981). The initial inoculated load was 3.5×10^2 spores/mL apple juice, activated at 80C for 10 min. The factor effects evaluation was carried out after 8 and 16 days of incubation (once the established incubation time by the determination of maximum nisin concentration was used in the optimum conditions of bacterial growth), by plating in K medium pH 3.7, followed by incubation at 43C for 5 days.

TABLE 1. GROWTH AND NO GROWTH RESPONSES OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* CRA7152 IN APPLE JUICE

X1	X2	X3	X4	R1*	R2*	R1**	R2**
5.5	70	50	11	0	0	0	0
5.5	70	43	19	0	0	0	0
5.5	50	43	13	1	1	1	1
5.5	50	35	15	0	0	1	1
5.5	30	35	13	0	1	1	1
5.5	30	25	11	0	0	0	0
5.5	0	50	19	0	0	0	0
5.5	0	25	15	1	1	1	1
3.5	70	43	15	0	0	0	0
3.5	70	35	11	0	0	0	0
3.5	50	50	13	0	0	0	0
3.5	50	35	19	0	0	0	0
3.5	30	50	11	0	0	0	0
3.5	30	43	15	0	0	0	0
3.5	0	25	19	0	0	0	0
5	70	25	15	0	0	0	0
5	70	25	13	0	0	0	0
5	50	50	15	0	0	1	1
5	50	25	19	0	0	0	0
5	30	43	19	0	0	0	0
5	30	43	11	1	1	1	1
5	0	50	13	1	1	1	1
5	0	35	11	1	1	1	1
4	70	50	19	0	0	0	0
4	70	35	13	0	0	0	0
4	50	43	11	0	0	0	0
4	50	25	11	0	0	0	0
4	30	50	15	0	0	1	1
4	30	35	19	0	0	0	0
4	30	25	13	0	0	0	0
4	0	43	15	1	1	1	1
4	0	43	13	1	1	1	1
3.5	0	35	11	0	0	0	0
4	0	35	11	0	1	1	1
5	0	43	11	1	1	1	1
5.5	70	50	19	0	0	0	0
3.5	0	25	11	0	0	0	0

* Eight days of incubation.

** Sixteen days of incubation.

X1 = pH, X2 = nisin concentration (IU/ml), X3 = temperature (C), X4 = soluble solids concentration (Brix), R1 and R2 (sample responses 1 and 2: 1 = growth, 0 = no growth).

Evaluation of Growth and Nongrowth

The assays were classified as positive for growth when the count of cells in the plates was larger than the number of activated spores inoculated at zero time (>3.5×10^2 UFC/mL); otherwise, they were classified as negative. That criterion was also used by Lopez-Malo and Palou (2000). The growth/nongrowth responses were analyzed through probabilistic modeling.

Probabilistic Model of Logistic Regression

The growth/nongrowth responses obtained in the different assays (Table 1) were adjusted using the logistic regression model, which described the growth probability of bacteria subjected to the combination of several studied factors. The logistic regression model describes the probability of *A. acidoterrestris* to grow or not, conditioned by a vector X (entrance variable such as pH, temperature, nisin concentration and solid soluble concentration – °Brix). Following, the specific logistic regression model is presented in Eq. (1) (Hosmer and Leweshow 2000),

$$P(x) = \frac{\exp\left[\sum \beta i x i\right]}{1 + \exp\left[\sum \beta i x i\right]} \quad (1)$$

where $P(x)$ it is the probability of growth or nongrowth.

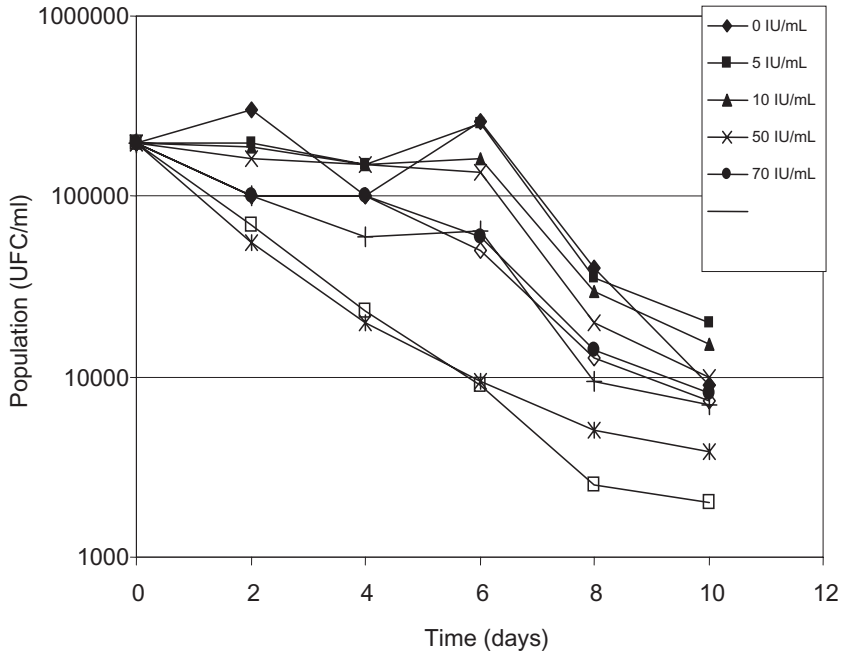

FIG. 1. NISIN INHIBITING EFFECT ON *A. ACIDOTERRESTRIS* CRA 7152 IN APPLE JUICE 11°BRIX, pH 4 AT 43C

The logit transformation of $P(x)$ is defined as:

$$Logit(P) = g(x) = Ln\left[\frac{p(x)}{1-p(x)}\right] = \sum \beta ixi \qquad (2)$$

In this research, soluble solids concentration, pH, nisin concentration, and incubation temperature were the independent variables, and the probability of *A. acidoterrestris* growing in the juice was the dependent variable. That answer was designated as "1" for growth and "0" for nongrowth under the studied conditions. Therefore, the following logit(P) model was chosen:

$$g(x) = \beta_0 + \beta_1 \mathrm{pH} + \beta_2 Ni + \beta_3 T + \beta_4 C + \beta_5 \mathrm{pH} \cdot Ni + \beta_6 \mathrm{pH} \cdot T + \beta_7 \mathrm{pH} \cdot C + \beta_8 Ni \cdot T + \beta_9 Ni \cdot C + \beta_{10} T \cdot C \qquad (3)$$

where $\beta_{0\ldots} \ldots \beta_{10}$ are the model coefficients estimated for the fitting of the experimental data with 0.95 probability; *Ni*, nisin concentration IU/mL; *T*, temperature C; and *C*, soluble solids concentration °Brix, using the SPSS 8.0 (Chicago, IL) procedure logistic regression.

After fitting the logistic regression model, critical predictions of the variables in the growth/nongrowth interface were made, with a 0.05% probability level, by substituting the logit value ($P[x]$) in the model of Eq. (1) and calculating the value of one independent variable keeping fixed the other independent variables. Also, growth probabilities were calculated using the logistic Eq. (1) for apple juice.

RESULTS AND DISCUSSION

Determination of the Maximum Nisin Concentration

Figure 1 shows that there was growth for the control (0) without nisin addition, and also with 5 IU/mL added into the juice demonstrated by the fast increase in the population initially inoculated until the sixth day of incubation. For 100, 80, 70, 50 and 10 IU/mL, a latent state of the bacterium was observed, because it can not grow, although there was not a significant reduction in the initial population. Concentrations of 250 and 500 IU/mL inhibited the growth in two logarithmic cycles at the most. However, in all cases, there was decrease in nisin effect after the eighth day of incubation, corroborated by the colony counts on the 10th day. Komitopoulou *et al.* (1999) found that 100 IU nisin/mL in apple juice was enough to inhibit the growth of *A. acidoterrestris* for 5 days of incubation at 45C. In another study of *A. acidoterrestris* survival in clarified apple juice, Yamazaki *et al.* (2000) reported that 600 IU/mL were inhibited for only 3 days of incubation at 40C; so after this period, growth took place. The differences found between the values obtained in the present study and values in the literature are caused by the strain of the microorganism and to factors that affect directly the bacteriocin activity such as pH, temperature, time of incubation and still the type of juice, which in the present study was pulpy, not clarified as in Yamazaki *et al.* (2000).

Thus 70 IU nisin/mL can be used as maximum concentration for this study.

Growth of *A. acidoterrestris* CRA7152 in Apple Juice

Growth took place in 26 treatments from 74 combinations (37 × 2 replicates) of temperature, pH, Brix and nisin concentration tested in the present study. There was no increase in the population initially inoculated in the other treatments.

Bacterial growth was inhibited in a higher concentration of soluble solids (19°Brix) and any pH, temperature and nisin (Table 1). In this condition, the bacterial viability decreases as a consequence of the low water activity. Splittstoesser *et al*. (1994) reported *A. acidoterrestris* growth inhibition when concentration of soluble solids exceeded 18.5°Brix, also corroborated by Peña and Massaguer (2006). For 0 IU nisin and 15°Brix, there was growth inhibition only at pH 3.5. This means that this type of product (pH ≤ 3.5) can be microbiologically stable for, at least, 16 days of incubation under the tested conditions. In several cases, there was growth for the longest time of incubation (16 days), but not for the shortest one (8 days), demonstrating the combined effect of factors on the lag time. A reduction in the initial inoculums count in cases of nongrowth occurred during the incubation period (data not shown). This is an important consideration since *A. acidoterrestris* viability was detected, although there was no increase in the population initially inoculated in 48 cases, indicating the ability of that microorganism to survive to tested conditions. It is also demonstrated the non-bactericidal activity of nisin but bacteriostatic, as described by Komitopoulou *et al*. (1999) and Yamazaki *et al*. (2000).

Synergistic combinations (Table 1) have also caused growth inhibition in favorable conditions of soluble solids and temperature. This is also important, because a minimum combination of factors to inhibit the bacterial growth can be found. Thus, depending on the level of the tested preservation factors, the survivors will be capable of multiplying themselves, increasing exponentially the initial number or simply surviving and keeping the initial count. Pinhatti *et al*. (1997) detected in orange juice counts of viable individuals up to 10^2 spores/mL. On the other hand, Eguchi *et al*. (2001) showed that even low initial populations, around 10^1–10^2 cells/mL, could germinate and grow in favorable conditions, making the juice susceptible to deterioration.

The time of incubation affected the activity of the bacteriocin depending on the conditions of storage temperature (Table 1), a fact that was also reported by Delves-Broughton (1990). Prolonging the time of incubation would mean therefore to obtain more positive growth cases.

TABLE 2. COEFFICIENTS OF THE SIGNIFICANT VARIABLES TO CONSTRUCT THE LOGISTCS MODEL FOR *A. ACIDOTERRESTRIS* GROWTH

Estimated variable	Coefficient	*P* value
Constant	–260.63	0.0446
pH	42.9825	0.0497
Nisin	–1.9023	0.046
Temperature	3.1839	0.0382
Brix	13.4063	0.0492
Brix*Nisin	0.1002	0.0463
Brix*Temp	–0.1688	0.0425

Probabilistic Modeling

The fitting of Eq. (3) to data in Table 1 (16 days), removing the nonsignificant terms ($p > 0.05$), resulted in the model of Eq. (4) to describe the bacterium growth probability (p(g)) as a response to the pH, temperature, nisin concentration and soluble solids factors. Table 2 shows the value and the significance of each model coefficient. All main effects and the interactions of Brix with each variable were significant.

$$P(cresc) = \frac{\begin{array}{c}\exp(-260.63 + 42.9825 * pH - 1.9023 * Ni + \\ 3.1839 * T + 13.4063 * Brix - 2.2139 * Brix * pH \\ +0.1002 * Brix * Ni - 0.1688 * Brix * T)\end{array}}{\begin{array}{c}(1 + \exp(-260.63 + 42.9825 * pH - 1.9023 * Ni + \\ 3.1839 * T + 13.4063 * Brix - 2.2139 * Brix * pH + \\ 0.1002 * Brix * Ni - 0.1688 * Brix * T))\end{array}} \quad (4)$$

The correct percentage of concordance was used as an indicative that the model is a good fit (Hosmer and Leweshow 2000). The determinations of concordance showed that 97.3% of the data were concordant with the model and only 2.7% were discordant (Table 3). In other studies, the concordance value was lower (Bolton and Frank 1999; Lopez–Malo *et al*. 2000).

Probabilistic models using logistic regression were reported for *Shigella flexneri* (Ratkowsky and Ross 1995) and *Saccharomyces cerevisiae* (Lopez–Malo *et al*. 2000), which demonstrate the flexibility in the construction of the logistic model, allowing the introduction of kinetic equations of

TABLE 3. CLASSIFICATION OF OBSERVED AND PREDICTED VALUES USING LOGISTIC MODEL FOR *A. ACIDOTERRESTRIS* CRA 7152 GROWTH IN APPLE JUICE (16 DAYS)

	Predicted response		
Response	No growth	Growth	%
Observed	0	(1)	Correct
No growth (0)	48	0	100
Growth (1)	2	24	92.31
Global			97.3

TABLE 4. GROWTH PROBABILITIES ($P = 0.05$) OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* CRA 7152 IN APPLE JUICE 11°BRIX

pH = 3.7	Nisin concentration (IU/mL)						
Temperature (C)	0	10	20	30	40	50	60
25	0.00002	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
30	0.01191	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
35	0.90179	0.00307	0.00000	0.00000	0.00000	0.00000	0.00000
40	0.99986	0.70095	0.00078	0.00000	0.00000	0.00000	0.00000
45	1.00000	0.99944	0.37433	0.00020	0.00000	0.00000	0.00000
50	1.00000	1.00000	0.99781	0.13248	0.00005	0.00000	0.00000
pH = 4.5	Nisin concentration (IU/ml)						
Temperature (C)	0	10	20	30	40	50	60
25	0.97916	0.01550	0.00001	0.00000	0.00000	0.00000	0.00000
30	0.99997	0.92305	0.00400	0.00000	0.00000	0.00000	0.00000
35	1.00000	0.99989	0.75380	0.00103	0.00000	0.00000	0.00000
40	1.00000	1.00000	0.99957	0.43869	0.00026	0.00000	0.00000
45	1.00000	1.00000	1.00000	0.99832	0.16631	0.00007	0.00000
50	1.00000	1.00000	1.00000	1.00000	0.99346	0.04845	0.00002

square root type (Ratkowsky and Ross 1995; Lanciotti *et al.* 2001) or polynomial models (Konstantinos *et al.* 2004; Peña *et al.* 2004). The present work was the first one in using this modeling tool with *A. acidoterrestris.*

By analyzing Table 2, one can see that all the variables individually and the interactions Brix*T, Brix*pH and Brix*Nisin affected the response. Therefore, the found model (Eq. 4) was used to predict growth probabilities of *A. acidoterrestris* (values between 0 and 1) for simple apple juice in two pH values, 3.7 and 4.5, at different temperatures and nisin concentrations. For pH 3.7, the growth probabilities were very small ($<10^{-5}$), with nisin concentrations above 50 IU/mL. When only 30 IU/mL is added in, low growth probabilities can be obtained ($<2.10^{-4}$) and it can withstand abuse temperatures up to 45C. It should be emphasized that these results are only applied for stated conditions with the maximum of 16 days of storage.

Increasing pH to 4.5, the growth probabilities also increase (Table 4), and 60 IU/mL become necessary to obtain probabilities of 2×10^{-5} for any tested temperature. In these conditions, 30 IU/mL would not be enough to keep the probabilities close to zero at temperatures above 40C. It is important to point out that under room temperature in the ranges of 25 to 35C, 10 to 20 IUI/mL and 30 to 40 IU/mL would be enough to keep the growth probabilities close to zero ($<10^{-5}$) in single strength juice with pH 3.7 and 4.5, respectively. Similar tables of probabilities for other values of soluble solids and pH can be made, since they are within the range of the studied factors.

Figures 2 and 3 represent the surfaces of some tested conditions, clearly showing the effect and the interaction of the studied variables on the growth probability of *A. acidoterrestris* in apple juice.

For pH 4.5 and 0 nisin (Fig. 2b), there was bacterial inhibition only in high concentrations of soluble solids. Reducing the pH to 3.7 (Fig. 2a), the number of combinations brix-temperature that inhibited the bacterial growth with 0.95 probabilities has gradually increased, occurring no growth at any value of Brix up to 30C. Above this temperature, the growth probability is close to zero only for juices around

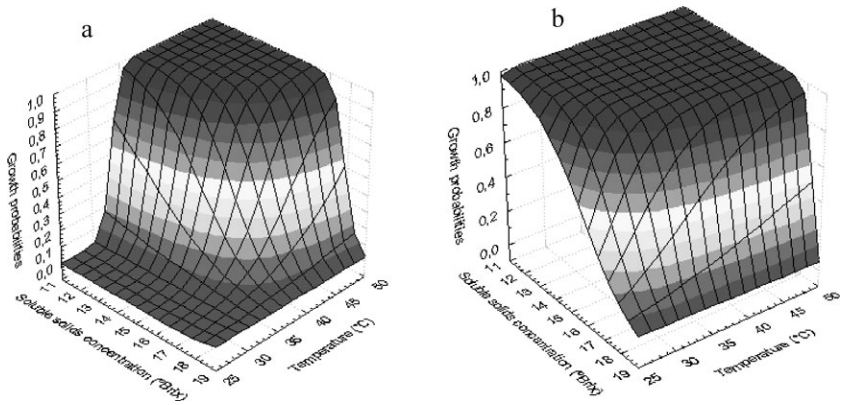

FIG. 2. GROWTH PROBABILITY OF *A. ACIDOTERRESTRIS* CRA 7152 IN APPLE JUICE WITH NISIN 0 IU/ML
(a) pH = 3.7. (b) pH = 4.5.

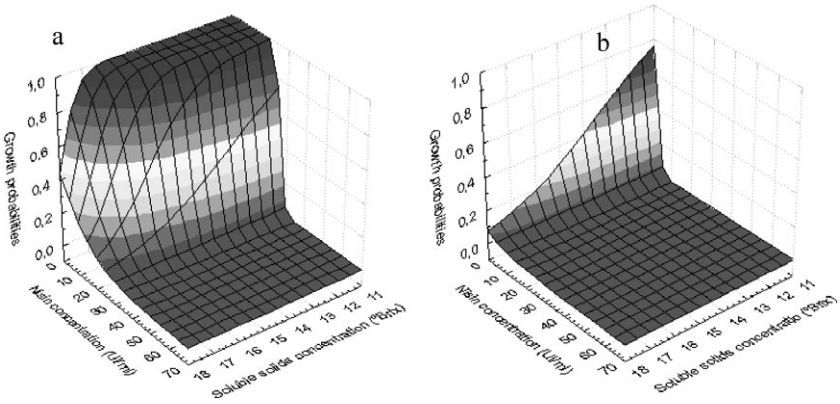

FIG. 3. GROWTH PROBABILITY OF *A. ACIDOTERRESTRIS* CRA 7152 IN APPLE JUICE WITH pH 4.0
(a) 45C. (b) 30C.

18°Brix, showing the reduced influence of water activity. This is also corroborated by Fig. 2b, even when increasing the pH to 4.5.

Figure 3 shows the growth probability as a function of Brix-nisin at pH 4.0. Even in optimum conditions of temperature (45C) (Cerny *et al.* 2000), it is possible to obtain growth probabilities close to zero only by adding in between 40 and 70 IU of nisin/mL. Figure 3a shows that, even without nisin, the model predicts growth for 18°Brix with $P = 0.4$, but this was not observed experimentally, which is important, because even with low growth probability the model would be predicting on the safe side.

Tables 5 and 6 present the critical values (from which inhibition or nongrowth could occur) predicted by the model of Eq. (4) for the different factors studied in apple juice with 11°Brix.

At 30C, the minimum pH values for growth occurrence at 0.05 probability would be 3.8 (0 IU/mL) and 4.6 (20 IU/mL) (Table 5). These critical pH values decrease as the temperature increases. It is known that the nisin activity is affected when exposed to temperatures above 20C during incubation (Delves-Broughton 1990). Temperatures between 40 and 45C are more favorable to *A. acidoterrestris* growth than between 25 and 30C (Walls and Chuyate 2000; Eguchi *et al.* 2001), being therefore necessary a reduction in pH to guarantee bacterial inhibition. As the nisin concentration increases, the minimum pH also increases. Minimum values around 4.5 can be obtained with 5, 20, 25, 30 and 40 IU/mL at incubation temperatures of 25, 30, 35, 40 and 45C, respectively, for 2 weeks of incubation.

Table 6 shows the required critical values of nisin concentration (minimum concentrations to inhibit bacterial growth) with 0.95 probabilities. These minimum values can be obtained depending on the temperature and the pH. At 35C, it is necessary 1.8, 13.4 and 22.8 IU nisin/mL juice at the pHs 3.5, 4.0 and 4.5, respectively, to avoid microbial growth. Although 250 IU/mL has been reported as required to inhibit up to two logarithmic cycles (Yamazaki *et al.* 2000), small amounts of nisin can be added in combination with other factors to cause some inhibition degree of *A. acidoterrestris* in apple juice.

Eq. (4) allows calculating other pH, T, and nisin concentration values for different values of the studied variables. These analyses should be taken into account for storage and transport, if there are signs of *Alicyclobacillus* occurrence. However, it is worth pointing out that these predictions are valid only for apple juice under the tested conditions, during the time period under study. Nevertheless, it is established that they can be used as an alternative to avoid apple juice deterioration by the development of this bacterium.

The probabilistic model is one of the most important tools for process control and even for the development of APPCC

TABLE 5. CRITICAL PH PREDICTED WITH $P = 0.05$ OF *A. ACIDOTERRESTRIS* CRA 7152 IN APPLE JUICE

	Temperature (C)				
Ni (IU/mL)	25	30	35	40	45
0	4.1	3.8	–	–	–
5	4.4	4.0	3.6	–	–
10	4.6	4.2	3.9	3.5	–
20	5.0	4.6	4.3	3.9	3.6
25	5.2	4.9	4.5	4.1	3.8
30	5.4	5.1	4.7	4.4	4.0
40	–	5.5	5.1	4.8	4.4
50	–	–	–	5.2	4.9

TABLE 6. CRITICAL NISIN CONCENTRATION FOR *A. ACIDOTERRESTRIS* CRA 7152 GROWTH AT $P = 0.05$ IN APPLE JUICE

	Temperature (C)				
pH	25	30	35	40	45
3.5	–	–	1.8	10.1	18.4
3.7	–	–	6.5	14.7	23.0
4	–	5.1	13.4	21.7	30.0
4.5	8.5	14.5	22.8	31.0	39.3
5	20.1	28.4	36.7	45.0	53.3

TABLE 7. VERIFICATION TRIALS OF RESPONSE MODEL FOR A PROBABILITY GROWTH OF *A. ACIDOTERRESTRIS* IN APPLE JUICE INCUBATED AT 30C FOR 16 DAYS

Trial no.	pH	Nisin concentration (IU/mL)	Soluble solids	Predicted[a] probabilities of growth	Observed response
1	3.7	5.0	11.5	0.00031	0[b] - 0 - 0 - 0 - 0
2	4.0	5.0	11.5	0.05541	0 - 0 - 0 - 0 - 1
3	5.0	5.0	11.5	1.00000	1 - 1 - 1 - 1 - 1
4	3.7	40.0	11.5	0.00000	0 - 0 - 0 - 0 - 0
5	4.0	40.0	11.5	0.00000	0 - 0 - 0 - 0 - 0
6	5.0	40.0	11.5	0.00001	0 - 0 - 0 - 0 - 0
7	3.7	5.0	12.5	0.00059	0 - 0 - 0 - 0 - 0
8	4.0	5.0	12.5	0.05477	1 - 0 - 0 - 0 - 0
9	5.0	5.0	12.5	0.99999	1 - 1 - 1 - 1 - 1

[a] Predicted for the Eq. (4).

[b] Responses (five sample): 1 = growth, 0 = no growth).

systems, considering that the risk determination can be in terms of probability. These models could bring benefits for food industry, since they describe conditions that can be applied to process control in order to minimize the risk of growth of pathogenic or spoiling microorganisms. Learning the limits of the growth/nongrowth interface allows the study of the physiological mechanism toward any side of the interface. In view of that, the results shown in this research have great importance as an alternative to control apple juice deterioration by *A. acidoterrestris.*

Model Evaluation

The model produced in the study was evaluated to predict the behavior of *Alicyclobacillus acidoterrestris* in apple juice by conducting 9 experiments under random combination of temperature, pH, nisin concentration and soluble solids (Table 7). All those experiments have verified that the model is valid. The results of experiments have shown random of the predictions. These experiments (Table 7) showed that the use of nisin in condition of temperature abuse can increase shelf life of apple juice and prevent of growth and spoilage by *Alicyclobacillus*.

CONCLUSIONS

The present study demonstrated that nisin plays an important role in the control of apple juice contamination by *A. acidoterrestris* and it can be used when in combination with pH, soluble solids concentration and temperature, and succeed in inhibiting microorganism development.

Predictive models can provide decisive support in different relevant areas at food industry. In many cases, models are empirical, interpreting only the response of the organism without understanding the mechanism of the response. However, when used in a proper manner, predictive probabilistic models are helpful tools for evaluating microbial responses and can help to identify potential problems for a product or process. Logistic regression is a useful tool to model the boundary between growth and nongrowth. Food development, formulation and processing based on the hurdle concept can find in probabilistic microbial modeling a practical to evaluate the effects of the combined factors.

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