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Combined Effects of Temperature, Water Activity, and pH on *Alicyclobacillus acidoterrestris* Spores

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ABSTRACT

A response surface model was developed to describe the effects of temperature (35 to 55°C), pH (3.5 to 5.5), and water activity (a_w, 0.960 to 0.992) on germination of *Alicyclobacillus acidoterrestris* spores. Germination and growth or viability loss depended, to varying extents, on the interactions among the independent variables and the complexity of the medium. In particular, maximum growth was achieved at temperatures between 35 and 42°C and at pH values from 3.5 to 4.5. The model was validated against data not used in its development. Bias factors of 0.999 and 0.817 for 2- and 7-day models, respectively, were obtained, indicating that the models were "fail safe." Results indicated that the model provided reliable predictions of growth of *A. acidoterrestris* spores.

Acidothermophilic bacilli belonging to the genus *Alicyclobacillus* have been isolated from a range of thermal environments *(1),* soils *(5),* and thermally processed commercial fruit products, including apple, grapefruit, lime, and orange juices *(3, 8, 24).*

The three validly named species of the genus *Alicyclobacillus* are *Alicyclobacillus acidocaldarius (4), Alicyclobacillus acidoterrestris (5),* and *Alicyclobacillus cycloheptanicus (6).* Moreover, Albuquerque et al. *(1)* have described and proposed an additional species: *Alicyclobacillus hesperidium* sp. nov.

All species have a pH optimum for growth of 3.5 to 4.0, a minimum pH of about 2.5, and a maximum pH of approximately 5.5 *(21, 22).* The species *A. acidoterrestris* and *A. cycloheptanicus* have optimum growth temperatures of 45 to 50°C, whereas *A. acidocaldarius* has a higher optimum growth temperature of about 60 to 65°C *(21, 22).*

Alicyclobacillus spores survive processing at high temperatures (85 to 90°C) and acidic environments *(8),* presenting a potential spoilage concern in hot-fill fruit and vegetable juices.

The only species of the genus linked to fruit juice spoilage is *A. acidoterrestris (12, 16, 20).* This microorganism has a unique cellular membrane composition containing ω-cyclohexyl fatty acids that contribute to its survival at low pH and high temperature *(10).*

Spoilage caused by this microorganism is difficult to detect. The juice appears normal or has a light sediment; often, the only evidence is an off-odor described as medicinal or antiseptic. The chemical responsible for this off-odor, identified as guaiacol, can be detected by smell in fruit juices at 2 ppb *(16).* Guaiacol was detected in orange and apple juices at about 10^5 cells per ml of *A. acidoterrestris.*

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Identification of environmental factors that can control the germination and outgrowth of *A. acidoterrestris* spores either alone or in combination could be fundamental in reducing the risk of guaiacol production and could be useful to the food processing industries.

Many researchers have investigated the environmental factors that influence the heat resistance of *A. acidoterrestris* spores *(8, 13, 15, 17, 23).* However, very little is known about the factors, or interaction of factors, that influence the germination and growth of *A. acidoterrestris* spores.

In this study, we modeled the growth of *A. acidoterrestris* from spores as a function of temperature, pH, and water activity (a_w); the individual effects and the interaction of these factors were analyzed by a quadratic response surface methodology.

MATERIALS AND METHODS

Bacterial strain. The *A. acidoterrestris* strain used in this study was isolated from spoiled pear juice.

Preparation of *A. acidoterrestris* spore suspension. The microorganism was grown on acidified malt extract agar (MEA, pH 4.5, Oxoid, Milan, Italy) at 45°C, then stored at 4°C as stock cultures. This medium was previously used for the detection of *A. acidoterrestris*; spores of this microorganism germinate and grow well on MEA adjusted to pH 4.0 *(19).*

Sporulation was achieved on acidified MEA. After incubation for 3 to 5 days at 45°C, spores were washed from the surface of the agar with cold, sterile distilled water and centrifuged at 3,000 × *g* for 15 min. The pellet was washed three times and suspended in sterile distilled water. To destroy the vegetative cells, the spore suspension was heat shocked for 10 min at 80°C. The number of spores was determined by microscopy and after plate counting on acidified MEA. Prepared spore suspensions were used immediately.

Experimental design. The composition of standard medium was 17 g/liter malt extract and 3 g/liter mycological peptone. The

TABLE 1. *Parameter combinations used in the central composite design*

Run	Temperature (°C)	pH	a_w (°Brix)
1	50	5.0	0.968 (31.0)
2	40	5.0	0.968 (31.0)
3	50	4.0	0.968 (31.0)
4	40	4.0	0.968 (31.0)
5	50	5.0	0.984 (22.0)
6	40	5.0	0.984 (22.0)
7	50	4.0	0.984 (22.0)
8	40	4.0	0.984 (22.0)
9	45	4.5	0.976 (27.7)
10	45	4.5	0.976 (27.7)
11	45	4.5	0.992 (12.5)
12	45	4.5	0.960 (38.7)
13	45	5.5	0.976 (27.7)
14	45	3.5	0.976 (27.7)
15	55	4.5	0.976 (27.7)
16	35	4.5	0.976 (27.7)
17	45	4.5	0.976 (27.7)

TABLE 2. *Best-fit equations describing the effect of independent variables on the growth or viability loss of* Alicyclobacillus acidoterrestris *spores*

Equations[a]
Two days
$\log N_t/\log N_0 = -8.6 - 0.018[T] - 0.0014[T]^2 + 10.69[a_w] - 0.15[pH] + 0.03[pH][T]$ (1)
$R = 0.77$ $F = 19.32$ $P = 0.000001$ SE = 0.076
Seven days
$\log N_t/\log N_0 = 288.28 + 4.8[pH] - 626.07[a_w] - 0.0001[T]^2 + 334.48[a_w]^2 - 0.26[pH]^2 + 2.5[pH][a_w]$ (2)
$R = 0.78$ $F = 16.35$ $P = 0.000001$ SE = 0.083

[a] [*T*], temperature (°C); [pH], pH value; [a_w], water activity; *R*, regression coefficient; *F*, *F* value; *P*, probability value; SE, standard error.

values of temperature, pH, and a_w varied according to a three-factor, five-level central composite design (CCD) *(2)*. Water activity and pH were adjusted with sucrose and citric acid, respectively. The amounts of sucrose (g/100 ml) needed to achieve the different a_w levels were: 11.76 (a_w 0.992), 25.53 (a_w 0.984), 35.29 (a_w 0.976), 47.06 (a_w 0.968), and 58.82 (a_w 0.960). The pH of the medium was adjusted after autoclaving to 3.5, 4.0, 4.5, 5.0, and 5.5 with appropriate amounts of a 1 M solution of citric acid and measured with a Crison pH meter model 2001 (Crison Instrument, Barcelona, Spain). The 17 variable combinations used are shown in Table 1; each combination was replicated twice. The °Brix values are reported in the same table. The CCD reduces the number of possible combinations to a manageable size because it uses only a fraction of the total number of factor combinations for experimentation. In statistical literature, this technique is known as confounding *(9)*.

Measurement of growth. Approximately 10^6 spores were added to 10 ml of media prepared according to the combinations of CCD. After thorough mixing, the inoculated samples were incubated at temperatures ranging from 35 to 55°C according to the CCD. Samples were analyzed at 2 and 7 days for viable counts (CFU ml^{-1}) representing a combined population of vegetative cells and spores of *A. acidoterrestris*. To determine this population, samples were serially diluted in quarter-strength Ringer's solution; 10-fold dilutions were then plated on acidified MEA (pH 4.5) and incubated for 48 h at 45°C.

The growth or viability loss of spores was calculated as

$$\log N_t/\log N_0$$

where N_0 are the original spores and N_t are the viable counts after 2 or 7 days.

Values less than 1 indicate decreased viability, whereas values more than 1 indicate growth.

Response surface modeling. The experimental data were modeled using a stepwise regression with the forward selection procedure (Statistica for Windows, Statsoft, Tulsa, Okla.). The system begins with no variable in the model and adds one variable at a time, as long as the new variable adds significance to the model. At each stage, the significance of variables is checked, and the system removes variables that become insignificant ($P \leq 0.05$).

The goodness-of-fit of the models obtained was evaluated by multiple determination coefficients (R^2) and Fisher's *F* test.

Model validation. Models were validated against data used for their development. In addition, growth of *A. acidoterrestris* spores was determined in real systems. Approximately 10^6 spores were added to 10 ml of six different commercial pasteurized acidic juices that were incubated at temperatures ranging from 35 to 50°C. For each juice, two repetitions were prepared and analyzed.

The water activity was measured with an AQUALAB Model CX2 water activity meter (Decagon Device Inc., Pullman, Wash.). The pH measurements were obtained with a Crison pH meter model 2001 (Crison Instrument) calibrated with two standard solutions buffered at pH 4.00 and 7.02.

Growth or viability loss of spores was calculated as described above.

Relative error (RE) of each prediction case was calculated with the following equation.

$$RE = [(X_p - X_o)/X_o]$$

where X_p is the predicted value and X_o is the observed value.

The median relative error of the model predictions was used as the measure of model prediction bias. The mean absolute relative error (MARE) of the model—the measure of model prediction accuracy—was calculated according to Delignette-Muller et al. *(7)*. Bias and accuracy factors were also calculated as described by Ross *(18)*.

RESULTS AND DISCUSSION

With the aim of evaluating the effects of the considered variables on the growth or viability loss of *A. acidoterrestris* spores, the ratio between the logarithm of cell loads after 2 and 7 days and the inoculum level (log CFU/ml) was calculated.

Values less than 1 indicate a viability decrease of inoculated spores, whereas values more than 1 indicate growth. The explanation of a decrease in viability is un-

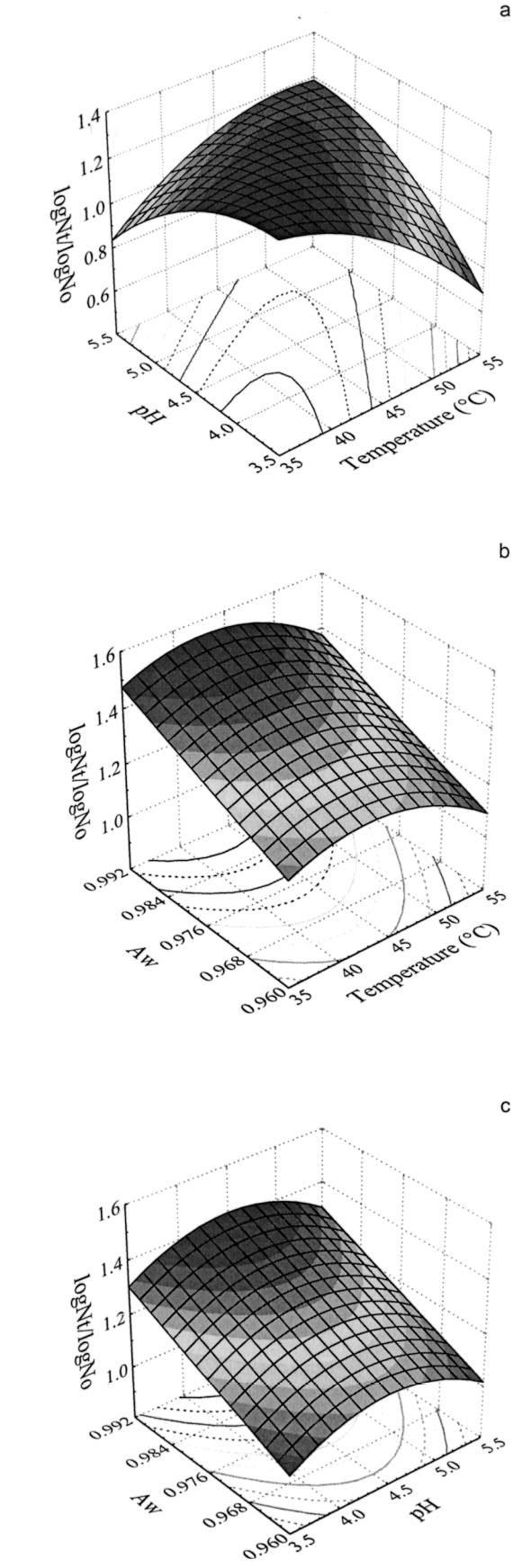

FIGURE 1. *Three-dimensional plot of the interaction of temperature × pH (a), temperature × a_w (b), and pH × a_w (c) on growth or viability loss of* Alicyclobacillus acidoterrestris *spores after 2 days.*

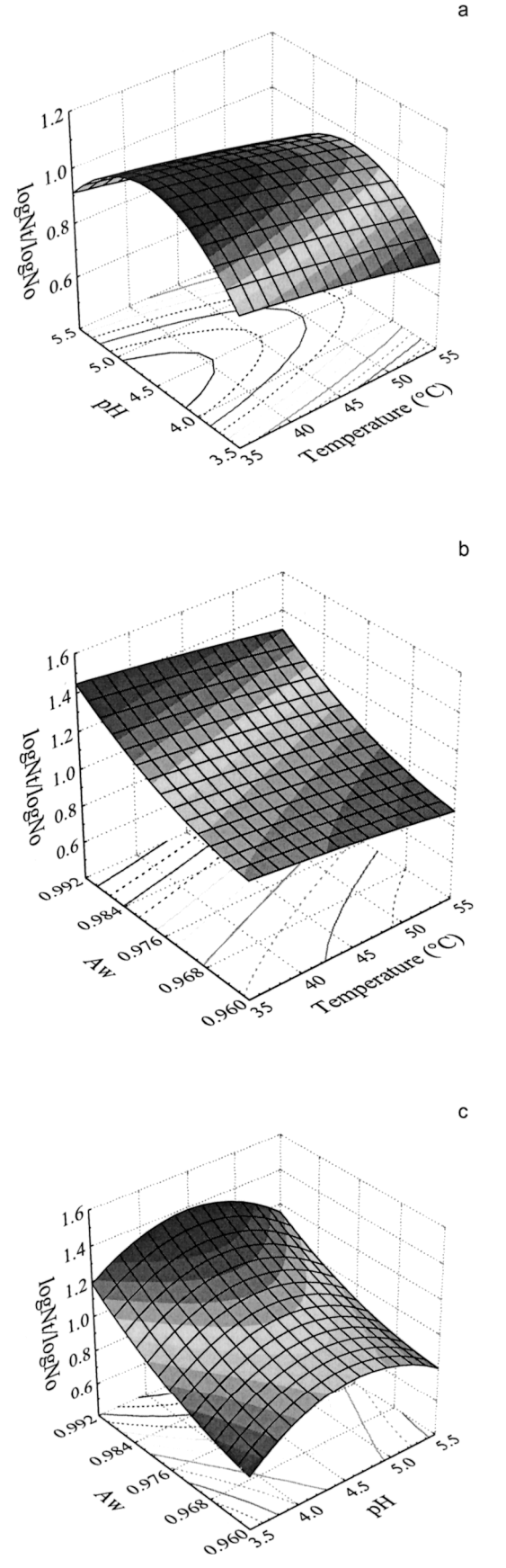

FIGURE 2. *Three-dimensional plot of the interaction of temperature × pH (a), temperature × a_w (b), and pH × a_w (c) on growth or viability loss of* Alicyclobacillus acidoterrestris *spores after 7 days.*

TABLE 3. *Growth or viability loss of* Alicyclobacillus acidoterrestris *spores inoculated in fruit juices and incubated at different temperatures*[a]

Incubation time (days)	Juice	Growth or viability loss			
		35°C	40°C	45°C	50°C
2	Orange		1.11	1.28	
		1.01	1.13	1.31	1.11
	Pineapple		1.21	1.27	0.93
		0.99	1.23	1.27	0.92
	Pear			1.32	0.94
		0.94	1.02	1.27	0.93
	Apple			1.08	0.91
		0.97	0.97	1.09	0.92
	Tomato		1.05	1.34	1.04
		1.06	1.08	1.33	1.06
	Apricot			1.26	0.84
		0.95	1.04	1.10	0.84
7	Orange		1.49		1.18
		1.15	1.56	1.67	1.17
	Pineapple	1.01		1.74	1.10
		1.03	1.66	1.66	1.12
	Pear		1.02		0.97
		1.04	1.00	1.61	0.96
	Apple	1.03	1.11		0.95
		1.09	1.06	n.d.	0.88
	Tomato	1.14	1.18	1.43	
		1.12	1.29	1.36	1.17
	Apricot	1.14	1.10		1.01
		1.08	1.03	n.d.	1.02

[a] Calculated as log N_t/log N_0, where N_0 are the original spores and N_t are the viable counts after 2 or 7 days (values <1 indicate loss of viability, whereas values >1 indicate growth). n.d., not determined.

certain; however, one could hypothesize that the inoculated spores can germinate and outgrow but vegetative cells do not survive when exposed to stress conditions such as suboptimal temperatures and a_w levels.

Such ratios were analyzed as a function of the variables of CCD. The best-fit equations describing the main, quadratic, and interactive effects of temperature, pH, and a_w on *A. acidoterrestris* growth or viability loss at 2 (equation 1) and 7 (equation 2) days are reported in Table 2. The R values indicate the adequacy of the polynomial model proposed. Moreover, the F values indicate a high level of significance ($P < 0.0001$).

According to equation 1 (Table 2), the ratio between log N_t and log N_0 after 2 days was positively affected by a_w and by the interaction of temperature with pH; it was negatively affected by temperature, both in its main and quadratic terms, and by pH in its quadratic term. As indicated by the negative sign of the quadratic term, growth increased up to a certain threshold with increasing temperature and pH, after which it decreased. Maximum growth was achieved at temperatures between 35 and 42°C and at pH values from 3.5 and 4.5.

The effects of the independent variables on growth or viability loss were better evaluated using three-dimensional plots obtained by imposing a constant value (i.e., the central point of CCD) to one variable at time.

As shown in Figure 1a through 1c, relative to the effects of $[T] \times [pH]$, $[T] \times [a_w]$, and $[pH] \times [a_w]$, respectively, maximum growth occurred at about 43°C, pH 4.5, and a_w 0.992, corresponding to about 12.5 °Brix. However, the highest viability losses were obtained at the higher temperatures and pH levels and at a_w 0.960 (38.7 °Brix).

According to equation 2 (Table 2), [log N_t/log N_0] after 7 days was significantly affected by pH and a_w individually, by all the independent variables in their quadratic terms, and by the interaction $[pH] \times [a_w]$.

As shown in Figure 2a relative to the effects of $[T] \times [pH]$, moderate growth occurred only at the lowest temperature and at pH 4.5; however, viability loss was observed in all other conditions.

Figure 2b and 2c shows the three-dimensional plots relating temperature and a_w and pH and a_w, respectively. Growth was at its maximum level at pH 4.5 and when the temperature was lowest and a_w highest (12.5 °Brix), whereas high viability losses were observed at the lowest a_w level (38.7 °Brix), even if temperature and pH were optimal.

Validating the ability of response surface models to interpolate values is a critical step in their development, but one that is rarely done in modeling experiments *(14).* The ability of our model to predict the growth or viability loss of *A. acidoterrestris* spores was validated against data that were not used in its development. Data used in model validation were collected by the same experimental protocol and with the same strain inoculated in various types of acidic foods.

TABLE 4. *Relative error of predictions for data used in development and data used in validation of predictive models for effects of temperature, pH, and a_w on growth or viability loss of* Alicyclobacillus acidoterrestris *spores*

	Cases	Relative error (%)			
		Mean absolute ± SD	Median	Minimum	Maximum
Two days					
Development	34	6.3 ± 0.03	−1.6	−12.2	13.9
Validation	38	14.4 ± 0.03	−5.8	−19.8	34.0
Seven days					
Development	34	20.0 ± 0.14	−9.3	−46.9	32.9
Validation	38	25.7 ± 0.22	−18.0	−69.5	11.1

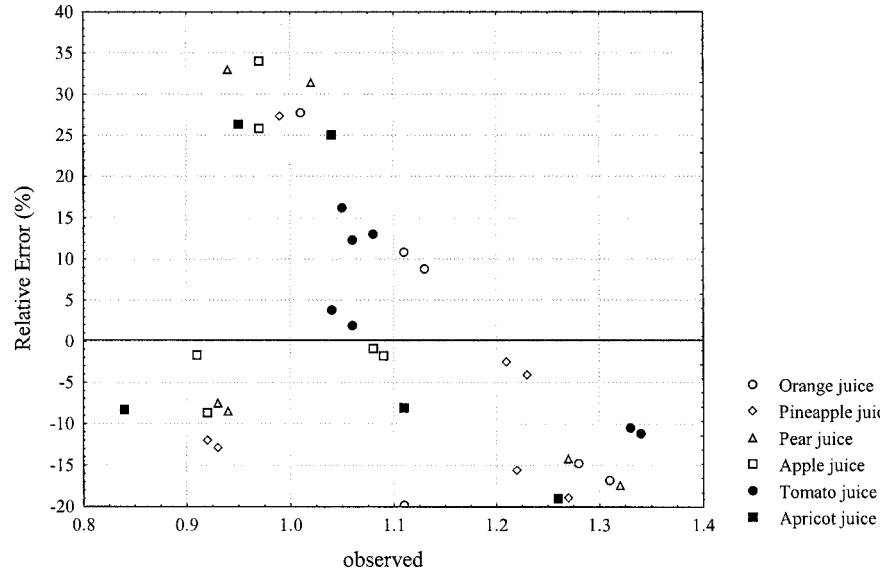

FIGURE 3. *Scatter plot of relative error (RE) of model predictions for growth or viability loss measured in different fruit juices after 2 days.*

Growth or viability loss of *A. acidoterrestris* spores after 2 and 7 days were determined in orange (six samples in triplicate), pineapple (seven samples in triplicate), pear (six samples in triplicate), apple (six samples in triplicate), and apricot (six samples in triplicate) juices, as well as in tomato sauce (seven samples in triplicate). The results obtained in the different juices at the five tested temperatures are reported in Table 3. It is possible to observe that after 2 days, the growth of *A. acidoterrestris* spores was greatly affected by temperature; in fact, only at 45°C can all juices support growth. At the other temperatures, the growth or viability loss depend on the type of juice; only orange juices (pH 3.78, 11.2 °Brix) and tomato sauce (pH 4.45, 7.0 °Brix) support growth at all tested temperatures. These products were characterized by the highest pH values and the lowest soluble solids (Brix) concentrations.

After 7 days, growth was observed in almost all conditions; a viability loss of inoculated spores was observed only in pear (pH 3.61, 11.0 °Brix) and apple (pH 3.64, 11.5 °Brix) juices incubated at 50°C. The inhibitory effect of these juices suggested that factors other than pH and a_w might affect the viability of the *A. acidoterrestris* cells.

These data were also used to calculate the relative error; the RE of each prediction case was used to calculate the MARE, a measure of prediction accuracy, and median relative errors, a measure of prediction bias (Table 4). For both models, the MAREs were relatively low. Moreover, the MAREs and median relative errors were similar for data used in model development and data used in model validation. Overall, prediction accuracy and prediction bias were better for the 2-day model than for the 7-day model. Oscar *(14)* calculated MARE for 16 predictive models representing 823 prediction cases and found that MARE ranged from 18.5 to 74.8%. In this study, MAREs were 14.4% in 2-day models and 25.7% in 7-day models; thus, compared with models developed in other laboratories, the current model had better prediction accuracy in almost all cases.

The bias and accuracy factors were also calculated as described by Ross *(18).* Bias factors of 0.999 and 0.817 for

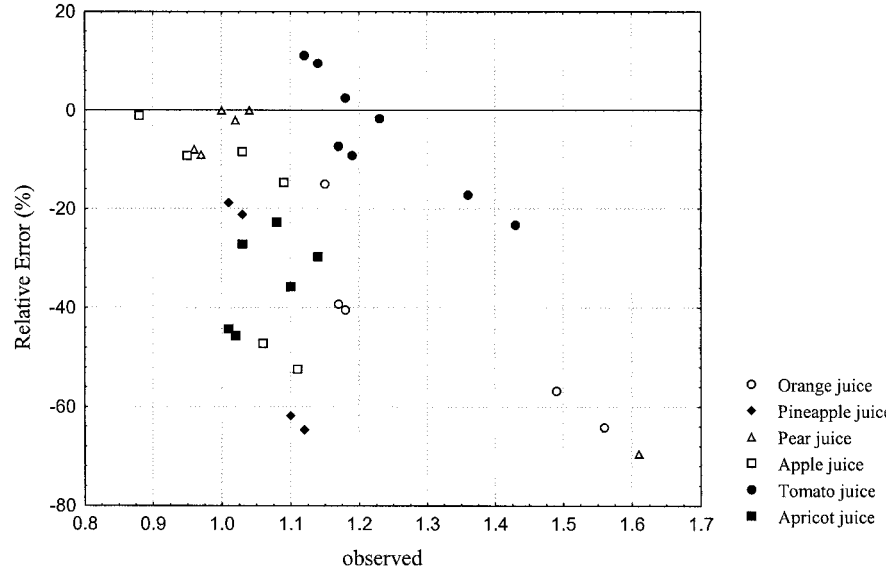

FIGURE 4. *Scatter plot of relative error (RE) of model predictions for growth or viability loss measured in different fruit juices after 7 days.*

2- and 7-day models, respectively, were obtained in this study, indicating that the models were "fail safe" *(18).* These bias factors also indicated that, on average, the models predicted growth or viability loss values at 99.9 and 81.7% of those actually observed. Accuracy factors of 1.15 and 1.24 for 2- and 7-day models, respectively, demonstrated very little degree of deviation between model prediction and experimentally observed values.

A scatter plot of RE for predictions after 2 days (Fig. 3) showed, in general, a random distribution of RE around 0 and, thus, a lack of systematic prediction bias. However, the results were strongly dependent on the products and temperature.

In contrast, a scatter plot of RE for prediction after 7 days (Fig. 4) showed a systematic bias; in fact, the values measured were generally lower than the predicted ones. However, because a model is generally regarded as safe when it predicts faster growth than is observed *(11),* the model can be regarded as safe.

Taking into account the differences observed between the *A. acidoterrestris* strains with regard to spore growth and thermal inactivation *(19),* the evaluation of a greater number of strains is required to confirm these findings. Moreover, in order to establish the influence of some fruit compounds, such as phenols, on germination and growth, more investigations are necessary. However, this study has provided information useful in determining the probability of growth of *A. acidoterrestris* under different environmental conditions.

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