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Development and validation of predictive models for the effect of storage temperature and pH on the growth boundaries and kinetics of *Alicyclobacillus acidoterrestris* ATCC 49025 in fruit drinks

Myrsini Kakagianni [a], Kelly Kalantzi [b], Evangelos Beletsiotis [b], Dimitrios Ghikas [b], Alexandra Lianou [c], Konstantinos P. Koutsoumanis [a, *]

[a] Laboratory of Food Microbiology and Hygiene, Department of Food Science and Technology, School of Agriculture, Faculty of Agriculture, Forestry and Natural Environment, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece
[b] Department of Molecular Microbiology, Quality Assurance Division, Delta Foods S.A., 23rd km National Road Athens-Lamia, 145 65 Agios Stefanos, Greece
[c] Laboratory of Microbiology and Biotechnology of Foods, Department of Food Science and Human Nutrition, School of Food, Biotechnology and Development, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece

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ABSTRACT

This study was undertaken to provide quantitative tools for predicting the behavior of the spoilage bacterium *Alicyclobacillus acidoterrestris* ATCC 49025 in fruit drinks. In the first part of the study, a growth/no growth interface model was developed, predicting the probability of growth as a function of temperature and pH. For this purpose, the growth ability of *A. acidoterrestris* was studied at different combinations of temperature (15–45 °C) and pH (2.02–5.05). The minimum pH and temperature where growth was observed was 2.52 (at 35 and 45 °C) and 25 °C (at pH ≥ 3.32), respectively. Then a logistic polynomial regression model was fitted to the binary data (0: no growth, 1: growth) and, based on the concordance index (98.8%) and the Hosmer-Lemeshow statistic (6.226, $P = 0.622$), a satisfactory goodness of fit was demonstrated. In the second part of the study, the effects of temperature (25–55 °C) and pH (3.03–5.53) on *A. acidoterrestris* growth rate were investigated and quantitatively described using the cardinal temperature model with inflection and the cardinal pH model, respectively. The estimated values for the cardinal parameters T_{min}, T_{max}, T_{opt} and pH_{min}, pH_{max}, pH_{opt} were 18.11, 55.68, 48.60 °C and 2.93, 5.90, 4.22, respectively. The developed models were validated against growth data of *A. acidoterrestris* obtained in eight commercial pasteurized fruit drinks. The validation results showed a good performance of both models. In all cases where the growth/no growth interface model predicted a probability lower than 0.5, *A. acidoterrestris* was, indeed, not able to grow in the tested fruit drinks; similarly, when the model predicted a probability above 0.9, growth was observed in all cases. A good agreement was also observed between growth predicted by the kinetic model and the observed kinetics of *A. acidoterrestris* in fruit drinks at both static and dynamic temperature conditions.

1. Introduction

For many years, heat processed fruit drinks were considered as microbiologically stable foods mainly due to their low pH (<4.0). During the 80's however, *Alicyclobacillus acidoterrestris* was identified as the causative agent of a large spoilage incident of apple juice in Germany (Cerny et al., 1984). Since then, this spore-forming bacterium has been recognized as a major quality problem by manufacturers and processors in the fruit industry (Huang et al., 2015; Steyn et al., 2011; Vieira et al., 2002; Wang et al., 2014).

The main characteristics of *Alicyclobacillus* spp. are the heat resistance of its spores and their ability to germinate and outgrow in acidic environments. After spore germination and outgrowth, the metabolically active cells can multiply up to critical cell concentrations and produce spoilage taint compounds leading to organoleptic rejection of the products with consequent large economic and credibility losses for the food industry (Gobbi et al., 2010). Undesirable effects on the sensory attributes of fruit juices and drinks are mainly attributed to the production of the metabolic

* Corresponding author.
E-mail address: kkoutsou@agro.auth.gr (K.P. Koutsoumanis).

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product guaiacol which causes "smoky, medicinal, phenolic, antiseptic disinfectant" off-flavors and/or off-odors (Bahçeci et al., 2005; Bevilacqua et al., 2009; Gocmen et al., 2005; Jensen, 2000), with normal or light sediment (Gocmen et al., 2005; Walker and Phillips, 2005). The lower limit of guaiacol detection in fruit juices by a trained sensory panel is 2 μg/l (2 ppb) while detectable off-odors in fruit juices and drinks are generally reported when the levels of *A. acidoterrestris* reach about 10^4–10^5 CFU/ml (Bahçeci et al., 2005; Sinigaglia et al., 2003).

Due to its high spoilage potential, *A. acidoterrestris* has been suggested as a possible target microbe in the design of pasteurization processes for acidic products such as fruit drinks (Vieira et al., 2002). However, standard pasteurization processes applied in the case of fruit drinks are not effective against *Alicyclobacillus* spores, while processing at higher temperatures is not feasible due to the negative effect on the quality of these products (Palop et al., 2000). As a result, control of *Alicyclobacillus* growth during distribution and storage is a key factor for the efficient risk management of fruit drinks' spoilage.

The fruit drink pH and the temperature during storage and distribution are the most important parameters affecting the growth of *A. acidoterrestris*. Research data have provided evidence that germination, outgrowth and subsequent vegetative growth of *A. acidoterrestris* spores would not be expected to occur when pasteurized fruit products of naturally low pH (<4.0) are stored below 20 °C (Bahçeci and Acar, 2007; Bahçeci et al., 2005; Spinelli et al., 2009). However, the conditions prevailing in the supply chain of pasteurized fruit drinks are out of the manufacturers' direct control and often deviate from specifications (Bahçeci et al., 2005; Heyndrickx, 2011), especially during the warmer months or in tropical and semitropical regions (Roig-Sagues et al., 2015). Thus, the estimation of the risk of spoilage constitutes a major target of quality managers, especially for products that are going to be distributed in hot climate countries. For assessing the risk of fruit drink spoilage caused by *A. acidoterrestris*, a growth model is required that is able to predict the microbial behavior during distribution and storage. However, within the domain of predictive microbiology literature, models for *A. acidoterrestris* growth kinetics are not available.

The objective of the present study was the development of predictive mathematical models for the description of the effects of temperature and pH on the growth of *A. acidoterrestris*, and the evaluation of their performance in predicting growth in fruit drinks under isothermal and non-isothermal conditions simulating transportation, distribution and storage of the products before delivery to the consumer. Such validated models could be used for an effective risk management of fruit drinks' spoilage.

2. Materials and methods

2.1. Bacterial strain

The type strain *A. acidoterrestris* ATCC 49025 was used for all experiments in the present study. The stock culture of the strain was stored frozen (−70 °C) onto Microbank™ porous beads (Pro-Lab Diagnostics, Ontario, Canada). The working culture was stored refrigerated (5 °C) on K Agar (2.5 g/l yeast extract; 5.0 g/l peptone; 1.0 g/l glucose; 1.0 g/l tween 80; 15 g/l agar) plates (IFU, 2007; Walls and Chuyate, 2000), and was renewed biweekly. After sterilization, the pH of the medium (K Agar) was adjusted to 4.0 with filtered 25% (w/v) citric acid (Merck, Darmstadt, Germany) using a digital pH meter with an epoxy refillable pH probe (Orion 3-Star pH Benchtop; Thermo Electron Corporation, Beverly, MA, USA). The microorganism was activated by transferring a loopful from the K Agar plates into 10 ml of K broth (2.5 g/l yeast extract; 5 g/l peptone; 1 g/l glucose; 1 g/l tween 80), adjusted to pH = 4.0 with filtered 25% (w/v) citric acid, and incubating at 45 °C for 48 h. The 48-h culture of the strain was then heat shocked at 80 °C for 10 min (IFU, 2007; Murray et al., 2007; Walls and Chuyate, 2000). The heat shock treatment was applied to *A. acidoterrestris* cultures in order to eliminate any vegetative cells and obtain uniform activation and germination of dormant endospores (Goto et al., 2008). Then, the heat shocked cultures were centrifuged (6000 rpm for 20 min) in a refrigerated centrifuge (4 °C) (model PK120R, ThermoElectron Corporation, Waltham, MA). The pellet was resuspended in 5 ml of quarter-strength Ringer's solution (Lab M, Limited, Lancashire, UK) and used for inoculation. The initial concentration of the inoculum was determined by surface plating on K Agar.

2.2. Development of the growth/no growth interface model

2.2.1. Experimental design

K broth was used as the basal medium for all experiments, while all experiments were performed using spores of *A. acidoterrestris* ATCC 49025 obtained as described in section 2.1. The growth ability of the spoilage microorganism was tested at different combinations of temperature (15, 18, 20, 25, 30, 35, 40 and 45 °C) and pH (2.02, 2.31, 2.52, 2.72, 3.05, 3.32, 3.60, 3.87, 4.22, 4.62 and 5.05), with five replicates for each combination. These values were selected based on pH measurements of nine different industrial fruit drinks used during this study and the biokinetic range of the microorganism's growth. For all conditions, the pH of the medium was adjusted to the appropriate values with filtered 25% (w/v) citric acid, and was measured both before and after autoclaving (prior to inoculation) using a digital pH meter with an epoxy refillable pH probe. The abovementioned pH values were the ones measured after autoclaving and used for the purpose of model development.

Portions of 180 μl of the modified K broth (K broth with modified pH) for each treatment were pipetted into wells of 100-well microtiter plates and 20 μl of the appropriate dilution of the inoculum were added to each well, with the initial inoculation spore level being approximately 10^4 CFU/well. In order to verify the exact inoculum density, immediately after inoculation 100 μl from each of the five wells were surface plated on K Agar (pH = 4.0) and colonies were counted after incubation of the plates at 45 °C for 48 h. The microtiter plates were then sealed with Parafilm (Parafilm 'M'; American National Can, Greenwich, CT, USA) to avoid evaporation, and were stored in high-precision (±0.2 °C) programmable incubators (model MIR 153, Sanyo Electric Co., Ora-Gun, Gunma, Japan) for 35 days. The temperature during storage of non-inoculated microplates was recorded using electronic temperature-monitoring devices (Cox Tracer data logger; Cox Technologies, Belmont, NC, USA).

2.2.2. Assessment of growth

During the 35-day storage, the microtiter plate wells were measured for growth on a weekly basis by recording the optical density (OD) of the medium using the automated turbidimetric system Bioscreen C (Oy Growth Curves Ab Ltd., Raisio, Finland) set to read at the wideband filter (420–580 nm). Prior to each measurement, the microtiter plates were shaken for 15 s. Five wells containing 200 μl of sterile K broth (pH = 6.73) served as negative controls. In order to define growth, the OD of a well was compared to the OD_{zero} which is the average of OD values recorded in all 100 wells at time-zero. A given well (corresponding to a certain temperature-pH combination) was considered as positive for growth, if the difference between its OD and the OD_{zero} was three times higher than the standard deviation of the OD_{zero} (Daelman et al., 2013). Data were processed using Microsoft® Excel (Microsoft Corp., Redmond, WA, USA).