Alicyclobacillus acidoterrestris in pasteurized exotic Brazilian fruit juices: Isolation, genotypic characterization and heat resistance

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A B S T R A C T
In this study, the population of Alicyclobacillus spp. was estimated in pasteurized exotic Brazilian fruit juices using the most probable number (MPN) technique followed by biochemical tests. Pasteurized passion fruit (n = 57) and pineapple (n = 50) juices were taken directly from Brazilian manufacturers. While Alicyclobacillus spp. was isolated from passion fruit juice, the microorganism was not found in any pineapple juice samples. A higher incidence of Alicyclobacillus was observed in samples taken in June and July (dry months in Brazil) in comparison to the other months (March, April, May and August), and the highest Alicyclobacillus counts were recovered from these samples (>23 MPN/100 mL). Sixteen (n = 16) Alicyclobacillus strains were typed using the randomly amplified polymorphic DNA method (RAPD-PCR). RAPD-PCR revealed great genetic similarity between the passion fruit juice strains and Alicyclobacillus acidoterrestris DSM 2498. The heat resistance of three isolates was determined, and the mean D95 (1.7 min) and z (7.6 °C) values in the passion fruit juice were not significantly different (p > 0.05) from those obtained for the DSM 2498 strain (D95 = 1.5 min and z = 7.1 °C). This is the first report on the isolation of A. acidoterrestris from exotic fruit juices such as passion fruit juice. It is worth pointing out the importance of applying good agricultural practices in the field and applying controls for the fruit selection and washing steps, as well as controlling the time/temperature conditions for pasteurization so as to reduce the incidence and chances of A. acidoterrestris spoilage in these juices.

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1. Introduction

Since its first association with the spoilage of apple juice in the early 80's, Alicyclobacillus spp. has been posed as a challenge for the microbiological stability of fruit juices worldwide (Tribst et al., 2009). Concern with Alicyclobacillus is related to the high heat (Bahçeci and Acar, 2007; Spinelli et al., 2010) and chemical resistance (Friedrich et al., 2009) of its spores. These characteristics provide this microorganism with the ability to survive the main lethal steps during fruit juice processing, i.e., fruit sanitization and juice pasteurization. The acidothermophilic behavior (Cerny et al., 1984; Wisotzkey et al., 1992) provides the Alicyclobacillus spp. spores with the ability to germinate in acidic environments like that of fruit juices and to grow at storage temperatures.

The Alicyclobacillus genus has currently been updated and there are at least 15 species (Tribst et al., 2009). Despite this, the spoilage of fruit juices/beverages has been associated with only four species: Alicyclobacillus acidoterrestris, Alicyclobacillus pomorum, Alicyclobacillus herbarius and A. acidiphilus (Cerny et al., 1984; Matsubara et al., 2002; Goto et al., 2003, 2007). However, due to the number of spoilage episodes and incidence, A. acidoterrestris is recognized as the most important species. The spoilage caused by A. acidoterrestris is characterized by a phenolic off-flavor associated with the presence of guaiacol, 2-6 dibromophenol and 2-6 di-chlorophenol (Chang and Kang, 2004).

On a worldwide basis, the incidence of Alicyclobacillus spp. has been reported mainly in apple and orange juices (Pettipher et al., 1997; Börlinghaus and Engel, 1997; Eiroa et al., 1999; Funes-Huacca et al., 2004, Luo et al., 2004; Groenewald et al., 2009), or in acidic beverages. (Goto et al., 2002, 2003, 2007; Matsubara et al., 2002) Apart from the isolation of Alicyclobacillus spp. from mango juice (Gouws et al., 2005) and pear concentrate (Groenewald et al., 2009), reports on the incidence of this microorganism in beverages, exotic fruit juices, or fruit juices other than orange and apple juices, are rare.

Exotic fruits are appreciated worldwide due to their pleasant flavor and their health and functional appeal (Oliveira et al., 2006; Rosso and Mercadante, 2007; Zanatta and Mercadante, 2007;
Hoffman-Ribani et al., 2009; Hassimotto et al., 2009). Amongst the several exotic tropical fruits found in Brazil, passion fruit (Passiflora edulis) and pineapple (Ananas comosus) show relevant economical importance, Brazil being, respectively, the first and second major producer of these fruits worldwide (Gonçalves and Souza, 2006; Food and Agriculture Organization Statistics, 2009). With respect to juice processing, pineapple and passion fruit present a challenge to processors because they both grow close to the soil and have a rough surface, thus presenting difficulties in the washing and disinfection of the fruits. In addition, throughout the ages the common field practices of workers in passion fruit plantations relied on harvesting the fruits when they fell to the ground, since this was an indication of the best point of maturation. Since the soil is the main source of Alicyclobacillus spp. (Eguchi et al., 2001a,b,c), the characteristics of the aforementioned fruits, the field practices used and the acidic nature of these fruits (pH ~ 4.0) may render pineapple and passion fruit juices more prone to Alicyclobacillus spp. contamination and further spoilage than other fruit juices. Thus, the present study aimed at: i) estimating the Alicyclobacillus spp. population in pasteurized passion and pineapple fruit juices sold in Brazil; ii) typing the isolates using Random-Amplified Polymorphic-DNA (RAPD) and iii) determining the heat resistance of the Alicyclobacillus spores that were isolated and identified.

2. Material and methods

2.1. Fruit juices

The pineapple and passion fruit juices used in this study were processed in the lab or obtained from fruit juice manufacturers. Fruit juices prepared in the lab were used in the experiments to determine the optimum conditions for the activation of the Alicyclobacillus spores, the optimum incubation time for the isolation of spores and to determine the heat resistance of the spores. Fruit juices obtained from manufacturers were used to evaluate the incidence of Alicyclobacillus in pasteurized pineapple and passion fruit juices.

For juice preparation in the lab, the fruits were purchased from wholesale outlets (CEASA – Centrais de Abastecimento de Campinas SA). The fruits were carefully washed with a sponge and neutral detergent, then rinsed and immersed in a hypochlorite solution (100 mg/L of free chlorine) for 15 min. They were then transported to a laminar flow chamber and rinsed in sterile distilled water. After wiping with sterile towels, the fruits were cut with disinfected knives and the juice extracted in a domestic juicer. The juices were filtered and stored frozen at −18 °C in sterile containers. The pH and °Brix values were determined and found to be 3.7 and 13 for passion fruit juice and 3.9 and 12 for pineapple juice, respectively.

Passion fruit juice samples (n = 57) and pineapple juice samples (n = 50) from 12 different batches (4–5 samples per batch) from a manufacturer located in the State of São Paulo, Brazil, were obtained during a single harvest period (March–August). The juices were pasteurized under aseptic conditions (97 °C for 15 s) and filled into paperboard packaging without the addition of chemical preservatives. Before evaluating the incidence of Alicyclobacillus spp in the juices, the chemical characteristics were determined: pineapple (pH = 3.8 ± 0.42 and °Brix = 13.5 ± 2.1) and passion fruit (pH = 3.1 ± 0.6 and °Brix = 12.5 ± 0.7).

2.2. Strains and spore suspensions

The A. acidoterrestris DSM 2498 type strain was used due to its recognized ability to produce guaiacol (Deinhard et al., 1987; Wisotzkey et al., 1992). Other reference strains were Alicyclobacillus acidocaldarius ATCC 43034 and A. cycloheptanicus DSM 4006.

The procedure for the preparation of Bacillus spore suspensions was previously described by Stumbo (1973). Briefly the A. acidoterrestris spore suspension was prepared after growth of the microorganism in formulated Bacillus acidocaldarius medium agar (BAM) (Darland and Brock, 1971) and incubated at 50 °C for 72 h. Distilled water (5 mL) was used to scrape off the growth at the point when more than 80% of spores were observed using phase contrast microscopy. The suspension was then transferred to a sterile Erlenmeyer flask containing glass beads and 100 mL of sterile distilled water. The suspension was then homogenized and an aliquot of 20 mL transferred to a screw-cap tube (20 by 180 mm) and heat-activated at 70 °C for 20 min. After quickly cooling in an ice bath, 1 mL portions were taken and spread onto a BAM agar surface, followed by incubation at 50 °C for 72 h. The spores (>80% of sporulation) were collected in a sterile Erlenmeyer flask containing 100 mL of distilled water after scraping the surface of the BAM agar using sterile glass rods. The spores were washed and resuspended in sterile distilled water after three centrifugations (16210 × g at 7 °C for 10 min). The A. acidoterrestris spores were maintained in a sterile flask containing glass bead at 4 °C until used. The spore population in the suspension was assessed by counting in a Petroff-Houser chamber, and by pour plate counting in BAM agar after incubating for 72 h. The spore suspension was prepared with a growth at 50 °C for 7 days. The time and temperature conditions for heat activation were estimated in previous experiments (data not shown). The suspensions of the three passion fruit juice isolates presenting great similarity with A. acidoterrestris DSM 2498, were prepared. The concentration of the suspensions was adjusted to 10^7 viable spores per mL.

2.3. Estimation and isolation of Alicyclobacillus in pineapple and passion fruit juice samples

The estimation and isolation of Alicyclobacillus in pasteurized juices was carried out using the most probable number (MPN) technique with three tube series. The pasteurized juice packages were first disinfected with a 70% alcohol solution. Ten portions of 10 mL juice were then aseptically withdrawn from each of the packages and inoculated into screw-cap tubes containing 10 mL of double strength BAM broth. The tubes were submitted to heat activation shock (70 °C for 20 min) and cooled in ice bath. Incubation was carried out at 50 °C for 24 h for the pineapple juice samples and for 48 h for the passion fruit juice samples. This variation in time was because the best spore recovery time depended on the juice, as indicated by previous experiments (data not shown). After incubation, BAM agar plates were inoculated by streaking the contents of each MPN tube. The BAM agar plates were then incubated at 50 °C for 7 days. In addition, each colony that grew on the BAM agar plates was streaked onto new BAM agar plates and again incubated at 50 °C for 48 h. Each isolate was evaluated with respect to its macroscopic and microscopic characteristics. Macroscopically the isolates were characterized according to their shape, size and color, while microscopically, they were characterized according to the shape of the cells, size, spore formation and the presence of swollen sporangium. The main macroscopic and microscopic characteristics of Alicyclobacillus spp, as described by Chang and Kang (2004) were considered in this evaluation. Using this approach, some samples only yielded one isolate sharing the same macroscopic and microscopic patterns as the other colonies in that sample. On the other hand, some samples yielded more than three colonies that varied according to their morphological patterns (Table 1). The isolates were maintained at 4 °C for further biochemical identification. The biochemical assays applied were Gram staining, catalase, benzidine, starch hydrolysis, acetoin production (pH adjusted to 5.0), carbohydrate fermentation,
citrate utilization, nitrate reduction, indole formation, ability to grow under anaerobic conditions in culture media at pH 3.5 and 6.8, and the motility test in semi solid BAM broth at 50 °C for 18 h (Gordon, 1973; Walls and Chuyate, 1998). The strains were also submitted to identification using the API CH 50 (Biomerieux, Montalien-Vericien, France) with the galleries being filled in and incubated at 50 °C for 48 h under aerobic conditions (Deinhard et al., 1987). The counts were expressed as MPN of A. acidoterrestris spores per mL considering the number of BAM positive tubes and strains confirmed by the biochemical tests.

2.4. Typing the Alicyclobacillus isolates from the pasteurized fruit juices by RAPD-PCR

For the RAPD typing of the Alicyclobacillus isolates, the total DNA was extracted according to Doyle and Doyle (1990) and quantified by comparison with DNA of a known concentration (Gibco-BRL). DNA amplification was carried out in a final volume of 10 μL containing 3 mM MgCl₂, 200 μL from each of the oligonucleotides (dATP, dCTP, dGTP and dTTP), 0.32 μL from the primers, 0.25 U of Taq DNA polymerase (Life Technologies) (Frederick, MD, USA) and 1 μL of the DNA from a bacterial cell lysate. Negative controls were prepared using water instead of bacterial DNA. In the present study, the two primers described by Yamazaki et al. (1997a,b) were used, i.e., BA-10 = 5′-AAC GCG CAA C-3′ and F-61 = 5′-CCGTGATGCGC-3′ (Life Technologies) (Frederick, MD, USA). Thermal cycling was carried out in a Perkin–Elmer model 2400 thermocycler (Waltham, MA, USA) using the conditions described by Yamazaki et al. (1997a,b). The amplification products were separated by electrophoresis (120 V/6 h) on 1.4% agarose gel containing 10 μg/mL of ethidium bromide immersed in TBE buffer. A DNA ladder of 500 bp was used (Gibco-BRL) (Frederick, MD, USA) and the DNA bands were visualized under UV light (254 nm) and their images captured using photography apparatus (Kodak 667).

The genetic similarity obtained from the Jaccard coefficient was used to prepare a dendogram showing the relationship amongst the isolates. A. acidoterrestris DSM 2498, A. acidocaldarius ATCC 43034 and A. cycloheptanicus DSM 4006. The UPGMA method was used to group them according to the NTSYS program, version 1.70 (Rohlf, 1998).

2.5. Determination of the heat resistance of the passion fruit juice isolates (Alicyclobacillus acidoterrestris)

The heat resistance was estimated for the three isolates that showed great genetic similarity (99%) with the A. acidoterrestris type strain (DSM 2498) using the RAPD technique. The heat resistance of these strains was compared with that of A. acidoterrestris DSM 2498. The D values were estimated at 87 °C, 90 °C and 95 °C using sealed TDT tubes (thermal death tubes) (ID 6 mm, ED 8 mm and 105 mm length). The lag times in the sealed TDT were measured using previously calibrated copper-constantan Omega Eng. N.36 thermocouples (Omega, CT, USA) connected to a temperature recorder (Omega, model CL511). The thermocouple was maintained in the position representing one-third of the volume occupied by the liquids (1.8 mL of passion fruit plus 0.2 mL of distilled water). The TDT tubes were filled with passion fruit juice containing 10⁴ spores/mL, sealed with the aid of a blowtorch (O2/LPG) and placed in a thermostatically controlled water bath (Polystat, Poly Science with ±0.1 °C of precision) previously set to the required temperature for the programmed time period. After the pre-determined period, the tubes were quickly cooled in an ice bath and opened aseptically. Successive decimal dilutions were prepared in sterile 0.1% peptone water and enumeration carried out using BAM agar plates. The plates were incubated at 50 °C for 7 days and the results expressed as CFU/mL. The survivor counts and respective heating times were used to draw the heat inactivation curves for the passion fruit juice A. acidoterrestris strains and for A. acidoterrestris DSM 2498. The D values (i.e. time in minutes to cause a one logarithmic cycle reduction in the microbial population at a specified temperature) were determined by linear regression of the data of log population against the time, while for the z value (i.e. variation in temperature in °C to cause a one logarithmic cycle reduction in the D value) was determined by regressing the temperature versus log D. All these determinations were performed in Excel and the results were repeated twice.

2.6. Statistical analyses

The analyses of variance and Tukey tests were performed using Statistica 7.0 (Statsoft, OK, USA) so as to compare the heat resistance of A. acidoterrestris DSM 2498 with that of the fruit juice isolates.

3. Results

A total of 90 Alicyclobacillus strains were isolated from the passion fruit juice samples (Table 1). No strains were isolated from the pineapple juice samples examined. The packages of the passion fruit juices (n = 16) from where the Alicyclobacillus were isolated showed no signs of spoilage when they were opened in the lab. The signs of spoilage checked for were the presence of medicinal or phenolic taints, swelling of the container and alterations of the pH or turbidity. A mean rate of incidence of Alicyclobacillus of 28% was obtained in the 57 samples of passion fruit juice analyzed. The occurrence of Alicyclobacillus varied from 20% for batches G and D, 40% for batches I and J and 100% for batches E and F. Higher incidences (5 positive samples out of 5 taken) were observed in the samples collected in June and July, and these samples also showed the highest MPN values (>23/100 mL).

When grown in culture media, all the isolated strains presented a strong, disagreeable, astringent and lightly acid aroma. The strains isolated from passion fruit juice could be divided into two main groups based on the spore morphology: 10 strains presented slightly rounded and centrally located endospores with slightly swollen sporangia, while the other 80 strains presented cylindrical endospores, subterminally located and with swollen sporangia.

Although A. acidoterrestris was isolated from passion fruit juice (Table 1), it is important to note that the simple presence of the microorganism does not necessarily indicate that the juice is spoiled, since spoilage also depends on the number of cells present in the juice.

Table 1 Occurrence of A. acidoterrestris in samples of pasteurized passion fruit juices taken from industries during the harvest season.

<table>
<thead>
<tr>
<th>Month</th>
<th>Batches</th>
<th>Samples collected (Positive Samples)</th>
<th>Number of strains isolated</th>
<th>A. acidoterrestris (MPN/100 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>March</td>
<td>A</td>
<td>4 (0)</td>
<td>0</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td>April</td>
<td>B</td>
<td>4 (0)</td>
<td>0</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td>April</td>
<td>C</td>
<td>4 (0)</td>
<td>0</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td>May</td>
<td>D</td>
<td>5 (1)</td>
<td>1</td>
<td>9.2/16.1/23.0/22.0/23.0</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>5 (5)</td>
<td>45</td>
<td>/23.0/23.0/23.0</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5 (5)</td>
<td>39</td>
<td>/23.0/6.9/12.0/23.0</td>
</tr>
<tr>
<td>June</td>
<td>G</td>
<td>5 (1)</td>
<td>2</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>5 (0)</td>
<td>0</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>5 (2)</td>
<td>2</td>
<td>1.1/1.1</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>5 (2)</td>
<td>2</td>
<td>2.2/1.1</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>5 (0)</td>
<td>0</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>5 (0)</td>
<td>0</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>57 (16)</td>
<td>90</td>
<td>–</td>
</tr>
</tbody>
</table>
The DNA amplification products of the isolates obtained using the primers BA-10 and F-61 are shown in Fig. 1 and 2, respectively. By analyzing the dendrogram (Fig. 3) and considering 20% of similarity, it was possible to separate the Alicyclobacillus strains into two groups (Table 2): (i) two strains of A. acidocaldarius ATCC 43034 (strains 17 and 18) and one A. cycloheptanicus DSM 4006 strain, (ii) all the other strains (2, 8, 9, 11, 12 and 13), that shared 95% of genetic similarity with A. acidoterrestris DSM 2498 (strains 20 and 21). Strains 4, 6 and 7 shared a genetic similarity of 99%. Reduced genetic similarity (60%) was shared by strain number 10. Overall, the passion fruit juice strains (1–16, except strains 10 and 15) showed high genetic similarity with the A. acidoterrestris DSM 2498 strain and low genetic similarity with the A. acidocaldarius and A. cycloheptanicus strains. The RAPD analysis suggested that A. acidoterrestris was the prevalent Alicyclobacillus species in the Brazilian passion fruit juices sampled.

Table 3 shows the heat resistance parameters (D and z values) of the three A. acidoterrestris strains isolated from the passion fruit juice and of the DSM 2498 type strain. The three isolates studied were from batch E, which was the batch with the highest number of isolates (n = 45) and highest counts (up to >23.0 MPN/100 mL) of this microorganism. All the inactivation curves presented a log-linear behavior.

From Table 2 it can be seen that the heat resistances of the passion fruit juice isolates were not significantly different (p > 0.05) when compared to the reference strain (DSM 2498) at 85 °C, 90 °C and 95 °C. A mean z value of 7.6 °C was obtained for the three passion fruit isolates, while the DSM 2498 strain showed a z value of 7.1 °C. It is known that the efficiency of the pasteurization process in log-linear inactivation kinetics can be determined by: $\gamma = F/D$, where $\gamma$ is the number of logarithmic reductions and F is the time of pasteurization process required. The passion fruit juices taken from the Brazilian manufacturers were pasteurized at 97 °C for 15 s. A D value at a different temperature than that estimated in the heat resistance experiments can be calculated using the equation: where: $D_{T2} = D_{T1} \times 10^{(T1-T2)/\gamma}$, $D_{T2} = D$ value at the highest temperature (97 °C), $D_{T1} = D$ value at the lowest temperature (95 °C), $T_1$ and $T_2$ is the lowest and highest temperatures. Considering $D_{DSM} = 1.7$ min and $z = 3.15$ °C for DSM 2498 and $D_{DSM} = 1.7$ min and $z = 7.6$ °C for the passion fruit strains, respectively, it can be seen that the industrial pasteurization conditions (97 °C for 15 s) would only cause 0.19 $\gamma$ and 0.23 $\gamma$ for A. acidoterrestris DSM 2498 and for the juice isolates respectively.

4. Discussion

In recent years, several studies have been carried out to develop methods to estimate the incidence and counts of Alicyclobacillus spp. (Lin et al., 2005; Chen et al., 2006; Al-Qadiri et al., 2006; Goto et al., 2008; Barrios Eguiluz et al., 2009), and to inactivate or inhibit the outgrowth of its spores (Lee et al., 2004, Pena and Massaguer, 2006; Bevilacqua et al., 2008; Podolak et al., 2009; Friedrich et al., 2009). However these studies were focused on either apple
or orange juices. The incidence and spoilage potential of other fruit juices by Alicyclobacillus spp. is either unknown or underestimated. In this study, the incidence and counts of Alicyclobacillus spp. in passion fruit and pineapple juices were determined.

The occurrence of Alicyclobacillus in passion fruit juice sampled from June to August could be due to the fact that in Brazil these months are characterized by a dry climate. Considering that the soil is the main source of A. acidoterrestris (Eguchi et al., 2001b), with low rain precipitations, dust is easily deposited on the surface of the fruits. Eguchi et al. (2001a) found similar results, obtaining higher thermoacidophilic sporeforming bacteria counts on orange fruit surfaces sampled in a season with low rain precipitation. This data reinforces the idea that fruits are the main source of A. acidoterrestris contamination in the juice industry (Parish and Goodrich, 2005), and highlights the relevance of studies aimed at improving the efficiency of the fruit washing and disinfection steps (Podolak et al., 2009; Orr and Beuchat, 2000; Lee et al., 2004, 2006; Rodrigues et al., 2007; Friedrich et al., 2009) and the application of good agricultural practices, avoiding the harvesting of fruits that have fallen to the ground.

Despite the high prevalence of Alicyclobacillus in specific months of the harvest season, the overall counts found in the passion fruit juices were low (between 1.1 and 23.0 spores/100 mL). Data on the levels of Alicyclobacillus spp. in apple and orange juices are scarce, but according to Pinhatti et al. (1997), the counts of Alicyclobacillus spp. in orange juice commonly range from 10⁶–10⁷ spores/mL. Nevertheless this information on the counts of alicyclobacilli present in the raw material and fruit juices is relevant, for an accurate design of thermal processing for example, in order to assure product stability during the shelf-life.

Alicyclobacillus were not isolated from any of the pineapple juice samples taken. Some studies have reported the inability of A. acidoterrestris to survive and grow in pineapple juice or beverages containing pineapple juice, where a decrease in the population of this microorganism could be observed (Splittstoesser et al., 1998; Walls and Chuyate, 2000). In fact, the ability of Alicyclobacillus spp. to survive and grow in juices is dependent on the initial contamination level and mainly on the juice characteristics, for example, the presence of natural inhibitors such as benzoaldehyde and phenolic compounds (McIntyre et al., 1995; Splittstoesser et al., 1998). A compound able to inhibit the growth or affect the survival of A. acidoterrestris may be present in pineapple juice, since this juice presents a pH value and soluble solids content in the range compatible with the growth of this microorganism. The enzyme bromelain could be one of the natural compounds found in the pineapple fruit showing an antimicrobial effect against Alicyclobacillus, however, studies must be carried out to confirm its effects on the spore germination and outgrowth of this microorganism.

Although none of the samples taken in this study presented signs of spoilage when opened in the lab, this does not mean that the isolated strains are unable to spoil the product, but only that the juice was not spoiled at the time the samples were taken. Reports on the isolation of Alicyclobacillus from fruit juices showing no signs of spoilage can be found in the literature (Previti et al., 1995; Eiroa et al., 1999; Pinhatti et al., 1997; Walls and Chuyate, 2000). In order to spoil a fruit juice, A. acidoterrestris requires adequate environmental conditions such as a temperature above 20 °C and acidic conditions (Spinelli et al., 2009). The possibility that not all the strains isolated from juices are able to produce off-flavors must also be considered, and the real prevalence of off-flavor-producing Alicyclobacillus strains is not known (Tribst et al., 2009).

RAPD-PCR can be used for species confirmation (Jeyaram et al., 2008), differentiation amongst species (Torriani et al., 2001) and to generate species-specific banding patterns (Ronimus et al., 1997). Groenewald et al. (2009) used a RAPD-PCR procedure to type 16 A. acidoterrestris and A. acidocaldarius strains and obtained 4 genotypically well-distinguished groups. Strains with identical banding patterns were present in: (i) the pear concentrate and soil outside the factory and (ii) in the factory environment and pear concentrate after pasteurization. The soil was thus implicated as the source of the Alicyclobacillus spores, highlighting the potential of RAPD to track the spread of Alicyclobacillus strains at the juice production site. In the present study, RAPD-PCR with the primers BA-10 and F-61 was successful in confirming the Alicyclobacillus strains isolated from the passion fruit juices, and to differentiate the A. acidoterrestris strains from other Alicyclobacilli that could contaminate the fruit juices. The RAPD results also indicated great genetic similarity amongst the Alicyclobacillus strains isolated from different batches of passion fruit juice, which were divided into two subgroups (Table 2). Of the 16 strains tested, 14 were allocated to subgroup I and 2 to subgroup II. All these strains shared great genetic similarity with the A. acidoterrestris DSM 2498 type strain, which is a cause for considerable concern to passion fruit juice producers, since A. acidoterrestris is the main Alicyclobacilli species associated with fruit juice spoilage.

The D₉₀°C and z values for the three A. acidoterrestris strains isolated from passion fruit juice (Table 3) were marginally inferior (D₉₀°C and z, of less than 2 min and 7.6 °C, respectively) to those previously described in the literature for A. acidoterrestris in fruit juices. The D₉₀°C values reported for A. acidoterrestris in orange, apple, mango and cupuaçu ranging from 2.7 to 3.6 min, 2.3–2.8 min, 8.3 min and 2.8 min, respectively, and the z values for these juices can range from 7.7 °C in apple juice (Splittstoesser et al.,

### Table 2

<p>| Groups and subgroups of A. acidoterrestris strains isolated from passion fruit juice according to the RAPD technique. |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Strains</th>
<th>Sources – Batches (identification of strain)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>I</td>
<td>1</td>
<td>E(1)</td>
</tr>
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<td></td>
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<td>2</td>
<td>E(2)</td>
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<td>3</td>
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<td></td>
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<td>E(15)</td>
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<td>11</td>
<td>F(59)</td>
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<td></td>
<td></td>
<td>12</td>
<td>G(83)</td>
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<td></td>
<td></td>
<td>13</td>
<td>I(85)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>J(87)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>D(90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>A. acidoterrestris DSM 2498</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>A. acidoterrestris DSM 2498</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>F(58)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>J(89)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>I</td>
<td>17</td>
<td>A. acidocaldarius ATCC 43034</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>A. acidocaldarius ATCC 43034</td>
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<tr>
<td></td>
<td>19</td>
<td>A. cycloheptanicus DSM 4006</td>
<td></td>
</tr>
</tbody>
</table>

* Strains isolated from pasteurized passion fruit juices, except for the DSM and ATCC strains, which were used as controls.

### Table 3

<table>
<thead>
<tr>
<th>Strains</th>
<th>D₉₀°C (min)</th>
<th>z (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSM 2498</td>
<td>21.4 ± 4.9*</td>
<td>4.9 ± 0.8*</td>
</tr>
<tr>
<td>A. acidoterrestris (E14)</td>
<td>20.6 ± 7.0*</td>
<td>6.7 ± 1.9*</td>
</tr>
<tr>
<td>A. acidoterrestris (E25)</td>
<td>21.2 ± 6.6*</td>
<td>4.3 ± 0.4*</td>
</tr>
<tr>
<td>A. acidoterrestris (E27)</td>
<td>20.7 ± 5.3*</td>
<td>4.6 ± 0.5*</td>
</tr>
</tbody>
</table>

* Same letters in the columns indicate no significant difference (p > 0.05) according to the Tukey test.
to 21.3 °C in mango pulp (Carvalho et al., 2008). These differences are the results of the main factors influencing the heat resistance of microorganisms such as the strain under study, the temperature and time of incubation and the heating medium characteristics (pH, acidity and soluble solids content) (Yamazaki et al., 1997a; b; Pontius et al., 1998; Palop et al., 2000; Leguérinel et al., 2006). The industrial pasteurization conditions (97 °C for 15 s), would only lead to γ values below 1. In order to assure 5 γ for the A. acidoterrestris DSM 24988 and for the passion fruit juice isolates, pasteurization of the juices should be applied at 95 °C for 7.5 and 8.5 min, respectively. Even an increase in the pasteurization temperature to 102 °C would require a time of approximately 1 min to assure 5 γ for the DSM and for the passion fruit juice isolates. However, these are deleterious conditions with respect to the sensory and nutritional properties of the product.

Knowledge of the incidence of Alicyclobacillus strains in exotic fruits, of the heat resistance of these strains, and of their ability to survive and grow in the product is of major importance in the design of control measures to be taken from field to factory. Although passion fruit and pineapple fruits present a rough surface (which imposes a technological barrier to efficient fruit washing), grow in close contact with the soil and have low pH values, the fact that Alicyclobacillus was not found in any of the pineapple samples demonstrates the juice-dependent survivability of this bacterium. This indicates that Alicyclobacillus may not be a threat for the microbiological stability of all types of acidic fruit juices, and studies must be carried out to investigate the reasons for this behavior in selected fruit juices. This could result, for example, in the recognition that in certain juices where Alicyclobacillus does not pose a threat, other microorganisms, such as heat resistant molds, should be focused on as the target for the thermal processes. The benefit of these changes is that with a reduction in the requirements for thermal processing, the nutritional and sensory aspects of the exotic fruits could be better protected.

With respect to passion fruit juice, the isolation of A. acidoterrestris reinforces the relevance of minimizing spoilage risks by preventing contamination with A. acidoterrestris by way of the application of good agricultural practices, mainly in the fruit selection and washing steps, as well as by applying adequate controls during fruit juice pasteurization and refrigeration during the product shelf-life.

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The authors are grateful to Capes and CNPq for their financial support of this study, and to M. N. U. Eiroa in memoriam.

References

Association of the Industries of Juices and Nectars from Fruits and Vegetables of the European Union (AIJN), 2007. Abacucylbacteria best practice guideline, incorporating comments from Hague Working Group. 2005. Version 10. p. 28. Al-Qadi, H.M., Lim, M., Cavinato, A.C., Rasco, B.A., 2006. Fourier transform infrared spectroscopy, detection and identification of Escherichia coli O157:H7 and Alicyclobacillus strains in apple juice. Int. J. Food Microbiol. 111, 73–80. Bahceci, K.S., Acar, J., 2007. Modeling the combined effects of pH, temperature and time of incubation and the heating medium characteristics (pH, acidity and soluble solids content) (Yamazaki et al., 1997a; b; Pontius et al., 1998; Palop et al., 2000; Leguérinel et al., 2006). The industrial pasteurization conditions (97 °C for 15 s), would only lead to γ values below 1. In order to assure 5 γ for the A. acidoterrestris DSM 24988 and for the passion fruit juice isolates, pasteurization of the juices should be applied at 95 °C for 7.5 and 8.5 min, respectively. Even an increase in the pasteurization temperature to 102 °C would require a time of approximately 1 min to assure 5 γ for the DSM and for the passion fruit juice isolates. However, these are deleterious conditions with respect to the sensory and nutritional properties of the product.

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