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Flavorings as new sources of contamination by deteriogenic *Alicyclobacillus* of fruit juices and beverages

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ABSTRACT

This study aimed to report the incidence of *Alicyclobacillus* and *Alicyclobacillus acidoterrestris* in apple and pear flavorings (n = 42) and to assess the effect of guaiacol-producing *A. acidoterrestris* strains on apple flavorings stored at 4, 20 and 45 °C. A real-time polymerase chain reaction (RT-PCR) method was used for simultaneous confirmation of alicyclobacilli. A total of six isolates were identified as *A. acidoterrestris*, and only one strain was not able to produce guaiacol. The storage of apple flavoring at the optimum growth temperature of *A. acidoterrestris* (45 °C) resulted in the reduction in the spores' counts within 30 days of storage. On the other hand, during chilling (4 °C) and ambient storage conditions (20 °C) the counts of spores that remained stable for up to 60 days. An *A. acidoterrestris* strain inoculated in flavoring and further added to apple juice was able to grow and produce guaiacol in high amounts between 1–7 days of storage at 30 °C. In the current study it was shown that flavorings may be contaminated by deteriogenic *A. acidoterrestris* strains that are able to survive during storage in a wide range of temperature for long periods and further contaminate and spoil formulated fruit juices and beverages. A novel potential source of fruit juices and beverages contamination by deteriogenic *Alicyclobacillus* was shown. To the best of the author's knowledge, this is the first report on the incidence and fate of *Alicyclobacillus* and *A. acidoterrestris* in flavorings.

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1. Introduction

Alicyclobacillus are strictly acidophilic and spore-forming microorganisms with markedly heat and chemical resistances (Silva and Gibbs, 2001; Peña et al., 2009; Bahçeci and Acar, 2007; Friedrich et al., 2009). Because of these properties, *Alicyclobacillus* has been able to survive through the processing and the species is able to grow in fruit juices during storage and, therefore, cause the spoilage of the final product.

The spoilage of fruit juices by *Alicyclobacillus* is characterized by off-flavors associated with the production of 2-methoxyphenol (guaiacol), 2,6-dibromophenol and 2,6-dichlorophenol (Siegmund and Pöllinger-Zierler, 2006, 2007; Concina et al., 2010). Despite this, guaiacol seems to be the main compound associated with juice spoilage (Tribst et al., 2009). Although several members of *Alicyclobacillus* have been described as guaiacol producers (Cerny et al., 1984; Matsubara et al., 2002; Goto et al., 2003; AIJN, 2007; Smit et al., 2010), *Alicyclobacillus acidoterrestris* has been considered the main challenge for fruit juices. This is because *A. acidoterrestris* is the most often species isolated from fruit juices and its presence in these products is directly linked with their spoilage (AIJN, 2007; Tribst et al., 2009).

Because of the economic losses associated with *Alicyclobacillus* spoilage, numerous studies have been focusing on its incidence in single strength or concentrated juices (Walls and Chuyate, 2000; Siegmund and Pöllinger-Zierler, 2006; Groenewald et al., 2009; Durak et al., 2010; McKnight et al., 2010; Danyluk et al., 2011; Oteiza et al., 2011; Steyn et al., 2011). Conversely, scarce information has been found regarding the incidence of *Alicyclobacillus* in juice by-products, such as aromas and essential oils, which have been used in the preparation of fruit-based products, such as flavorings (Eguchi et al., 1999; Bicas et al., 2011).

Flavorings are concentrated preparations intended to impart food flavor and not to be consumed as such. They are prepared using a combination of fruit aromas, flavor carriers (such as ethanol and propylene glycol), and other minor ingredients (Anon., 2008), thus being high value-added products. Liquid flavorings may contain up to 90% of flavor carriers (Matheis, 1999) and various substances depicting inhibitory activities against microorganisms (Ayala-Zavala et al., 2011; Arcan and Yemenicioğlu, 2011; Côté et al., 2011; Bajpai et al., 2012). Therefore, food flavorings are recognized to present bactericidal or, at least, bacteriostatic activities (Taylor, 2007). Nonetheless, it has been shown that spore-forming microorganisms can survive through long-term exposure (up to 12 months) in some flavor carriers, such as ethanol (Thomas, 2012). Thus, as flavorings are widely used in the manufacture of juices and beverages, the knowledge of the incidence and survival of

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A. acidoterrestris in these products is of foremost importance for ensuring shelf-stable foods. This is because fruit flavorings are normally added to fruit juices and beverages after heat treatment steps.

Given the above, in this study we report on the incidence of *Alicyclobacillus* spp. in fruit flavorings. A real-time polymerase chain reaction (RT-PCR) method was used to identify the isolates to the species level, *i.e.*, *A. acidoterrestris*. Finally, the fate of guaiacol-producing *A. acidoterrestris* strains intentionally inoculated in flavorings, stored at different temperature conditions was also studied.

2. Material and methods

2.1. Samples

A total of 42 samples of apple and pear flavorings from different lots were collected in Argentina. The flavorings collected were produced by different companies of Argentina. Apple and pear flavorings contained 9–10% ethanol, density of 1.04 g/L at 4 °C and pH = 3.88 ± 0.55, and no sucrose was added to the product.

2.2. Detection of *Alicyclobacillus* spp.

Apple and pear flavoring samples were analyzed for the presence of *Alicyclobacillus* spp. based on the method described by the International Federation of Fruit Juice Producers (IFU, 2007) with slight modifications. Briefly, 50 mL of each sample was filtered through a 0.45 μm pore size membrane (Merck Millipore, Darmstadt, Germany). Then, the membranes were placed in flasks containing 50 mL of YSG (yeast extract, 2 g; soluble starch, 2 g; and glucose, 1 g) broth (pH 3.7 ± 0.1) that were gently homogenized during 1 min. Flasks were placed in a thermostatic controlled water bath (Model Masson 1203-Vicking, Buenos Aires, Argentina) previously adjusted at 80 ± 1 °C. Come-up time was determined and considered in the heat shock. A heat shock of 10 min was applied, following a cooling-down procedure to 40–45 °C in an ice-water bath and incubation at 45 °C for 5 days. Then, a loop of enriched samples was streaked onto YSG agar (pH = 3.7 ± 0.1) plates, following incubation at 45 °C for 3–5 days. All the colonies grown on YSG agar were confirmed as described in IFU (2007). Then, the presumptive colonies were further identified to the species level by real-time PCR.

2.3. Confirmation of *Alicyclobacillus* genus and simultaneous identification of *A. acidoterrestris* by real-time PCR

The confirmation of presumptive colonies was performed by Real-Time PCR using the foodproof® *Alicyclobacillus* Detection Kit-5′Nuclease (BIOTECON Diagnostics GmbH, Potsdam, Germany). This kit is based upon hydrolysis (5′-nuclease) probes or TaqMan® technology which allows the rapid detection of *Alicyclobacillus* DNA including the simultaneous identification of *A. acidoterrestris*. The kit consisted of premade master mix containing ready-to-use primers and probes, an enzyme solution that contained blocked “HotStart” *Taq* DNA polymerase and the enzyme Uracil-DNA N-Glycosylase (UNG) for preventing false-positive results due to carry-over contamination of internal amplification control (IAC). Furthermore, a positive control of DNA is also provided with the kit. DNA was extracted from previously cultures grown in YSG broth at 45 °C/3 days using the foodproof® ShortPrep II Kit (BIOTECON Diagnostics GmbH, Potsdam, Germany) following the manufacturer's instructions.

Real-time PCR experiments were run on the Mx3005P QPCR Systems (Agilent Technologies, Waldbronn, Germany). For each reaction, 18 μL of master mix were mixed with 1 μL of enzyme solution and 1 μL of IAC. Then, 20 μL of master mixes were transferred into PCR tubes, together with 5 μL of PCR-grade water (negative control), 5 μL of positive control or 5 μL of DNA, respectively. PCR conditions were as follows: initial pre-incubation at 37 °C for 4 min and 95 °C for 5 min, followed by 50 cycles of amplification at 95 °C for 5 s and 60 °C for 60 s. Fluorescence signals were measured after the second step of the amplification program. The results were assessed as described by the manufacturer of the kit: amplification of channel VIC/HEX was considered positive for *Alicyclobacillus* spp., while amplification of channel FAM was considered positive for *A. acidoterrestris*.

2.4. Guaiacol production by *A. acidoterrestris* strains isolated from flavorings

The spoilage potential of *A. acidoterrestris* strains isolated from apple and pear flavorings was assessed using a peroxidase enzyme-based colorimetric method. This method is based upon the acidic reaction of guaiacol produced in broth containing vanillic acid added with peroxidase enzyme in the presence of H_2O_2, thus releasing tetraguaiacol (brown component) (Niwa and Kawamoto, 2003). Briefly, a loopful of each *A. acidoterrestris* was inoculated into 2 mL of YSG broth (pH 3.7 ± 0.1) containing 100 mg/L of vanillic acid, following incubation at 45 °C/5 h. Then, 1 mL of 50 mM potassium hydrogen phthalate buffer, 8.5 μL of 3% H_2O_2, and 1 μL of peroxidase solutions were added to the medium and the formation of a brown color was visually monitored, which indicated the presence of guaiacol-producing *A. acidoterrestris* strains. *A. acidoterrestris* (DSM 2498) was used as the positive control.

2.5. Fate of *Alicyclobacillus* spp. in flavorings

2.5.1. Microorganisms and spore suspensions

Alicyclobacillus spp. (CIATI T278) and *A. acidoterrestris* (CIATI T300) isolated from apple (AF) and pear flavorings (PF), respectively, were used in this step of the study. *Alicyclobacillus* spp. (CIATI T278) was used in order to gather information on the fate of another non-*A. acidoterrestris* species in the flavorings. The cells from stock culture were incubated on YSG agar (pH 3.7 ± 0.1) at 45 °C for up to 5 days. The presence of spores was confirmed by microscope examination, after staining the plates with (pH 3.7 ± 0.1) malachite green solution and safranin. When at least 80% cells sporulated, the surface of the agar was gently rubbed with a sterile cotton swab and spores were washed with 4 mL of sterile distilled water. The resulting suspension was transferred into sterile tubes and centrifuged at 4000 ×g for 20 min. After the first centrifugation, the supernatants were discarded and the pellets were washed with sterile distilled water. Washing and centrifugation were repeated twice, and the final suspension was stored at −20 °C and used within 2 months. The population of spores (N_0) in each suspension was quantified by using YSG agar (pH 3.7 ± 0.1) and the solution was standardized at 10^6 spores/mL.

2.5.2. Determination of volatile compounds in apple and pear flavorings

2.5.2.1. Standards. The chemical standards 1-butanol and 1-pentanol were from Merck (Merck KGaA, Darmstadt, Germany). The following chemical standards were from Sigma (Sigma-Aldrich, Saint Louis, USA): 2-methyl-1-butanol, c-3-hexenol, t-2-hexenol, 1-hexanol, 3-methyl-1-butanol, n-hexanal, nonanal, 2-furaldehyde, t-2-hexenal, ethyl acetate, ethyl propanoate, propyl acetate, propyl propanoate, butyl acetate, ethyl-2-methyl butyrate, 2-methyl butyl acetate, propyl butyrate, butyl propanoate, amyl acetate, ethyl hexanoate, hexyl acetate, butyl-2-methyl butyrate and propyl hexanoate. Benzaldehide and ethyl butyrate were acquired from Fluka (Fluka Buchs, Schweiz, Switzerland).

2.5.2.2. Samples and preparation. A liquid–liquid procedure was used to extract the volatile compounds of fruit flavorings. Briefly, 3 mL of each fruit flavoring were placed in test tubes, following addition of 1 g of NaCl and 3 mL of a diethyl ether solution containing 40.8 mg/L and 34.2 mg/L of 4-heptanol and n-heptanol, respectively. Tubes were closed and gently shaken for 5 min, following repose for 1 h at 5–10 °C. Then, the ether phase was separated and transferred to a vial containing

anhydrous Na_2SO_4. The tubes were closed, sealed and kept at −20 °C until gas-chromatography analysis.

2.5.2.3. Gas chromatography (GC-FID). The analyses were performed in a HP 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a DB-5 capillary column (60 m × 0.32 mm I.D., 1 mm film thickness; J&W Scientific, Folsom, CA, USA). The temperature of the injector was 230 °C and the oven temperature was set at 40 °C (7 min holding time), and programmed to increase at 1 °C/min up to 70 °C, following a rate of 10 °C/min up to 200 °C (3 min holding time), then an increase of 10 °C/min up to 220 °C (4 min holding time) and a final increase of 15 °C/min to 290 °C (15 min holding time). Helium was the carrier gas and analysis was performed at 91.7 kPa. Injection volume was 2 μL in split mode (26:1). Stock solutions of each standard were prepared in diethyl ether and used for calibration and quantification. A linear regression method with internal standard was used (using three equidistant concentration levels). The determinations were performed in triplicate and the volatile compounds were identified by comparing retention times of samples with those of pure standard compounds.

2.5.3. Fate of Alicyclobacillus in fruit flavorings

A total of 100 mL of non-contaminated AF, PF and 10% ethanol solution disposed in sterile glass flasks were used in this step. AF, PF and 10% ethanol solution were separately inoculated with *Alicyclobacillus* spp. and *A. acidoterrestris* spores in order to achieve a population of approximately 5 spores/mL of flavoring. Then, flasks were gently shaken and stored at 4 °C, 20 °C and 45 °C during 60 days. The experimental control (10% ethanol solution) was selected to evaluate if the inhibitory effects of flavorings on *A. acidoterrestris* spores were due to other compounds present in these ingredients or due to ethanol. The concentration of ethanol in the solution was the same found in the fruit flavorings from which *Alicyclobacillus* spp. and *A. acidoterrestris* were isolated.

During storage, enumeration of spores was carried out every other 10 days. Duplicate samples of 1 mL each were submitted to heat shock for 80 °C/10 min to allow activation of spores. Then, heat-shocked samples were filtered through 0.45 μm pore size membranes (Merck Millipore, Darmstadt, Germany). Membranes were placed onto YSG agar plates (pH 3.7 ± 0.1), following incubation at 45 °C/5 days. Colonies grown on YSG agar were enumerated and the results expressed as CFU/mL. Regression models describing the behavior of *Alicyclobacillus* in flavorings and ethanol were constructed based on N/N_0 relation (log N/log N_0), where: N_0 is the initial concentration of spores in flavorings and N is the number of viable spores at a determined storage period.

The initial inoculum used in the experiments was based on the counts of *Alicyclobacillus* spp. found in fruit aromas (Eguchi et al., 1999). The lower storage temperature assessed (4 °C), represented the condition during shipping transportation, while 20 °C represented the ambient conditions. Storage at 45 °C was used to assess the behavior of *Alicyclobacillus* at its optimum growth temperature (Tribst et al., 2009). A total of twelve experiments conducted in duplicate were performed and replicated twice.

2.5.3.1. Evaluation of guaiacol production in apple juice prepared with flavorings contaminated by A. acidoterrestris. The ability of *A. acidoterrestris* (CIATI T300) to grow and produce guaiacol in apple juice formulated with a contaminated flavoring was evaluated at 30 °C for 7 days. Apple flavoring was inoculated with *A. acidoterrestris* (CIATI T300) spores to obtain final counts of approximately 5 spores/mL. The inoculated apple flavoring was homogenized and allowed to stand for 8 h. Then, 1 mL of flavoring was added to 100 mL of previously pasteurized apple juice (105 °C/5 min). The amount of flavoring added to the apple juice is within the range applied industrially, *i.e.*, 0.1–2%. Then, the apple juice was stored at 30 °C for 7 days and samples were collected on a daily basis for enumeration of *A. acidoterrestris* spores and determination of concentration of guaiacol. Guaiacol was quantified in the same samples collected for enumeration of *A. acidoterrestris* spore counts. The counts of spores followed the same method as described in Section 2.5.3. The determination of guaiacol was performed using a GC Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA) equipped with a quadrupole mass spectrometry detector (Model 5975C). The GC–MS was equipped with a J&W HP5-MS chromatographic column (30 m × 0.25 mm × 0.25 μm) (J&W Scientific, Folsom, CA, USA).

Guaiacol was extracted from apple juice samples through headspace solid-phase microextraction (SPME) using polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB) (Supelco, Bellefonte, PA, USA). Four mL of samples or standards were transferred to a 20 mL headspace vial. Control samples were apple juices added of non-inoculated flavorings and spiked samples with phenol. The extraction was performed in the headspace at 70 °C during 30 min under constant agitation. After each extraction the fiber has been cleaned in the second injection port of the GC at 280 °C for 10 min.

The determination of guaiacol was done using a GC Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA) equipped with a quadrupole mass spectrometry detector (Model 5975C) and a Combi Pal auto sampler for SPME fibers. The GC–MS was equipped with a HP-5ms (30 m × 0.25 mm I.D.) coated with a 0.25 μm film of (5% phenyl)-methylpolysiloxane (Agilent). The oven temperature was held at 50 °C for 5 min, and then increased in a rate of 4 °C/min up to 102 °C, and 20 °C/min up to 260 °C. This temperature was kept constant for 10 min. Splitless injection mode at 260 °C was applied and helium was used as carrier gas with a flow rate of 1.4 mL/min. MS spectra were recorded at 70 eV and the identification of guaiacol and phenol was carried out using the National Institute of Standards and Technology (NIST) library and the reference substances. Stock solutions of each standard were prepared in diethyl ether and used for calibration and quantification. The parameters of the MS detector were: temperature of ion source = 230 °C, temperature of quadrupole = 150 °C, temperature of transference line = 280 °C. Ions 109 and 81 were used as qualifiers for guaiacol, while ion 94 was used as qualifier for phenol. The ions 124 and 66 were used for quantification of guaiacol and phenol, respectively. Phenol was used as the internal standard.

2.5.4. Statistical analysis

Firstly, the conformity to the normal distribution of experimental data was assessed by using the Shapiro–Wilk test. Counts of *Alicyclobacillus* spores exposed at different temperature conditions throughout the storage were verified for significant statistical differences using one-factor analysis of variance (ANOVA) followed by Scott–Knott's test when data were normally distributed or the Kruskal–Wallis analysis of variances followed by the Kruskal–Wallis multiple comparison Z test was applied to results that did not follow a normal distribution. Statistical analysis was performed using Assistat version 7.6 free software (Campina Grande, Brazil) (Silva and Azevedo, 2002). p-Values below 5% were regarded as significant.

3. Results and discussion

Alicyclobacillus has been isolated from a variety of fruit juices and beverages, such as apples, citrus, blueberry, mango, passion fruit, pear, tomato, banana, apricot, peach, grape, guava, grapefruit and kiwi juices (Walls and Chuyate, 2000; Siegmund and Pöllinger-Zierler, 2006; Groenewald et al., 2009; Durak et al., 2010; McKnight et al., 2010; Danyluk et al., 2011; Oteiza et al., 2011; Steyn et al., 2011). Regardless of this fact, few studies have reported the incidence of this bacterium on farm, in processing environment, and in fruit juice by-products. In the first comprehensive study focusing on the ecology of *Alicyclobacillus* from field to final products, Eguchi et al. (1999) reported soil as the habitat of this bacterium. These authors found *Alicyclobacillus* populations between 10^4–10^6 CFU/g in soil samples of orange orchards. From soil, spores are transferred to fruit peels through dust (Eguchi et al., 1999), and then, to the processing environment. Several measures to reduce

Table 1
Incidence of *Alicyclobacillus* spp., *A. acidoterrestris* and guaiacol-producing *A. acidoterrestris* in apple and pear flavorings[a].

Flavorings	*Alicyclobacillus* spp.	*A. acidoterrestris*	Guaiacol-producing *A. acidoterrestris*
	n (%)	*n* (%)	*n* (%)
Apple (n = 28)	9 (32.1)	2 (7.1)	2 (7.1)
Pear (n = 14)	8 (57.1)	4 (28.6)	3 (21.4)
Total (n = 42)	17 (40.5)	6 (14.3)	5 (11.9)

[a] Where: *n* is the number of samples, % is the percentage of positive samples.

contamination of fruits by *Alicyclobacillus* have been reported (Tribst et al., 2009; Friedrich et al., 2009; Danyluk et al., 2011). However it is known that none is able to eliminate all spores from fruit surfaces. Thus, during fruit peeling, any *Alicyclobacillus* spores contaminating fruits can potentially be transferred to the pulp. Once in the pulp, spores may be then carried from juice to aroma concentrates during the evaporative concentration (Taylor, 2007). As fruit aromas are usually restored to juices after pasteurization or used in the preparation of fruit flavorings, the presence of *Alicyclobacillus* in these compounds is of great concern as it can impact on the microbiological stability of foods and beverages.

Table 1 shows that 40% of the flavorings analyzed were contaminated by *Alicyclobacillus* spp. Eight out of fourteen and nine out of 28 samples of pear and apple flavoring were contaminated with *Alicyclobacillus* spp., respectively. The total amount of samples analyzed (n = 42) corresponds to the quantity it was possible to collect because of the high costs of these ingredients. Through a RT-PCR assay, *A. acidoterrestris* was confirmed in two and four samples of pear and apple flavoring, respectively. Still, according to the RT-PCR assay data, all these isolates would have the potential to produce guaiacol, except for one strain recovered from pear flavoring (Table 1). The presence of guaiacol-producing *A. acidoterrestris* strains in apple (7%) and pear (21%) flavorings (Table 1) is of major concern because these products are normally added to fruit juices, beverages and foods after pasteurization. Therefore, taking into account that *A. acidoterrestris* is able to grow at temperatures above 20 °C (Spinelli et al., 2009), this finding suggests flavorings as potential sources of fruit juices and beverages contamination.

The detection of *Alicyclobacillus* spp. and identification of *A. acidoterrestris* were adequately performed as the isolates were previously grown in YSG broth at 45 °C/3 days (Table 1). This incubation period was sufficient for the isolates to grow and reach populations above the limit of detection of RT-PCR (>10^2–10^3 CFU/mL). Therefore, RT-PCR can be considered a rapid and easy tool to detect *Alicyclobacillus* and *A. acidoterrestris* simultaneously. According to the manufacturer, the foodproof® *Alicyclobacillus* detection kit has been tested for inclusivity with 38 strains of *Alicyclobacillus* genus, including *A. acidoterrestris*, with all of them being detected in channels FAM (*A. acidoterrestris*) and VIC/HEX (all *Alicyclobacillus*). All strains of *Alicyclobacillus* species, except for *A. acidoterrestris*, were detected in channel VIC/HEX. On the other hand, the exclusivity assay indicated that the 40 strains of closely related genera were neither detected in channel FAM nor in channel VIC/HEX (Biotecon, 2011).

Despite the results reported above, the simple presence of *Alicyclobacillus* strains in flavorings might not lead to fruit juices and beverages spoilage. Therefore, it was necessary to test whether fruit juices and beverages added with contaminated flavorings could be spoiled by *A. acidoterrestris* or not. Our approach was to evaluate both the survival of this microorganism in the flavorings under different storage conditions and its ability to produce guaiacol in fruit juices added with these ingredients. The fate of two *Alicyclobacillus* strains (*Alicyclobacillus* spp. CIATI T278 and *A. acidoterrestris* CIATI T300) inoculated in fruit flavorings stored at 4, 20 and 45 °C/60 days is shown in Fig. 1. As shown, the behavior of different *Alicyclobacillus* species was not significantly different ($p > 0.05$). The populations of *Alicyclobacillus* and *A. acidoterrestris* remained stable during 60 days when fruit flavorings were stored at 4 and 20 °C ($p > 0.05$). Nonetheless, a decline in spores' populations of these microorganisms was observed when fruit flavorings were stored at 45 °C (Fig. 1) ($p < 0.05$). The populations of *Alicyclobacillus* and *A. acidoterrestris* declined from zero to 30 days of storage ($p < 0.05$). After 30 days of storage, however, the decline stabilized and it was not significant according to Kruskal–Wallis analysis of variances followed by the Kruskal–Wallis multiple comparison Z test ($p > 0.05$). It is noteworthy that storage of this product at 45 °C is impracticable and not recommended by manufacturers due to potential chemical and sensorial changes. However, this condition was evaluated as it relates to the optimum growth condition of most *Alicyclobacillus*

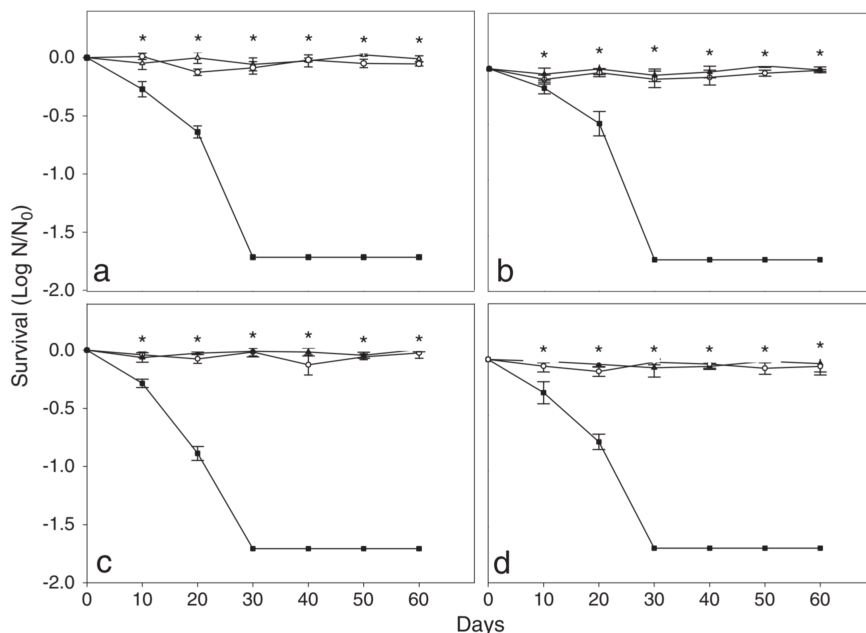

Fig. 1. Fate of *Alicyclobacillus* spp. spores in apple (a) and pear (c) flavorings and of *A. acidoterrestris* in apple (b) and pear (d) flavorings stored at 4 °C (Δ), 20 °C (○), and 45 °C (■) during 60 days. Asterisks indicate significant differences among the counts of *Alicyclobacillus* spores at different temperatures for the same storage time ($p \leq 0.05$) according to Skott–Knott's test.

spp. species (Tribst et al., 2009). The survival of *Alicyclobacillus* and *A. acidoterrestris* spores in fruit flavorings stored at 4 and 20 °C suggests their protection from the deleterious effects of ethanol and antimicrobial compounds present in the flavorings by low temperature (4 °C), as well observed in Fig. 1. This behavior represents a major concern for flavoring industries as well as for food and beverage industries because this bacterium is able to survive in contaminated flavorings for long-term storage period.

Volatile compounds found in apple and pear flavorings were mainly alcohols, ester and aldehydes. In apple flavoring, 27 compounds were found, while in pear flavoring, ethyl-2-methyl butyrate, 2-furaldehyde, butyl propanoate, propyl hexanoate and nonanal were not detected (Table 2). All the compounds found in the flavorings are common components of fruits (Table 2). These compounds are generated during fruit ripening or processing and have been recognized as descriptors of unique flavors of fruit and fruit products, such as fruity, green, floral, sweet and leafy, among others (Boylston, 2010). In addition, some of these compounds present antimicrobial activity (Bisignano et al., 2001; Baliga et al., 2011a,b; Côté et al., 2011; Dembitsky et al., 2011; Bajpai et al., 2012). In order to ensure that the decline in population of spores observed at 45 °C was due to antimicrobial compounds and not to ethanol present in the flavorings, we performed experiments to evaluate the survival of *A. acidoterrestris* (CIATI T300) in 10% ethanol solution. As can be seen in Fig. 2, the population of *A. acidoterrestris* (CIATI T300) spores remained stable during 60 days, regardless of the storage conditions (4, 20 and 45 °C) ($p > 0.05$). Therefore, the decline of *Alicyclobacillus* spores inoculated in flavorings after approximately 10 days of storage at 45 °C (Fig. 1) was due to the combination of antimicrobial compounds of flavorings and storage temperature. On the other hand, the survival of *Alicyclobacillus* spores at low storage temperature might be due to protection of spores from the deleterious effects of antimicrobial compounds present in the flavorings. This protection effect may be similar to the response of *Escherichia coli* O157:H7 when exposed to acidic environments at low temperature conditions (Conner and Kotrola, 1995).

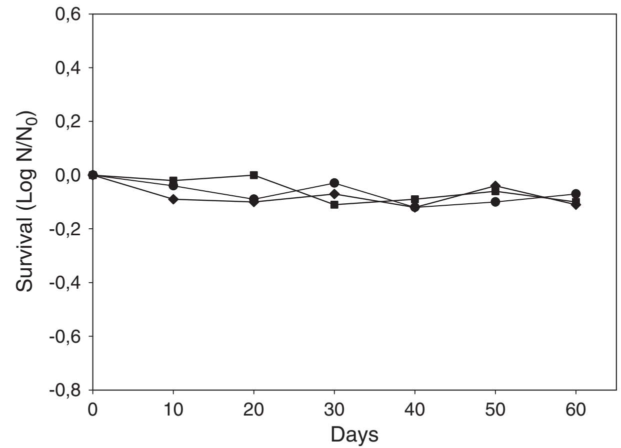

Fig. 2. Fate of *Alicyclobacillus acidoterrestris* (CIATI T300) spores in 10% ethanol stored at 4 °C (•), 20 °C (■), and 45 °C (◆) during 60 days. Statistical analysis indicated no significant differences regarding the counts of *Alicyclobacillus* spores at different temperatures for each storage time ($p \leq 0.05$) according to Skott–Knott's test.

In Table 3, it is shown that the use of flavorings contaminated with up to 50 spores of deteriogenic *A. acidoterrestris* per mL led to apple juice spoilage. For example, after 1 day of storage at 30 °C, 4.5 μg/L of guaiacol were found in the apple juice whereas after 7 days the total amount of guaiacol reached 157.0 μg/L (Table 3). Thus, in the current study a new source for fruit juices and beverages contamination by deteriogenic *Alicyclobacillus* has been shown. To the best of the authors' knowledge this is the first study on the incidence of *Alicyclobacillus* and *A. acidoterrestris* in apple and pear flavorings as well as on the fate of this bacterium in flavorings. Hence, this study provides very relevant information for industries regarding the role of fruit flavorings as potential sources of contamination of their products by deteriogenic *Alicyclobacillus*.

As the soil is the main source of *Alicyclobacillus* spores contaminating factory environment as well as final products and by-products (Eguchi et al., 1999), efforts to ensure raw materials (fruits) with low contamination by *Alicyclobacillus* spp. and the implementation of measures during processing should be taken. Steps such as fruit washing should be highly efficient in removing contamination of fruits as it is known that *Alicyclobacillus* spp. spores may not only contaminate fruit juices, but also be carried to fruit flavorings that are used in a wide range of foods and beverages. Flavorings contaminated by *Alicyclobacillus* spp. may play an important role in post-pasteurization steps of fruit juices, fruit products and beverages in which they are added. The addition of contaminated flavorings may lead to spoilage of foods by *Alicyclobacillus* spp.

Table 2
Volatile compounds (mg/L) in apple and pear flavorings as determined by GC-FID.

Volatile compounds	Apple flavoring	Pear flavoring
Alcohols		
1-Butanol	292.9 ± 36.8	139.3 ± 32.8
2-Methyl-1-butanol	303.2 ± 120.3	29.9 ± 12.0
1-Pentanol	10.2 ± 1.6	0.9 ± 1.6
c-3-Hexenol	0.6 ± 0.7	0.4 ± 0.6
t-2-Hexenol	26.7 ± 9.9	5.7 ± 4.2
1-Hexanol	140.9 ± 15.2	40.5 ± 8.6
3-Methyl-1-butanol	22.2 ± 8.1	34.9 ± 5.7
Aldehydes		
n-Hexanal	53.9 ± 27.5	38.9 ± 13.1
Nonanal	0.2 ± 0.1	Nd
Benzaldehide	27.1 ± 7.1	1.9 ± 1.2
2-Furaldehyde	1.2 ± 2.1	Nd
t-2-Hexenal	228.3 ± 118.0	69.4 ± 24.8
Esters		
Ethyl acetate	292.7 ± 55.3	383.7 ± 157.3
Ethyl propanoate	37.9 ± 9.1	0.2 ± 0.4
Propyl acetate	21.6 ± 8.4	6.7 ± 0.9
Ethyl butyrate	35.4 ± 3.7	0.3 ± 0.6
Propyl propanoate	3.3 ± 1.2	0.1 ± 0.2
Butyl acetate	37.4 ± 6.6	65.4 ± 38.3
Ethyl-2-methyl butyrate	12.5 ± 1.8	Nd
2-Methyl butyl acetate	36.8 ± 9.0	1.8 ± 1.3
Propyl butyrate	3.5 ± 0.4	0.1 ± 0.1
Butyl propanoate	0.6 ± 0.2	Nd
Amyl acetate	1.5 ± 0.4	1.4 ± 0.4
Ethyl hexanoate	3.5 ± 2.2	0.2 ± 0.4
Hexyl acetate	14.3 ± 5.8	24.3 ± 21.5
Butyl-2-methyl butyrate	0.5 ± 0.2	0.1 ± 0.2
Propyl hexanoate	0.2 ± 0.2	Nd

Nd = not detected.

Table 3
Counts of *Alicyclobacillus acidoterrestris* spores and guaiacol concentration (μg/L) in single strength apple juice (11°Brix) stored at 30 °C during 7 days. Apple flavoring intentionally inoculated with *A. acidoterrestris* (CIATI T300) was added at concentration of 1% to single strength juice.

Storage time (days)	Spore count (log CFU/mL)	Guaiacol (μg/L)
0	0.7 ± 0.09	<3
1	2.3 ± 0.04	4.5 ± 1.2
2	3.7 ± 0.02	34.1 ± 1.3
3	4.6 ± 0.01	55.8 ± 2.2
4	5.7 ± 0.01	62.3 ± 1.7
5	6.0 ± 0.04	92.7 ± 1.9
6	6.7 ± 0.01	122.2 ± 2.5
7	7.0 ± 0.01	157.0 ± 3.1

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