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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.

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