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Use of a multivariate approach to assess the incidence of *Alicyclobacillus* spp. in concentrate fruit juices marketed in Argentina: Results of a 14-year survey

Juan Martìn Oteiza [a,*], Gastón Ares [b], Anderson S. Sant'Ana [c], Silvina Soto [a], Leda Giannuzzi [d]

[a] *Centro de Investigación y Asistencia Técnica a la Industria Agroalimentaria (CIATI AC), Neuquén, Argentina*
[b] *Departamento de Ciencia y Tecnología de Alimentos, Facultad de Química, Universidad de la República (UdelaR), Montevideo, Uruguay*
[c] *Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil*
[d] *Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CCT-La Plata, Facultad Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina*

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ABSTRACT

The purpose of this study was to determine the incidence of *Alicyclobacillus* spp. in fruit/vegetable juices (concentrated pulps and clarified and non-clarified juices) marketed in Argentina between 1996 and 2009. The presence of *Alicyclobacillus* was determined in a total of 8556 samples of fruit and vegetable juices (apple, pear, grape, peach, blend of juices, tangerine, pineapple, orange, mango, plum, guava, apricot, lemon, banana, kiwi, carrot, strawberry, grapefruit, and beetroot) collected in seven Argentinean provinces. Multiple factor analysis (MFA) was carried out on a data matrix that contained the percentage of positive samples, type of juice, raw material and production year.

Except for kiwi and orange, *Alicyclobacillus* was found in juices from all the evaluated raw materials. The highest percentage of positive samples was found for beetroot, strawberry, banana, peach, mango, carrot and plum juices. The percentage of positive samples for these juices ranged from 100% to 24%.

Furthermore, the application of multivariate techniques provided an insight on the relationship between the incidence of *Alicyclobacillus* and production variables. This approach enabled the identification of the most relevant variables that increased the percentage of positive samples among the juices, which could help in developing strategies to avoid the incidence of this bacterium.

By means of hierarchical cluster analysis seven groups (clusters) of juices which showed different percentages of positive samples for *Alicyclobacillus* spp. were identified. This analysis showed that pineapple, peach, strawberry, mango and beetroot juices had higher rates of positivity for *Alicyclobacillus* than the rest of the evaluated juices. MFA analysis also showed that some clear relationships could be highlighted between the percentage of samples positive for *Alicyclobacillus* and five types of fruit juices (strawberry, beetroot, grapefruit, pineapple and mango). It was observed that a large proportion of juices produced in 2000, 2005 and 2008 were located in clusters with higher incidence of *Alicyclobacillus* spp., whereas a larger proportion of clarified concentrate juice and concentrate pulp samples showed higher probability of incidence of *Alicyclobacillus* in these products. Data presented in this study brings a contribution to the ecology of *Alicyclobacillus* in fruit/vegetable juices marketed in Argentina. This information would be useful to enhance the microbiological stability of fruit juices regarding the presence of *Alicyclobacillus* spp.

1. Introduction

Alicyclobacillus is a major challenge for fruit juice industries (Tribst et al., 2009). The microorganisms within the *Alicyclobacillus* genus present as main characteristics high chemical resistance (Silva and Gibbs, 2001; Friedrich et al., 2009), acidothermophilic behavior (Spinelli et al., 2009), and high heat resistance (Peña and Massaguer, 2009; Bahçeci and Acar, 2007). These characteristics allow the survival of *Alicyclobacillus* to pasteurization treatments used for processing fruit juices (Spinelli et al., 2010) and its growth under storage conditions, which leads to the spoilage of fruit juices (Spinelli et al., 2009).

The spoilage caused by *Alicyclobacillus* is mainly characterized by smoky and medicinal off-flavors due to the generation of compounds like 2-methoxyphenol (guaiacol), 2,6-dibromophenol and 2,6-dichlorophenol (Siegmund and Pöllinger-Zierler, 2006; Siegmund and Pöllinger-Zierler, 2007; Concina et al., 2010). Currently, more than 15 species have been described within the *Alicyclobacillus* genus (Tribst et al., 2009). However, few of them (*A. acidoterrestris*, *A. pomorum*, *A. herbarius*, *A. acidiphillus*, *A. hesperidum*, *A. cycloheptanicus* and *A. acidocaldarius*) have been shown to spoil fruit juices (Cerny et al.

* Corresponding author at: Calle Chile y Primeros Pobladores, Centenario (8309), Neuquén, Argentina. Tel./fax: +54 299 4899680.
E-mail address: juano@ciati.com.ar (J.M. Oteiza).

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1984; Matsubara et al. 2002; Goto et al. 2003; AIJN, 2007; Smit et al. 2010). Although these seven species may represent a risk for fruit juice spoilage (Smit et al. 2010), most published studies have been focused on the incidence of *A. acidoterrestris*, since this is the species most commonly involved in juice spoilage (AIJN, 2007; Tribst et al., 2009).

The incidence of *A. acidoterrestris* has been reported to be restricted to few types of fruit juices such as apples (Siegmund and Pöllinger-Zierler, 2006; Durak et al., 2010; Steyn et al., 2011), citrus (Durak et al., 2010), blueberry (Durak et al., 2010), mango (Durak et al., 2010), pear (Walls and Chuyate, 2000; Groenewald et al., 2009), tomato (Walls and Chuyate, 2000), passion fruit (McKnight et al., 2010), coconut cream, and grapefruit concentrate (Danyluk et al., 2011).

Argentina has an important international participation in producing, elaborating, and exporting concentrated lemon, grape, pear and apple juices. The country is the second largest exporter of concentrated apple and pear juices in the world and the leading producer and exporter of concentrated lemon and grape juices. Thus, the knowledge about the prevalence of *Alicyclobacillus* spp. in a wide variety of juices is of major importance to add more information on the ecology of the microorganism and on the susceptibility of juices to *Alicyclobacillus* spoilage. Additionally, this information might be useful to design pasteurization processes for new fruit products that guarantee products as shelf-stable as possible.

The worldwide demands with respect to the presence of *Alicyclobacillus* spp. in juices is variable and approximately 60% of importers require their absence for purchasing the product. The detection of *Alicyclobacillus* spp. in a container of fruit juice received in the destination country may result in economic losses, and leads to distrust by buyers to suppliers. Due to the time required for isolation and identification of *Alicyclobacillus* at species level and the limited availability of quick, simple and cost-effective methods for differentiating strains with spoilage potential, analysis during commercialization among industries is carried out mainly based on presence/absence approach of the microorganism at the genus level, i.e., *Alicyclobacillus*. Although it might be considered a conservative policy, presence/absence approach is necessary to overcome the previously mentioned issues in order to speed up the commercialization process. In this context, in this study the incidence of *Alicyclobacillus* spp. in fruit/vegetable juices (concentrated pulp, concentrate clarified and non-clarified juices) marketed in Argentina during 14 years was investigated using a multivariate approach.

2. Material and methods

2.1. Samples

A total of 8556 samples of different types of fruit or vegetable juices (concentrate pulps, clarified concentrate and non-clarified concentrate) were collected in different Argentinean provinces (n = 7) between 1996 and 2009. Clarified concentrate and non-clarified juices were: apple, pear, grape, peach, blend of juices (orange, banana, mango, kiwi and strawberry), tangerine, pineapple, orange, mango, plum, guava, apricot, lemon, banana, kiwi, carrot, strawberry, grapefruit, and beetroot. Concentrate fruit pulps were: plum, peach, pear and apricot. pH and ^{o}Brix values of samples were measured using pH-meter (Selecta, model pH-2005, Spain) and refractometer (Bellingham-Stanley Ltd, model RFM 330+, United Kingdom) and the ranges are presented as a supplementary material. The distribution of samples analyzed through the years is shown in Table 1.

2.2. Juice collection and detection of Alicyclobacillus spp.

Juices were collected under aseptic conditions, disposed in pouches or other plastic sterile flasks and transported to the lab under refrigeration. Samples were analyzed for the presence or absence of *Alicyclobacillus* spp. based on the method described by the International Federation of Fruit Juice Producers (IFU, 2007). A 10 ± 0.1 g portion of each fruit juice was aseptically diluted in 90 mL of either BAT (pH 4.0 ± 0.2) or YSG (pH 3.7 ± 0.1) enrichment broths and gently homogenized during 1 min. If necessary, the pH of the media containing the juices was adjusted with HCl/H_2SO_4 or NaOH 1N to the reference values (4.0 or 3.7).

Then, flasks containing juice and culture media were set 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. Heat shock was applied for 10 min and the flasks were promptly cooled down to 40–45 °C in an ice-water bath and incubated at 45 ± 1 °C for 5 days. Then, a loop of enriched samples was streaked either onto BAT (4.0 ± 0.2) (Merck, Darmstadt, Germany) or YSG (pH = 3.7 ± 0.1) agar plates. BAT/YSG agar plates were incubated at 45 ± 1 °C for 2–5 days and checked for the presence of typical colonies of *Alicyclobacillus* and at least 5 colonies were selected for further confirmation. 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 and spore formation with an optical microscope (Leica DM750, Swiss).

After this, the isolated colonies were streaked onto a neutral pH medium (Plate Count Agar) (Merck, Darmstadt, Germany), incubated for 2 days at 45 °C and for 4 days at 25 °C.

Moreover, the isolates were further tested for growth in *Sulfobacillus* broth medium (pH 2.0) (DSMZ, 2007) to differentiate between *Alicyclobacillus* and *Sulfobacillus* (Parish and Goodrich, 2005). The presence of at least one colony of gram-positive (or gram variable), spore-forming rods on BAT/YSG incubated aerobically at 45 °C, coupled with the lack of growth on PCA at 45 and 25 °C and the lack of growth in *Sulfobacillus* medium was recorded as positive for *Alicyclobacillus* spp. In all the other cases the results were reported as negative for *Alicyclobacillus* spp.

2.3. Statistical analysis

The average percentage of positive samples was determined for each raw material.

Multiple factor analysis (MFA) was carried out in order to study the relationship between the incidence of *Alicyclobacillus* and juice characteristics. This analysis was performed on a data matrix that contained the percentage of positive samples, type of juice (qualitative variable indicating if clarified concentrate juice, non-clarified concentrate juice or concentrate pulp was considered), raw material (qualitative variable which indicated if the juice was from apple, pear, grape, peach, tangerine, pineapple, orange, mango, plum, guava, apricot, lemon, banana, kiwi, carrot, strawberry, grapefruit, beetroot, or blend of fruits) and production year (qualitative variable).

In order to identify groups of juices with similar characteristics and incidence of *Alicyclobacillus*, hierarchical cluster analysis was performed on sample coordinates in the first five dimensions of the MFA considering Euclidean distances and Ward agglomeration criterion. Statistical analyses were carried out in R language (R Development Core Team, 2007). Multiple factor analysis was performed using FactoMineR (Husson et al., 2007; Lê et al., 2008).

3. Results and discussion

This is the first throughout report on the incidence of *Alicyclobacillus* spp. in fruit and vegetable juices marketed in Argentina. Table 1 shows the number and types of juices collected during 14 years in Argentina and analyzed for presence of this bacterium.

As shown in Table 2, except for kiwi and orange, *Alicyclobacillus* spp. was found in juices from all the evaluated raw materials. Therefore, the increasing consumption of mixed beverages made of different types of