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Short communication

Fate of *Alicyclobacillus* spp. in enrichment broth and in juice concentrates

Juan Martin Oteiza [a,*], Silvina Soto [a], Verônica O. Alvarenga [b], Anderson S. Sant'Ana [b,**], Leda Gianuzzi [c]

[a] Centro de Investigación y Asistencia Técnica a la Industria (CIATI AC), Neuquén, Argentina
[b] Department of Food Science, Faculty of Food Engineering, University of Campinas, Campinas, SP, Brazil
[c] 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

In this study, the fate of *Alicyclobacillus acidoterrestris* spores in different types of juice concentrates stored under different conditions was investigated. In addition, the impact of dilution procedures during the enrichment step for the detection of *Alicyclobacillus* in lemon juice concentrates was studied. Pear, red grape, mango, tangerine, carrot and lemon juice concentrates (50–69.4°Brix, pH 1.7–4.3) were inoculated with *A. acidoterrestris* spores (10^3 spore/mL) and stored at 4 °C and 20 °C, after which the spores were counted at 0, 2, 5, 9, 17, 21, 28, 36, 43, and 50 days. No significant differences in the number of *Alicyclobacillus* spores were observed at storage temperatures of 4 °C and 20 °C ($p > 0.05$). The results also indicated that the number of spores of *A. acidoterrestris* remained stable in all types of juice concentrates during the storage period, except in lemon juice concentrate. In lemon juice concentrate, a decline in *A. acidoterrestris* spore populations of 0.3–0.8 log CFU/mL was observed within 5–10 days of storage. The decline in *A. acidoterrestris* spore populations was more pronounced in cloudy lemon juice concentrate, which contained higher concentrations of flavonoids (mainly eriocitrin and hesperidin) than clarified lemon juice concentrate. It was also found that dilution of lemon juice concentrate samples in the proportion of 1:19 allowed the germination of *A. acidoterrestris* spores and the growth of populations of up to 10^7 CFU/mL. In contrast, the proportion (1:9) recommended in internationally recognized methods led to a reduction in the population of this microorganism that would yield false negative results. Data presented in this study demonstrated that *Alicyclobacillus* spores remain stable in most juice concentrates during storage, but that natural antimicrobial compounds present in some of them may decrease spore counts and inhibit their recovery by detection procedures.

1. Introduction

Alicyclobacillus acidoterrestris is an acidothermophilic spore-forming bacterium of great concern for juice industries (AIJN et al., 2007; Friedrich et al., 2009; Spinelli et al., 2009; Tribst et al., 2009). This microorganism causes spoilage of fruit juices by producing compounds responsible for off-flavors such as 2-methoxyphenol (guaiacol), 2,6-dibromophenol and 2,6-dichlorophenol (Concina et al., 2010; Siegmund and Pöllinger-Zierler, 2006, 2007).

Spoilage episodes or the spoilage potential of single strength juices and juice-based beverages by *Alicyclobacillus* have been reported in the literature (Bevilacqua et al., 2008a,b; Borlinghaus and Engel, 1997; Sinigaglia et al., 2003; Splittstoesser et al., 1994). Because of their high soluble solid contents that prevent the germination and outgrowth of spores, juice concentrates are unlikely to be spoiled by *Alicyclobacillus* (Peña and Massaguer, 2006; Peña et al., 2011). Nonetheless, these substrates constitute an important source of contamination by this bacterium in juice-processing environments, in single-strength juices and in juice-based beverages. Since its first association with apple juice spoilage in the 1980s (Cerny et al., 1984), incidence of *A. acidoterrestris* in juices and juice concentrates has been assayed in several studies (Danyluk et al., 2011; Durak et al., 2010; Groenewald et al., 2009; McKnight et al., 2010; Oteiza et al., 2011; Steyn et al., 2011; Siegmund and Pöllinger-Zierler, 2006; Walls and Chuyate, 2000). The spoilage of single-strength juices and juice-based beverages by *Alicyclobacillus* will also be dependent upon the fate of its spores during juice concentrate storage. Therefore, the assessment of spore's viability in juice concentrate as affected by storage conditions may provide useful information to improve single-strength juices' stability.

Given the importance of juice concentrate as potential sources of *Alicyclobacillus* spores, several studies have been conducted to assess the effectiveness of strategies to reduce spore loads in raw materials (Pinhatti et al., 1997). In addition, studies have been done to assess strategies to avoid germination of *Alicyclobacillus* spores during juice storage and commercialization (Bevilacqua et al., 2008a,b; Peña and Massaguer, 2006; Spinelli et al., 2009). The prevalence of *Alicyclobacillus* spp. was shown to vary depending on the type of juice, processing

* Corresponding author.
** Corresponding author at: Rua Monteiro Lobato, 80, Cidade Universitária Zeferino Vaz, CEP: 13083-862, Campinas, SP, Brazil.
E-mail addresses: juano@ciati.com.ar (J.M. Oteiza), and@unicamp.br (A.S. Sant'Ana).

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season, harvesting practices, etc. (Danyluk et al., 2011; Pinhatti et al., 1997). In a recent survey, it was reported that beetroot, strawberry, banana, peach, mango, carrot and plum juices were positive for *Alicyclobacillus* spp. in levels ranging from 24 to 100%. In contrast, lemon juice concentrates were amongst the concentrates with the lowest prevalence of *Alicyclobacillus* (Oteiza et al., 2011). The reason for such lower levels could be due to the presence of components in this juice that are inhibitory for *Alicyclobacillus* spp. (McNamara et al., 2011). Several importers require the absence of *Alicyclobacillus* spp. in juice concentrates aiming to reduce the probability of final product spoilage (Oteiza et al., 2011). However, for a successful quality assessment program of these products, adequate methods should be used. Many methods used to detect the presence of *Alicyclobacillus* spp. in juice concentrates rely upon a preliminary enrichment step to amplify the number of *Alicyclobacillus* for detection (IFU and of F.J.P., 2007a,b; AIJN et al., 2007). Typically, these methods require enrichment of a 1 in 9 dilution of sample of the juice concentrate. Given the presence of *Alicyclobacillus*-inhibitory substances in some juice concentrates such as lemon juice concentrate, such dilution may not be sufficient to eliminate this inhibitory effect. In these cases, a negative result may be recorded when, in reality, viable spores of *Alicyclobacillus* may be present. Consequently, defective batches of juice concentrate may enter into the process line. Therefore, the fate of *Alicyclobacillus* spores in enrichment broth and in juice concentrates, particularly in lemon juice concentrates, is worthy of investigation. In this study, the fate of *A. acidoterrestris* spores inoculated into pear, red grape, mango, tangerine, carrot and lemon juice concentrates stored at 4 °C and 20 °C was studied. In addition, the fate of *A. acidoterrestris* in enrichment broth as affected by the dilution procedures considering recommendations of standard analytical methods was also investigated.

2. Material and methods

2.1. Juice concentrates

Six different types of juice concentrates, including clarified (pear, red grape, mango, and lemon) and cloudy (tangerine, carrot and lemon) juice concentrates were used in the study. Juice concentrates were collected from tanks in factories located in Argentina under aseptic conditions, dispensed into pouches or plastic sterile flasks and transported to the laboratory under refrigeration. The juice concentrates were analyzed for the presence of *Alicyclobacillus* spp. according to the method described by the International Federation of Fruit Juice Producers (IFU and of F.J.P., 2007a) and proved to be free of this bacterium. The pH and °Brix values of the juice concentrates were measured using a pH meter (Selecta, model pH-2005, Barcelona, Spain) and refractometer (Bellingham-Stanley Ltd, model RFM 330+, United Kingdom), respectively.

2.2. Fate of A. acidoterrestris spores in juice concentrates during simulated storage

2.2.1. Microorganisms and preparation of spore suspensions

A. acidoterrestris DSM 2498, obtained from the DSMZ Culture Collection (Germany), was used in this study. The microorganism was streaked onto plates of yeast starch glucose agar (YSG) (in g/L: yeast extract, 2.0; glucose, 1.0; soluble starch, 2.0 and agar, 15.0) (pH 3.7 ± 0.1), followed by incubation at 45 °C for up to 5 days (IFU and of F.J.P., 2007a,b). During this period, the presence of spores was verified through microscopic examination after staining with malachite green solution and safranin. The plates were washed for spore collection once at least 80% of the area of the microscopic slide was covered by spores. Washing of YSG agar plates was performed by gently rubbing with a sterile cotton swab with 4 mL of sterile distilled water. The suspension was placed into sterile tubes and centrifuged at 4000 ×*g* for 20 min and the supernatants were discarded. The remaining spores in pellets were washed twice with sterile distilled water, and the final suspension was stored at −20 °C until use. Sterile distilled water was used to standardize the concentration of spores in the suspension at 10^6 spores/mL. The concentration of spores was verified through enumeration with YSG agar (pH 3.7 ± 0.1) after heat shock at 80 °C for 10 min.

2.2.2. Inoculation, storage conditions, and fate of A. acidoterrestris in juice concentrates

Sterile glass flasks containing 250 mL of each juice concentrates were separately inoculated with *A. acidoterrestris* spores to achieve a population of approximately 10^3 spores/mL. After this, flasks were gently shaken and stored at 4 °C and 20 °C for up to 60 days. The initial inoculum used in the experiments was based on counts of *Alicyclobacillus* spp. previously found in juice concentrates (Eguchi et al., 1999; Pinhatti et al., 1997). The lower storage temperature assessed (4 °C), represented a condition recorded during juice concentrate transportation by ships, while 20 °C represented storage at ambient conditions. Non-inoculated juice concentrates stored at 4 °C and 20 °C for 50 days were used as controls.

The counts of *A. acidoterrestris* spores in fruit and vegetable concentrates were determined at different time intervals through the enumeration in YSG agar (pH 3.7 ± 0.1). For this purpose, duplicate samples of 1 mL each were submitted to heat shock at 80 °C for 10 min to allow spore activation. Then, clarified juice concentrates (pear, red grape, mango, and lemon) were filtered through 0.45 μm pore size membranes (Merck Millipore, Darmstadt, Germany) and placed onto YSG agar plates. For cloudy juice concentrates (tangerine, carrot and lemon), an aliquot of 0.1 mL of the juices was spread plated onto two YSG agar plates. The inoculated YSG agar plates were incubated at 45 °C for 5 days, following the enumeration of colony growth. The results were expressed as counts of spores per mL of juice concentrate (spores/mL) and further plotted in graphs describing the N/N_0 relationship ($\log N/\log N_0$), where N_0 is the initial concentration of spores in juice concentrates and N is the number of viable spores at a determined storage time period. Two separate sets of experiments in triplicate were performed on two different days.

2.3. Assessment of the fate of A. acidoterrestris during the enrichment step of the detection procedure

The suspension of *A. acidoterrestris* DSM 2498 was used to inoculate clarified and cloudy lemon juice concentrates to reach 10^4 spores/mL of concentrate as described in Section 2.2.2. The juice concentrates were diluted at proportions of 1:9 and 1:19 in YSG broth (i.e., 10 g of juice concentrate added to 90 mL and 190 mL of YSG broth, respectively). The pH of these solutions was adjusted to 3.75, followed by heat shock at 80 °C for 10 min and incubation at 45 °C for up to 6 days (IFU and of F.J.P., 2007a,b). Every 24 h, the concentration of vegetative cells of *A. acidoterrestris* DSM 2498 was determined using a spread plate method with YSG agar. Additionally, the concentration of spores at the end of incubation period (6 days) was determined by applying a heat shock of 80 °C for 10 min, plating in YSG agar and incubation at 45 °C for 5 days. This procedure was adopted to confirm that spores were truly inactivated. The colonies grown in YSG agar were counted and the results expressed as CFU/g. These experiments were performed in duplicate and repeated twice.

2.4. Chemical analysis of clarified and non-clarified lemon juice concentrates

2.4.1. Concentration of the main organic acids

The L-malic, citric and D-isocitric acid contents were determined by enzymatic methods using commercially available test kits (Boehringer Mannheim catalogues nos. 139.068, 139.076 and 414433) (Boehringer Mannheim, Germany). The differences in absorbance between the

blank and juice concentrate samples were determined using a UV–vis spectrophotometer (Shimadzu model 1800, Shimadzu, Japan).

2.4.2. Concentration of flavonoids and limonin

The chemical standards hesperidin and naringin were obtained from Fluka (Fluka Buchs, Schwiez, Switzerland), whereas the neohesperidin dihydrochalcone, eriocitrin, neoeriocitrin, narirutin and limonin were obtained from Sigma (Sigma-Aldrich, Saint Louis, USA).

Juice concentrate samples were prepared for these analytical determinations as indicated by IFFJP (1991), Shaw and Wilson (1984) and Widmer (1991). HPLC analyses were performed in a Shimadzu SCL-10AVP liquid chromatograph (Shimadzu, Singapore) equipped with a LC20AD pump, column oven CTO 20AC, controller CBM 20A,

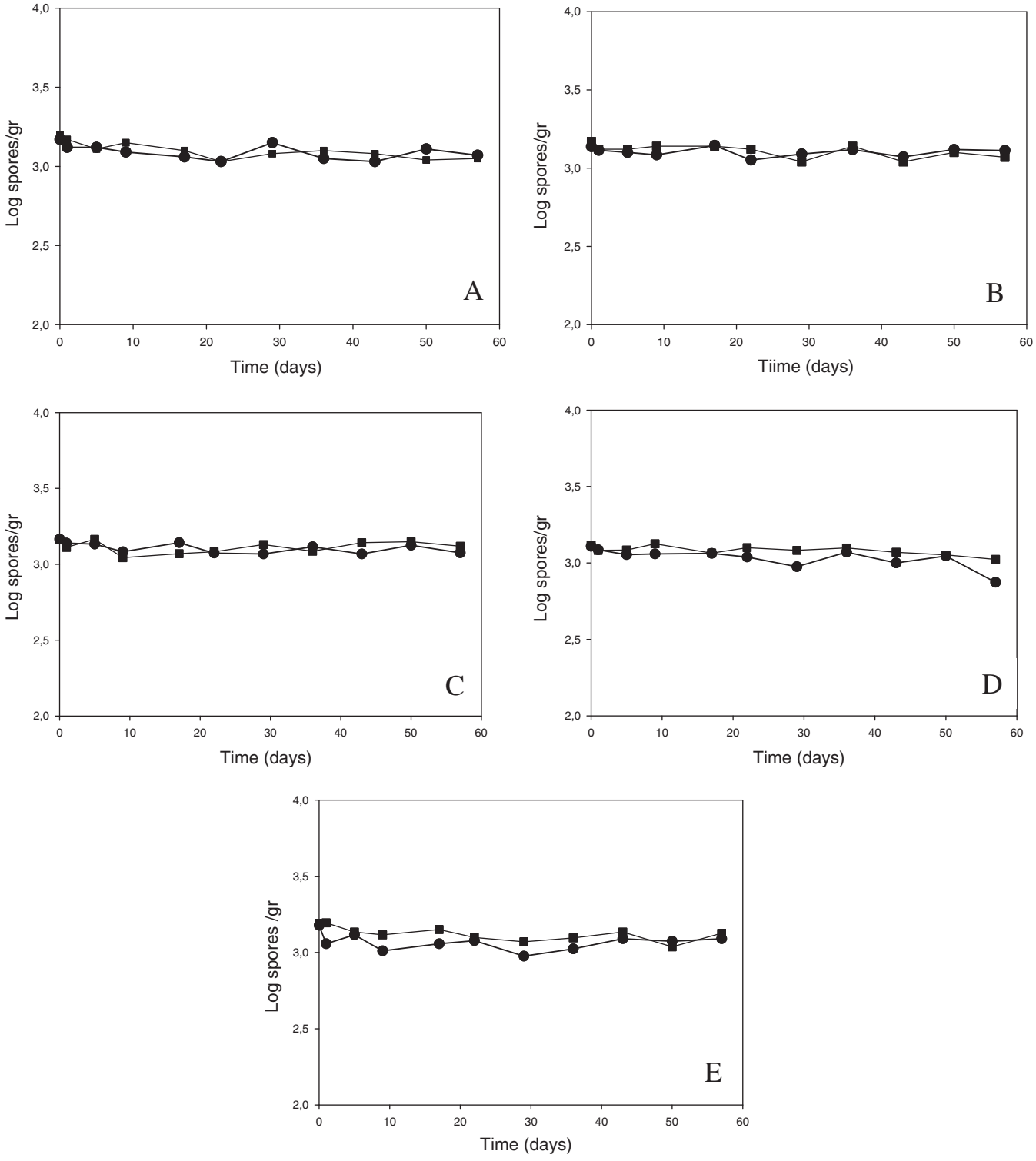

Fig. 1. Fate of *A. acidoterrestris* (DSM 2498) spores inoculated in clarified [grape (A), pear (B) and mango (C)] and cloudy [tangerine (D), and carrot (E)] juice concentrates stored at 4 °C (■) and 20 °C (•) during up to 60 days.

autosampler SIL 20 AHT, and SPD-M20A diode array detector at 280 and 350 nm. Phenomenex (5 μm C18(2) 100 Å, LC Column 250 × 4.6 mm, Ea, Phenomenex, USA) and Cyano (80 Å, 5 μm, 4.6 mm × 250 mm, Spherisorb Cyano (CN) Column, Waters Corporation, USA) columns were used to determine concentrations of flavonoids and limonin, respectively. The column temperature was held at 30 °C for both columns. The mobile phase components were acetonitrile:water:acetic acid (200:800:0.5 v/v/v) at a flow rate of 1 mL/min for flavonoid determination, and 37% solution of acetonitrile in water at a flow rate of 1.5 mL/min for limonin. The injection volume was 20 μL (see Supplemental material for chromatograms).

2.5. Statistical analysis

Significant statistical differences ($p \leq 0.05$) were examined using a one-way analysis of variance (ANOVA) followed by Scott–Knott's test using Assistant version 7.6 free software (Campina Grande, Brazil) (Silva and Azevedo, 2002).

3. Results and discussion

In this study, the fate of *A. acidoterrestris* spores in lemon, red grape, pear, mango, tangerine and carrot juice concentrates stored at different conditions and the impact of dilution procedures in the enrichment broth on the detection of this bacterium were investigated. This information is of foremost relevance in developing effective control strategies and accurate screening programs directed to prevent the spoilage of fruit juices and beverages by this bacterium.

3.1. Fate of A. acidoterrestris spores in fruit and vegetable concentrates during simulated storage

pH values of clarified pear, clarified grape, clarified mango, clarified lemon, cloudy carrot cloudy tangerine and cloudy lemon juice concentrates were 3.5, 3.3, 3.5, 1.7, 4.3, 3.4 and 1.9, respectively. The °Brix values of these juices were 69.4, 67.2, 60.2, 50.8, 68.0, 62.5 and 56.5, respectively. All juice concentrates, except for the lemon juice concentrates, had pH values of >3.0, and soluble solid contents were always above 50° Brix. Both lemon juice concentrates used in this study presented pH values of approximately 1.90. Taking into account these characteristics, it is known that the growth of most microorganisms is unlikely to occur in juice concentrates. In fact, the microbiological stability of juice concentrates has been attributed to high soluble solid content (>40° Brix) and the presence of organic acids and natural antimicrobial compounds in sufficient amounts to inhibit the germination of *Alicyclobacillus* spores (Bevilacqua et al., 2008a,b; Borlinghaus and Engel, 1997; Maldonado et al., 2013; Pinhatti et al., 1997; Splittstoesser et al., 1994, 1998; Walls and Chuyate, 2000). Whether the effectiveness of these compounds as antimicrobials in the juice concentrates is enhanced or not by storage conditions is unknown.

No significant differences ($p > 0.05$) were observed in the counts of *A. acidoterrestris* spores in clarified red grape, pear and mango juice

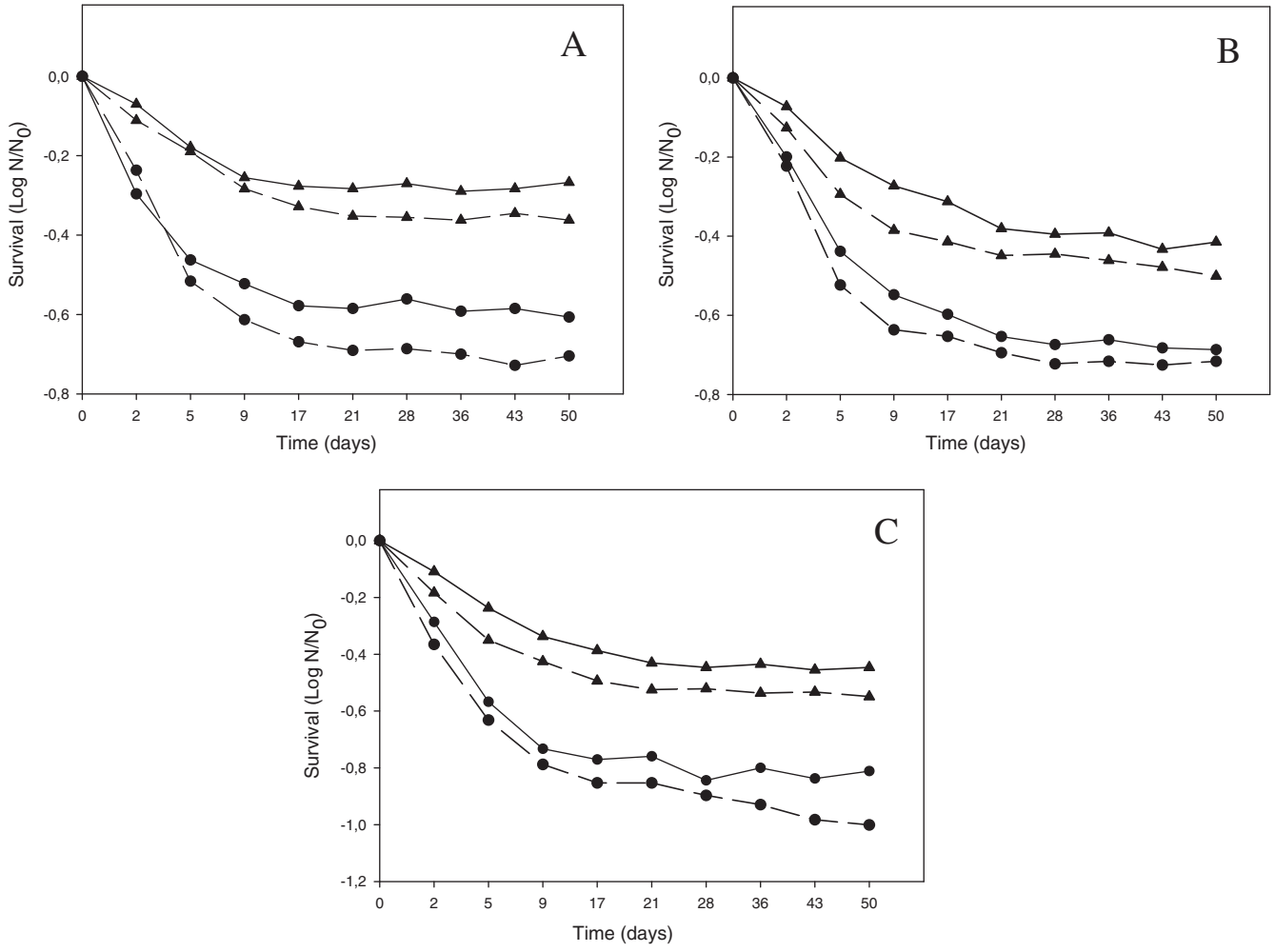

Fig. 2. Survival of *A. acidoterrestris* (DSM 2498) spores inoculated in clarified (▲) and cloudy (•) lemon juice concentrates provided by company A (A), company B (B) and company C (C) stored at 4 °C (—) and 20 °C (— —) during 50 days.

concentrates or in cloudy tangerine and carrot juice concentrates stored at 4 °C and 20 °C for approximately 60 days (Fig. 1). Nonetheless, an initial decline in the counts of *A. acidoterrestris* spores in lemon juice concentrates (independent of juice processor) was observed within 5–10 days of storage, followed by stabilization of spore populations up to the end of the storage period (Fig. 2). Although the counts of spores decreased in lemon juice concentrates, a marked difference was observed when clarified and cloudy lemon juice concentrates were compared ($p < 0.05$). The mean decimal reductions observed in clarified and cloudy lemon juice concentrates were 0.8 and 0.3 log CFU/mL, respectively (Fig. 2). This difference may be a cause of concern for some industries and importers and it can result in false negative results that may further lead to large economic losses and to mistrust of producers by importers and consumers (Oteiza et al., 2011). A potential explanation for these observations would be that compounds with antimicrobial activity naturally present in these juice concentrates could inhibit *Alicyclobacillus*. In addition, it is not clear whether these compounds could also affect the sensitivity of detection methods used in quality control procedures in juice concentrate industries. Thus, considering that both cloudy and clarified lemon juice concentrates presented very similar soluble solid contents and the same adjusted pH, a further investigation of their composition in terms of minor compounds with potential antimicrobial properties was performed to understand whether these compounds could play a role in inhibiting the germination and/or outgrow of *A. acidoterrestris*. It is known that many phytochemicals such as flavonoids, essential oils and organic acids possess antimicrobial proprieties and have been identified in different fruits and juices (Gattuso et al., 2007; Igual et al., 2013; Tripoli et al., 2007). As seen in Table 1, both types of lemon juice concentrates used in this study exhibited different amounts of malic, citric and D-isocitric acids and limonin, but these differences were not significant ($p > 0.05$). In contrast, higher concentrations of eriocitrin and hesperidin were found in the cloudy juice concentrate compared with the clarified lemon juice concentrate. Eriocitrin and hesperidin are two flavonoids commonly found in citrus fruits and juices (Cano et al., 2008; Kanaze et al., 2003; Theodoridis et al., 2006; Tripoli et al., 2007). Although there are few published reports regarding the antimicrobial activities of eriocitrin and hesperidin, it is known that these compounds possess antimicrobial activity against both Gram-positive and Gram-negative bacteria (Bakar et al., 2012; Basile et al., 2000; Kawaguchi et al., 2004). Therefore, this finding could explain the differences in the reduction of *A. acidoterrestris* spores observed between these types of lemon juice concentrates (Table 1).

3.2. Assessment of the fate of A. acidoterrestris during the enrichment broth of the detection procedures

Currently, the International Federation of Fruit Juice Producers (IFU) and Japan Fruit Juice Association (JFJA) methods for *Alicyclobacillus* detection recommend that juice concentrates should be diluted at the proportion of 1:9 in YSG broth, followed by heat shock, plating on appropriate agar and incubation (IFU and of F.J.P., 2007a,b; Japanese Fruit Juice Association, JFJA, 2003). Thus, to investigate the impact of juice dilution on the fate of *A. acidoterrestris*, lemon juice concentrates were diluted at the proportions of 1:9 and 1:19 with YSG broth, followed by heat shock and incubation. As seen in Fig. 3, a decrease in the counts of *A. acidoterrestris* spores, which reached levels below the limit of quantification (<100 spores/g or mL), was observed in samples diluted at the proportion of 1:9 in YSG broth ($p > 0.05$). In contrast, when the proportion of 1:19 was used, *A. acidoterrestris* was able to germinate and outgrow to reach levels as high as 10^7 CFU/g in both cloudy and clarified juice concentrates (Fig. 3). The practical implications of these findings are that, depending on the type of fruit, the juice concentrate samples might need to be diluted at higher proportions (for instance, 1:19) to overcome the inhibitory effects of the natural antimicrobials present. This is particularly critical if one considers that the spore load of *Alicyclobacillus* present in juice concentrates is approximately 10^2 spores/g or mL (Pinhatti et al., 1997; Spinelli et al., 2009). In this case, the chances to report false negative results are high if a proportion of 1:9 is used for the detection of *Alicyclobacillus*.

Table 1
Organic acids, limonin and flavonoids (mg/kg) present in clarified and cloudy lemon juice concentrate[a].

Natural compounds	Clarified	Cloudy
L-Malic	3.8 ± 2.0[a]	2.9 ± 1.4[a]
Citric	53.6 ± 7.6[a]	56.1 ± 4.2[a]
D-Isocitric	279.5 ± 77.2[a]	232.4 ± 60.3[a]
Limonin	88.8 ± 38.2[a]	91.25 ± 2.8[a]
Eriocitrin	383.3 ± 39.4[a]	1059.7 ± 58.8[b]
Hesperidin	122.7 ± 40.9[a]	2135.2 ± 101.1[b]

[a] Values followed by same letter do not differ significantly according to Skott–Knott's test.

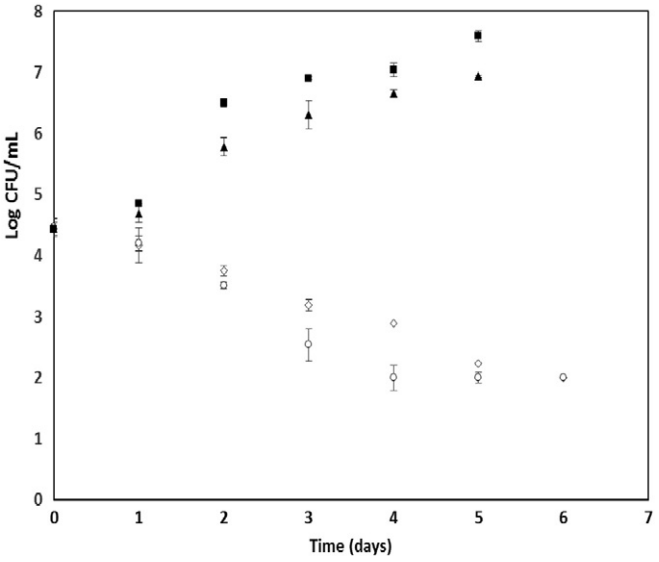

Fig. 3. The fate of *An acidoterrestris* (DSM 2498) inoculated in clarified and cloudy lemon juice concentrates diluted at the proportions of 1:9 and 1:19 with YSG broth. The assessment was done during simulated broth enrichment of a detection procedure. Where: (◇) and (■) are clarified lemon juice concentrates diluted at proportions of 1:9 and 1:19, respectively; (○) and (▲) are cloudy lemon juice concentrates diluted at proportions of 1:9 and 1:19, respectively.

In conclusion, we demonstrated that the number of *Alicyclobacillus* spores remained stable in most types of juice concentrates during storage under the most common conditions employed for their shipping and storage. However, the presence of natural antimicrobials, most likely eriocitrin and hesperidin, caused a decline in the counts of *Alicyclobacillus* spores in lemon juice concentrates. This reduction was more pronounced in cloudy lemon juice concentrates. It is suggested that natural antimicrobials present in lemon juice concentrate can inhibit germination and outgrowth of *Alicyclobacillus* spores if a dilution of 1:9 is used during detection procedures. Thus, a modification in the dilution proportion used in the detection procedures from 1:9 to 1:19 is suggested mainly for juice concentrates in which natural compounds presenting antimicrobial activity may inhibit the germination and outgrowth of *Alicyclobacillus* spp. These findings have relevance for the juice industry and may impact the success of screening and quality control programs as well as the costs resulting from spoilage episodes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2015.05.021.

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