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Prevalence, concentration, spoilage, and mitigation of *Alicyclobacillus* spp. in tropical and subtropical fruit juice concentrates☆

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ABSTRACT

The presence of *Alicyclobacillus* in fruit juices and concentrates poses a serious problem for the juice industry. This study was undertaken to determine the (i) prevalence, concentration, and species of *Alicyclobacillus* in tropical and subtropical concentrates; (ii) efficacy of aqueous chlorine dioxide in reducing *Alicyclobacillus* spp. spores on tropical and subtropical fruit surfaces; and (iii) fate of and off-flavor production by *Alicyclobacillus acidoterrestris* in mango and pineapple juices. One hundred and eighty tropical and subtropical juice concentrates were screened for the presence and concentration of *Alicyclobacillus* spp. If found, the species of *Alicyclobacillus* was determined by 16S rDNA sequencing and analysis with NCI BLAST. Of these samples, 6.1% were positive for *Alicyclobacillus*, and nine *A. acidoterrestris* strains and two *Alicyclobacillus acidocaldarius* strains were identified. A five-strain cocktail of *Alicyclobacillus* spp. was inoculated onto the surface of fruits (grapefruit, guava, limes, mangoes, oranges and pineapple), which were then washed with 0, 50, or 100 ppm aqueous chlorine dioxide. Significant reductions due to chlorine dioxide were only seen on citrus fruits. A five-strain cocktail of *A. acidoterrestris* was inoculated into mango and pineapple juices. Microbial populations were enumerated over a 16-day period. Aroma compounds in the juice were analyzed by GC–olfactometry (GC–O) and confirmed using GC–MS. GC–O of mango juice identified previously reported medicinal/antiseptic compounds. GC–O of pineapple juice revealed an unexpected "cheese" off-aroma associated with 2-methylbutyric acid and 3-methylbutyric acid.

1. Introduction

The presence of *Alicyclobacillus* in acidic fruit juices poses a serious problem for the juice industry. Standard pasteurization processes utilizing temperatures of 85–95 °C that are used within these industries are aimed at the destruction of pathogens such as *Escherichia coli* O157:H7 and *Salmonella*, and are not effective against thermotolerant spore-forming spoilage bacteria (Splittstoesser et al., 1994). Thermal processes stringent enough to destroy heat-resistant *Alicyclobacillus* spores in these products are not feasible as they are potentially damaging to the product quality (Walls and Chuyate, 1998; Palop et al., 2000). Since the 1982 discovery of apple juice spoiled by a thermoacidophilic, spore-forming bacteria (Cerny et al., 1984), *Alicyclobacillus* has been isolated from many juices and concentrates, including: apple, cherry, cranberry, grapefruit, mango, orange, pear, tomato, and white grape (Cerny et al., 1984; Splittstoesser et al., 1994, 1998; Wisse and Parish, 1998; Eiroa et al., 1999; Gouws et al., 2005).

Spoilage by *Alicyclobacillus* lacks the typical gas production, turbidity, and heavy sediment classically associated with other bacterial spoilages of juices (Walls and Chuyate, 1998). In apple and orange juice, a smoky, medicinal, antiseptic off-odor associated with the chemical guaiacol, which can be detected by humans at as low a concentration as 2 ppb, is typically the primary sign of spoilage (Splittstoesser et al., 1998; Pettipher et al., 1997; Orr et al., 2000; Jensen and Whitfield, 2003; Gocmen et al., 2005). Other compounds, including 2,6-dibromophenol and 2,6-dichlorophenol,

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have also been associated with *Alicyclobacillus* contamination (Jensen and Whitfield, 2003; Gocmen et al., 2005). Bacterial levels capable of producing these compounds at or above the human sensory threshold can be well below that visibly detectable in juice (Pettipher et al., 1997), thus necessitating control of *Alicyclobacillus* on incoming raw product and in the processing environment.

Little work has been done to thoroughly analyze the prevalence of *Alicyclobacillus* in the juice industry and no published work establishes potential contamination levels. Thirty-five percent of companies responding to a 1998 survey by the National Food Processors Association (primarily apple juice producers) had seen spoilage consistent with *Alicyclobacillus* contamination; however contamination events were not always verified (Walls and Chuyate, 1998). *Alicyclobacillus* contamination has been documented in 3 of 33 commercial juice products (Splittstoesser et al., 1998), 4 of 4 U.S. apple juice samples, 1 of 4 U.S. apple concentrate samples, 0 of 56 U.K. ultrapasteurized apple juice samples, and 5 of 18 U.K. fresh apple juice products (Splittstoesser et al., 1998), and 11 of 75 orange juice samples from 11 Brazilian producers (Eiroa et al., 1999). In addition to being found in juice and juice products, *Alicyclobacillus* has been isolated from raw products and in raw product production and juice processing environments (McIntyre et al., 1995; Wisse and Parish, 1998; Parish and Goodrich, 2005; Chen et al., 2006; Groenewald et al., 2009; Wang et al., 2010). These studies have lead to the perception that *Alicyclobacillus* contamination is a fairly widespread problem within the industry; however they do not adequately address the prevalence of this contamination.

Given the lack of information related to *Alicyclobacillus* spp. in non-orange and apple juice and juice production environments, this study was undertaken to determine the (i) prevalence, most probable number (MPN), and species of *Alicyclobacillus* in tropical and subtropical concentrates; (ii) efficacy of aqueous chlorine dioxide in reducing *Alicyclobacillus* spp. spores on tropical and subtropical fruit surfaces; and (iii) fate of and off-flavor production by *Alicyclobacillus acidoterrestris* in mango and pineapple juices.

2. Materials and methods

2.1. Isolation of Alicyclobacillus from tropical concentrates

One hundred and eighty tropical and subtropical fruit concentrates were collected from juice processing facilities in central Florida, USA and included both domestic and imported concentrates. Samples were tested for the presence of *Alicyclobacillus* by adding 50 g of concentrate to 450 ml of *Alicyclobacillus* broth (AB; Wisse and Parish, 1998), and heating to 75 °C in a water bath with a 20 min hold time. The formulation for AB includes in g/L distilled water: 0.2 $(NH_4)_2SO_4$; 0.5 $MgSO_4 \times 7H_2O$; 0.25 $CaCl \times 2H_2O$; 3.0 KH_2PO_4; 1 glucose; 2 soluble starch; and 2 yeast extract. The pH was adjusted to 3.5 with a (1 N) H_2SO_4 solution before autoclaving. Samples were held at 75 °C for 20 min. Immediately after heat treatment, samples were placed on ice to cool. Heat-shocked samples were incubated for 5 days at 45 °C then were streaked onto *Alicyclobacillus* agar (AA; Wisse and Parish, 1998), Acidified Potato Dextrose Agar (APDA, Difco Laboratories, Detriot MI) and Plate Count Agar (PCA, Difco Laboratories, Detriot MI). The formulation for AA includes in g/L distilled water: 0.4 $(NH_4)_2SO_4$; 1.0 $MgSO_4 \times 7H_2O$; 0.5 $CaCl \times 2H_2O$; 6.0 KH_2PO_4; 2 glucose; 4 soluble starch; and 4 yeast extract. The pH was adjusted to 3.5 with a (1 N) H_2SO_4 solution before autoclaving. An equal volume of Bacto-agar (Difco Laboratories, Detriot MI) was prepared to a final concentration of 3.5%. The two solutions were autoclaved and tempered to 50 °C before mixing and pouring (Wisse and Parish, 1998). Plates were incubated at 45 °C, with an additional PCA plate incubated at 25 °C. Cells from colonies growing on AA and APDA after up to 5 days were Gram stained.

2.2. Presumptive Alicyclobacillus

When colonies grew on AA, and/or APDA, while no growth was visible on corresponding PCA plates, and cells from colonies on AA and/or APDA were positive upon Gram staining, samples were considered presumptive *Alicyclobacillus* positive and selected for further examination.

2.3. Genotypic identification

Presumptive *Alicyclobacillus* isolates were grown 18 ± 2 h in AB at 45 °C. DNA lysates for PCR amplifications were prepared from these isolates as described by Durak et al. (2010). Briefly, the 16s rRNA gene was amplified by PCR using universal forward (5′-AGAGTTTGATCCTGGCTCAG-3′) primer (Weisburg et al., 1991) and a reverse primer (5′-GGTATCTAATCCTGTTTGC-3′) which was the complementary sequence of a previously published primer (Rothman et al., 2002). PCR products were purified using Millipore Montage PCR (Bedford, MA) and sequenced using an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems) at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR), DNA Sequencing Core (Gainesville, FL). Resulting 16s rRNA gene sequences were used for homology comparison with NCI BLAST nucleotide search database.

2.4. Most probable number of Alicyclobacillus in concentrates

A 3-tube MPN technique was used. One gram of confirmed *Alicyclobacillus* positive concentrate was weighed into 9 ml sterile Phosphate Buffered Saline (PBS). Following heat shock at 75 °C for 20 min (described above) serial dilutions were made in PBS. One milliliter aliquots of appropriate dilutions were added to 3 tubes of 9 ml AB and incubated at 45 °C for 5 days. Growth, determined by visual cloudiness, in AB following 5 days was considered a positive tube. MPN of *Alicyclobacillus* spores per gram was determined using FDA Bacteriological Analytical Manual statistical tables (US FDA, 2007).

2.5. Selection of test strains

Five-strain cocktails of *A. acidoterrestris* and *Alicyclobacillus* spp. isolated from fruit concentrates or soils were used for fate and off-flavor production in mango and pineapple juices and efficacy of aqueous chlorine dioxide on tropical and subtropical fruit surfaces, respectively. Strains of *Alicyclobacillus* acidoterresteris (identified by their strain reference number and source) included: MDD268 (frozen concentrated orange juice from the Netherlands); MDD267 (frozen concentrated orange juice from Brazil) MDD180 (soil from a citrus grove in Brazil); MDD35 (frozen pineapple concentrate from the Philippines); and, MDD31 (frozen mango puree from Mexico). Strains used for aqueous chlorine dioxide (identified by their strain reference number and their sources) included: *A. acidoterrestris* (MDD268; frozen concentrated orange juice from the Netherlands); *A. acidoterrestris* (MDD267; frozen concentrated orange juice from Brazil); *A. acidoterrestris* (MDD180; soil from a citrus grove in Brazil); *Alicyclobacillus acidocaldarius* (MDD266; frozen mango concentrate from Mexico); and *A. acidocaldarius* (MDD 265; frozen coconut cream from the Dominican Republic).

2.6. Inoculum preparation

Prior to the experiment, a frozen stock culture of each *Alicyclobacillus* strain was inoculated by streaking onto AA and incubating at 45 °C until 90% sporulation was achieved (5–10 days). Sporulation was determined using a hemocytometer. Spores were then harvested