EVALUATION OF THE DISTRIBUTION AND ACCUMULATION OF SPECIES OF *ALICYCLOBACILLUS* IN THE FRUIT CONCENTRATE PROCESSING ENVIRONMENT

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DECLARATION

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ABSTRACT

Alicyclobacillus species are thermo-acidophilic bacteria that produce highly resistant endospores able to survive the processing temperatures of fruit concentrate manufacturing, including evaporation and conventional pasteurisation (86 °- 96 ℃ for ± 2 min). *Alicyclobacillus* endospores retain their viability in juice concentrates which, under favourable conditions, could germinate and multiply to numbers high enough to cause spoilage and product deterioration through the production of chemical taint compounds. This thesis reports on the distribution of *Alicyclobacillus* in the fruit concentrate processing environment and the effect of current manufacturing practices on the accumulation of *Alicyclobacillus* in fruit concentrates. These practices include the recirculation (recycling) of flume water as a means of water conservation, as well as continuous process running times when facilities operate at full capacity. This thesis also reports on the effect of fruit variety and skin type on the occurrence of *Alicyclobacillus* in fruit concentrates.

Alicyclobacillus was monitored at nine processing stages of fruit concentrate manufacturing during the functioning of either a recirculating or a one-pass (not recirculated) flume water system. Significantly higher *Alicyclobacillus* levels were recovered in fruit mash, single strength juice, concentrate and the final pasteurised product (± 30 ℃Brix) during the functioning of a re circulating flume system, compared to when a one-pass flume system was functional ($P < 0.05$). Irrespective of the flume system, high *Alicyclobacillus* levels were recovered from the concentrate and condensate water (a by-product of juice concentration) from the evaporator, which makes this a point of concern during concentrate manufacturing. Manufacturing practices such as the recirculation of flume water and the recovery of condensate water for fruit washing purposes pose a potential risk of *Alicyclobacillus* contamination and accumulation in fruit concentrates and the processing environment.

The effect of continuous process running times on *Alicyclobacillus* was monitored in a facility that was operating at full capacity. Sampling occurred every 12 h at four processing stages, during a processing tempo of 1.8 - 2.0 t h^{-1} for 108 h. Vegetative cells increased significantly ($P < 0.05$) in single strength juice and condensate water after 84 h of processing, with 3.15 and 3.85 $\log_{10}$ cfu mL^{-1} recovered, respectively. Similar accumulation patterns of vegetative cells were observed in concentrate and the final pasteurised product. Endospores in single strength juice, concentrate and the final product were also the highest after 84 h of processing with 1.32, 1.59 and

1.64 $\log_{10}$ cfu mL^{-1}, respectively. When fruit concentrate manufacturing facilities process at full capacity, a restriction in the continuous process running time to under 84 h in between CIP procedures, along with good manufacturing practices, can minimise *Alicyclobacillus* accumulation in fruit concentrates.

The effect of fruit skin type, specifically hairy-skinned stone fruits (peach and apricot) and smooth-skinned pome fruits (apple and pear) on the occurrence of *Alicyclobacillus* in concentrates were examined. Apple concentrate samples had the highest occurrence (average %) of vegetative *Alicyclobacillus* cells (50%), followed by apricot (40%), peach (15%) and pear (10%) concentrates. The occurrence of *Alicyclobacillus* endospores in fruit concentrate samples were also the highest in apple (50%), followed by pear (25%), apricot (20%), and peach (10%) concentrates. The occurrence of *Alicyclobacillus* vegetative cells and endospores did not differ significantly ($P > 0.05$) between concentrates from hairy-skin and smooth-skin fruit varieties. Thus it was concluded that fruit washing steps prior to processing was more critical for the control of *Alicyclobacillus* than the type of fruit skin being processed.

UITTREKSEL

Alicyclobacillus spesies is termo-asidofiliese bakterieë wat hoogs bestande endospore produseer met die vermoë om prosesseringstemperature, insluitend verdamping en konvensionele pasteurisasie temperature (86 ° - 96 ℃ vir ± 2 min), tydens die vervaardiging van vrugtekonsentraat te oorleef. *Alicyclobacillus* endospore behou hul lewensvatbaarheid in vrugtekonsentrate en kan in gunstige toestande ontkiem en vermeerder tot getalle wat wansmake in produkte kan veroorsaak weens die produksie van chemiese verbindings. Hierdie tesis doen verslag oor die verspreiding van *Alicyclobacillus* in die vrugtekosentraat prosesseringsomgewing en oor die effek van huidige produksie praktyke op die akkumulasie van *Alicyclobacillus* in vrugtekonsentrate. Die praktyke sluit in, die hersirkulering van leigeut (transport) water as ‘n wyse van waterbesparing, asook aaneenlopende prosesseringstye wanneer vrugtekosentraat fabrieke teen ‘n volle kapasiteit prosesseer. Daar word ook verslag gedoen oor die effek van verskillende vrug variëteite en vel tipes op die voorkoms van *Alicyclobacillus* in vrugtekonsentrate.

Alicyclobacillus was gemonitor by nege verskillende stadiums van ‘n vrugtekosentraat prosesseringsfabriek tydens die funksionering van óf 'n hersirkulerende óf ‘n deurlopende (nie-hersirkulerende) leigeut waterstelsel. *Alicyclobacillus* vlakke was beduidend hoër in gemaalde vrugte, enkelsterkte sap, konsentraat en die finale gepasteuriseerde produk (± 30 ℃Brix), gedurende die funksionering van 'n hersirkulerende leigeutstelsel, in vergelyking met die funksionering van ‘n deurlopende leigeutstelsel ($P < 0.05$). Ongeag van die leigeutstelsel, is hoë vlakke *Alicyclobacillus* gevind in konsentraat en kondensaat water ('n by-produk van die sap konsentrasie porses) vanuit die verdamper, en maak dit dus ‘n punt van belang tydens die vervaardiging van vrugtekonsentraat. Daar is gevind dat vervaardigingspraktyke soos die hersirkulasie van leigeut water en die herwinnig van kondensaat water moontlike risiko’s inhou vir die besoedeling en akkumulasie van *Alicyclobacillus* in vrugtekosentrate en die prosesseringsomgewing.

Die effek van aaneenlopende prosesseringstye op *Alicyclobacillus* was gemonitor in 'n vrugtekosentraat prosesseringsfabriek wat teen volle kapasiteit prosesseer. Steekproefneming het elke 12 h by vier prosesseringsstadiums geskied, tydens 'n prosesseringstempo van 1.8 - 2.0 t h^{-1} vir 108 h. Vegetatiewe selle het beduidend toegeneem ($P < 0.05$) in die enkelsterkte sap en kondensaat water na 84 uur van prosessering, met 3.15 en 3.85 $\log_{10}$ kve mL^{-1}, onderskeidelik verhaal. Soortgelyke

akkumulasiepatrone vir vegetatiewe selle was waargeneem in konsentraat en die finale gepasteuriseerde produk. Endospore in enkelsterkte sap, konsentaat en die finale produk was ook die hoogste na 84 uur van prosessering, met 1.32, 1.59 en 1.64 $\log_{10}$ kve mL^{-1}, onderskeidelik. Wanneer vrugtekonsentraat fabrieke teen volle kapasiteit prosesseseer, kan 'n beperking in aaneenlopende prosesseringstye tot onder 84 h tussen CIP prosedures, gepaard met goeie vervaardigingspraktyke, die akkumulasie van *Alicyclobacillus* in vrugte konsentate verminder.

Die effek van verskillende vrug variëteite se vel tipes, spesifiek harige-vel steenvrugte (perske en appelkoos) en gladde-vel kernvrugte (appel en peer) op die voorkoms van *Alicyclobacillus* in vrugtekonsentrate was ondersoek. Appel konsentaat monsters het die hoogste voorkoms van vegetatiewe *Alicyclobacillus* selle gehad (gemiddelde %), met (50%), gevolg deur appelkoos (40%), perske (15%) en peer (10%) konsentraat. Die voorkoms van *Alicyclobacillus* endospore in vrugte konsentraat monsters was weer die hoogste in appel (50%), gevolg deur peer (25%), appelkoos (20%), en perske (10%) konsentraat. Die voorkoms van *Alicyclobacillus* vegetatiewe selle en endospore het nie betekenisvol tussen konsentrate van harige-vel en gladde-vel vrug variëteite verskil nie ($P > 0.05$). Die gevolgtrekking was dat vrugte wasstappe, voor die prosessering van vrugtekonsentraat, van meer belang is vir die beheer van *Alicyclobacillus* as die vel tipe van die vrug variëteit wat geprosesseer word.

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CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

Preserved and packaged fruit products have a significant place in modern consumption markets. Strong growth in emerging middle-class markets are indicated by the demand for industrially packaged fruit concentrates, pulps and purees (Ross, 2007). These concentrated fruit products are valuable semi-prepared food components to the bakery, dairy, confectionary, canning, baby food, frozen food, distilling and beverage industries. Not only do they contribute to the functional properties of food products, due to a high pectin and fibre content, they also enrich products with characteristic colours, valuable extracts, tastes and aromas (Hegenbart, 1994). In the beverage industry, fruit concentrates, pulps and purees ensure the availability of a continuous supply of fruit juice and fruit juice-containing beverages globally (Hui *et al*., 2006; Ross, 2007; Anon., 2010). However, there are continuous pressures to improve the quality of processed fruit products in order for reconstituted fruit beverages to be competitive with fruit beverages that are made from fresh fruits (Hui *et al*., 2006; Ross, 2007).

Due to their intrinsic physical and chemical properties, including a high sugar content, low pH, high viscosity, reduced water activity and low oxygen and nitrogen contents, concentrated fruit products are considered commercially sterile and have significantly improved transportation and storage properties (Eiroa *et al.*, 1999; Palop *et al.*, 2000; Maldonado *et al.*, 2008). Upon dilution, fruit beverages and fruit based products are susceptible to spoilage by acid tolerant, low heat resistant microbes such as yeasts, mycelial fungi and lactic acid bacteria. Consequently, these products undergo a pasteurisation treatment to prevent spoilage (Walls & Chuyate, 1998; Eiroa *et al.*, 1999; Deák, 2008). Whilst it is unlikely that bacterial endospores will be destroyed by conventional pasteurisation treatments (86 ° - 96 ℃ for ± 2 min), it is believed that the natural acidic environment (pH < 4.0) of fruit products will act as a control measure against bacterial spoilage (Cerny, 1980; Pontius *et al.*, 1998; Silva & Gibbs, 2004). For this reason, it was generally assumed that pasteurisation of high acid food products would allow for an extended shelf-life under ambient conditions (Silva & Gibbs, 2004).

In recent years, *Alicyclobacillus* has been isolated from several fruit concentrates that formed part of various spoilt high-acid, shelf-stable food products (Pinhatti *et al*., 1997; Wisse & Parish, 1998; Eiroa *et al.*, 1999; Silva & Gibbs, 2004; Gouws *et al*., 2005;

Chen *et al.,* 2006; Durak *et al.,* 2010) and *Alicyclobacillus* accordingly become a major concern to the food industry worldwide (Chang & Kang, 2004; Walker & Philips, 2008). Species of *Alicyclobacillus* are thermo-acidophilic, non-pathogenic, endospore-forming and have an exceptional ability to survive thermal processing applications applied during fruit concentrate production. They favour acidic environments and are unaffected by the high soluble solid content of concentrated fruit products (Yamazaki *et al.*, 1996; Murakami *et al.,* 1998; Pontius *et al.,* 1998). The high soluble solid content of fruit concentrates (> 20 °Brix) increases the thermal resistance of *Alicyclobacillus* endospores and inhibit their growth and germination. These endospores will retain their viability and under favourable conditions could germinate and multiply to numbers high enough to cause spoilage and product deterioration (Chang & Kang, 2004; Maldonado *et al.*, 2008; Ceviz *et al.*, 2009). A combination of factors are attributed to creating a favourable environment for product spoilage to occur, including the *Alicyclobacillus* species present, the contamination level, temperature, available oxygen, the medium constituents, soluble solid content, oxidation-reduction potential (Eh) and pH (Borlinghaus & Engel, 1997; Walker & Philips, 2008).

Spoilage caused by *Alicyclobacillus* in fruit beverage products is widely reported to be flat sour type spoilage with distinct offensive smelling medicinal or antiseptic characteristics. Furthermore, spoilage have been attributed mainly to the formation of chemical taint compounds, specifically 2-methoxyphenol (guaiacol) and the halogenated phenolic compounds, 2,6-dichlorophenol and 2,6-dibromophenol (Borlinghaus & Engel, 1997; Jensen & Whitfield, 2003; Gocmen *et al.*, 2005).

The ultimate source of *Alicyclobacillus* in the processing environment is soil that adheres to unwashed or poorly washed fruit, soil that is carried into the manufacturing facility from the vicinity, as well as contaminated processing water (McIntyre *et al.,* 1995; Wisse & Parish, 1998; Groenewald *et al.,* 2009). The elimination and control of *Alicyclobacillus* from the processing environment prove to be very difficult and little information is available on how certain manufacturing practices and processing treatments affect *Alicyclobacillus* during fruit concentrate production (Bahçeci *et al.,* 2005; AIJN, 2008; Walker & Philips, 2008).

The aim of this study was to assess the distribution and extent of contamination of *Alicyclobacillus* within the fruit concentrate processing environment. Another aim was to monitor the effect of current manufacturing practices such as the recirculation of flume water and continuous process running times on the accumulation of

Alicyclobacillus in the final processed product. The study also examined the effect of fruit varieties and skin types on the occurrence of *Alicyclobacillus*.

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CHAPTER 2

LITERATURE REVIEW

A. Background

Most fruit beverage products are susceptible to the growth of yeasts, mycelial fungi and lactic acid bacteria due to their pH growth range and ability to grow in high-acid environments (pH < 4.0) (Pontius *et al.*, 1998; Eiroa *et al.*, 1999; Hui *et al*., 2006). The fruit beverage industry applies a hot-fill-hold pasteurisation process, where the product is held at 86 °- 96 ℃ for approximately 2 min to sufficiently destroy mesophilic spoilage microbes (Chang & Kang, 2004; Jay *et al*., 2005a). Thermal treatments during the production of fruit concentrates, along with several inherent physiochemical characteristics, including high sugar concentrations (65 °- 80 ℃Brix), low pH (3.5 - 4.0), the presence of organic acids, reduced water activity (0.85 - 0.99) and reduced oxygen and nitrogen concentrations are strong inhibitors of the growth of most deteriogenic and pathogenic microbes. Spoilage in reconstituted fruit beverages from concentrates is restricted to a small number of endospore-forming, Gram-positive bacteria and heat resistant mycelial fungi that are able to survive the concentration and pasteurisation process (Pontius *et al.*, 1998; Silva & Gibbs, 2004; Jay *et al*., 2005a; Deák, 2008).

Spoilage incidents in high-acid food products often involve anaerobic bacteria such as *Clostridium butyricum* and *Clostridium pasteurianum* that grow and produce gas and butyric odours in canned foods (pH < 4.5). Aerobic bacilli such as *Bacillus coagulans* and *Bacillus megaterium* are known to cause flat-sour type spoilage in acidic fruit beverages (Brown, 2000; Silva & Gibbs, 2004), whereas *Lactobacillus plantarum* var. *mobilis*, *Lactobacillus brevis*, *Leuconostoc mesenteroides* and *Leuconostoc dextranicum* are known to cause vinegary, buttermilk off-odours and off-flavours in frozen concentrated orange juice (33 ℃Brix) (Hays & Riester, 1952). Additionally, heat resistant species of mycelial fungi such as *Byssochlamys fulva*, *Byssochlamys nivea*, *Neosartorya fischeri* and *Talaromyces flavus* are reported to spoil a number of foodstuffs including fruit juice, pulps, concentrates and canned fruits (Yamazaki *et al.*, 1996; Silva & Gibbs, 2004; Deák, 2008).

In 1982, a new type of spoilage bacterium emerged during a large-scale spoilage incident in Germany (Cerney *et al.*, 1984). Flat-sour type spoilage with offensive smelling medicinal or antiseptic characteristics was noted in commercial pasteurised

apple juice. The microbe responsible for the incident was a thermo-acidophilic, endospore-forming bacterium that was subsequently identified as *Alicyclobacillus acidoterrestris* (Cerny *et al*., 1984; Deinhard *et al*., 1987a; Wisotzkey *et al*., 1992).

Since then, spoilage incidents by members of the genus *Alicyclobacillus* have been reported frequently and include a diverse range of high-acid, shelf-stable fruit and vegetable products that were either hot-filled, pasteurised, canned, ultra heat-treated or carbonated (Chang & Kang, 2004; Gouws *et al.*, 2005; Walker & Philips, 2008a). A 2005 survey by the European Fruit Juice Association (AIJN) showed that 45% of the 68 participants from the fruit processing industry experienced *Alicyclobacillus* related problems in the three years prior to the survey, including 33% experiencing problems more than once. The survey concluded that *Alicyclobacillus* has a considerable impact in processed fruit products, especially in raw materials (beverage bases) and fruit juice concentrates (Howard, 2006).

B. Historical perspective

Prior to the large-scale German spoilage incident, the presence of thermo-acidophilic, endospore-forming bacteria was inconceivable. The earliest reports of these bacteria date back to 1967 in Tokohu, Japan when they were isolated by Uchino & Doi from acid springs (pH 2.0 - 3.0) with water temperatures reaching 75 °- 80 ℃. These bacteria showed close similarities to *B. coagulans* and they were tentatively categorised as an obligate thermophillic strain of this species as they had the ability to grow at 55 ℃ (Uchino & Doi, 1967). Studies from all over the world have confirmed the presence of thermo-acidophilic, endospore-forming bacteria from geothermal sites, including Yellowstone National Park (USA) (Darland & Brock, 1971), Volcano National Park (Hawaii), Piciarelli (Italy) (De Rosa *et al.,* 1971), and **Kunashir Island** (Russia) (Loginova *et al.*, 1978). However, unlike the *B. coagulans* strain from Uchino & Doi*,* these bacteria possessed unique membrane lipids with hopanoids and up to 65% ω-cyclohexane fatty acids (De Rosa *et al.,* 1971), their optimum growth pH was lower and the DNA composition indicated a higher G+C content of approximately 62 mol%, compared to 45 - 50 mol% for *Bacillus* spp. Consequently a new species was proposed, *Bacillus acidocaldarius* (Darland & Brock, 1971).

In a search for relatives of *B. acidocaldarius*, Hippchen *et al*. (1981) isolated several thermo-acidophilic, endospore-forming bacteria with similar membrane properties from a variety of neutral (non-acidic), non-thermal environments. Cerney *et*

al. (1984) reported that the German apple juice spoilage incident in 1982 was due to the presence of a *Bacillus* species with unique ω-cyclohexane fatty acids and hopanoids that closely resembled those of *B. acidocaldarius* and the strains isolated from neutral environments by Hippchen *et al*. (1981). Ultimately, Deinhard *et al.* (1987a) identified the relationships among ω-cyclohexane fatty acid possessing bacilli. Their taxonomic investigations led to the proposal of two new species namely, *Bacillus acidoterrestris,* the name referring to the acid and soil environments from which it has been isolated from, and *Bacillus cycloheptanicus*, named on account of the unique ω-cycloheptane fatty acids that the cells contain (Deinhard *et al.*, 1987a; b). Contrary to its relatives, *B. acidoterrestris* had the ability to utilise erythritol, sorbitol and xylitol as a carbon source and produce acid. The growth temperature of *B. acidoterrestris* (35 °- 53 ℃) was lower than that of *B. acidocaldarius* (45 °- 70 ℃), whereas *B. cycloheptanicus* (40 °- 53 ℃) had the narrowest growth range. In addition, DNA-DNA hybridisation revealed that *B. cycloheptanicus* had a low similarity to *B. acidocaldarius* and *B. acidoterrestris* and that it contained an obligate nutrient requirement for methionine, isoleucine and pantothenate (Deinhard *et al.*, 1987a; b).

In 1992, comparative sequence analysis of the 16S ribosomal ribonucleic acid (rRNA) of these bacteria revealed that *B. acidocaldarius, B. acidoterrestris* and *B. cycloheptanicus* are genetically unique and differ significantly from the traditional *Bacillus* species (Wisotzkey *et al*., 1992). It was then proposed that these three bacilli are reclassified into a new genus, *Alicyclobacillus* gen. nov., in the family *Bacillaceae*. As a result, the species were renamed *Alicyclobacillus acidocaldarius*, *Alicyclobacillus acidoterrestris* and *Alicyclobacillus cycloheptanicus* (Wisotzkey *et al.*, 1992). To date, 20 species and 2 subspecies that belong to the genus *Alicyclobacillus* have been identified (Table 1) (Euzéby, 2010).

C. The genus *Alicyclobacillus*

General characteristics

Alicyclobacillus colonies are generally round, non-pigmented or creamy white, translucent to opaque and between 0.3 - 5.0 mm in diameter on growth media (Chang & Kang, 2004; Murray *et al*., 2007). *Alicyclobacillus* comprise of small rod-shaped (9 - 4.3 µm x 0.3 - 1.0 µm), Gram-positive to Gram-variable, catalase positive bacteria that have the ability to growth under aerobic to facultative anaerobic conditions. A distinctive characteristic of *Alicyclobacillus* is the cell membrane, which consists mainly

Table 1 Species and subspecies belonging to the genus *Alicyclobacillus*

Species	Source	Reference
Alicyclobacillus acidiphilus	Acidic beverages	Matsubara *et al*., 2002
Alicyclobacillus acidocaldarius	Acid thermal water	Darland & Brock, 1971; De Rosa *et al.,* 1971; Loginova *et al*., 1978; Wisotzkey *et al*., 1992
Alicyclobacillus acidocaldarius subsp. *acidocaldarius*	Acid thermal habitats	Darland & Brock, 1971; Wisotzkey *et al*., 1992
Alicyclobacillus acidocaldarius subsp. *rittmannii*	Geothermal soil (Mount Rittmann, Antarctica)	Nicolaus *et al*., 1998
Alicyclobacillus acidoterrestris	Soil, Apple juice	Deinhard *et al*., 1987a; Wisotzkey *et al*., 1992
Alicyclobacillus aeris	Copper mine	Guo *et al*., 2009
Alicyclobacillus contaminans	Soil from crop fields (Fuji city, Japan)	Goto *et al*., 2007
Alicyclobacillus cycloheptanicus	Soil	Uchino & Doi, 1967; Deinhard *et al*., 1987b; Wisotzkey *et al*., 1992
Alicyclobacillus disulfidooxidans	Oxidisable lead-zink ores, Waste water sludge	Dufresne *et al*., 1996; Karavaiko *et al*., 2005
Alicyclobacillus fastidiosus	Apple juice	Goto *et al*., 2007
Alicyclobacillus ferrooxydans	Solfataric soil	Jiang *et al*., 2008
Alicyclobacillus herbarius	Herbal tea	Goto *et al*., 2002
Alicyclobacillus hesperidum	Solfataric soil (São Miquel, Greece)	Albuquerque *et al*., 2000
Alicyclobacillus kakegawensis	Soil from crop fields (Kakegawa city, Japan)	Goto *et al*., 2007
Alicyclobacillus macrosporangiidus	Soil from crop fields (Fujieda city, Japan)	Goto *et al*., 2007,
Alicyclobacillus pohliae	Geothermal soil (Mount Melbourne, Antarctica)	Imperio *et al*., 2008
Alicyclobacillus pomorum	Mixed fruit juice	Goto *et al*., 2003
Alicyclobacillus sacchari	Sugar	Goto *et al*., 2007.
Alicyclobacillus sendaiensis	Soil (Sendai city, Japan)	Tsuruoka *et al*., 2003
Alicyclobacillus shizuokensis	Soil (Shizuoka city, Japan)	Goto *et al*., 2007
Alicyclobacillus tolerans	Waste water sludge	Karavaiko *et al*., 2005
Alicyclobacillus vulcanalis	Coso hot springs (California, USA)	Simbahan *et al*., 2004

of ω-alicyclic fatty acids that contain six to seven closely packed carbon rings (Deinhard *et al.*, 1987a; b; Wisotzkey *et al.*, 1992). Growth temperatures, depending on the species, range between 20 ° - 70 ℃ with optimum te mperatures from 42 ° - 60 ℃ (Wisotzkey *et al.*, 1992; Chang & Kang, 2004). *Alicyclobacillus* are obligate acidophilic, with growth reported between pH 2.0 - 6.0 (Wisotzkey *et al.*, 1992; Yamazaki *et al*., 1996; Walls & Chuyate, 1998). Under adverse conditions, oval shaped endospores (1.5 - 1.8 µm x 0.9 - 1.0 µm) are formed, and the sporulation process is tolerant of oxygen. Endospores are located terminally or sub-terminally and can cause the sporangium to swell (Deinhard *et al.*, 1987a; b; Wisotzkey *et al.*, 1992). Furthermore, *Alicyclobacillus* species have more than a 92% sequence similarity based on the rRNA genes and the G+C DNA content range between 48.7 - 62.7 mol% (Wisotzkey *et al.*, 1992; Karavaiko *et al.*, 2005).

Pathogenicity

Pathogenicity was a natural concern for food and beverage industries worldwide, since *Alicyclobacillus* had the potential to spoil a variety of processed, high-acid fruit and vegetable products (Walls & Chuyate, 2000; Chang & Kang, 2004; Walker & Philips, 2008a). In a study to test the pathogenicity of *Alicyclobacillus*, mice were directly injected with *A. acidoterrestris* endospores and guinea pigs were fed spoiled juice containing > 5 x 10^6 cfu mL^{-1} *A. acidoterrestris* endospores. No illnesses or deaths were reported in the mice or guinea pigs at the specific levels (Walls & Chuyate, 2000). Unlike other spoilage microbes in high-acid fruit products, the growth of *A. acidoterrestris* do not raise the product pH above 4.5 and consequently there are no risk of secondary growth by pathogenic food-borne bacteria such as *Clostridium botulinum*, *C. perfringens, Bacillus cereus, B. subtilis* and *B. licheniformis* (Brown, 2000; Lusardi *et al.*, 2000). Researchers have concluded that although *Alicyclobacillus* has a major economic impact on the fruit juice industry, it is not a food safety concern (Walls & Chuyate, 2000; Chang & Kang, 2004).

Heat resistance

Alicyclobacillus is resistant to the pasteurisation treatments normally applied to high-acid concentrated fruit products (Wisotzkey *et al.*, 1992; Silva *et al.*, 1999; Chang & Kang, 2004). Depending on the species of *Alicyclobacillus*, 15 - 91% of the total fatty acid content in the cell membranes comprises of ω-cyclohexane fatty acids (Hippchen

et al., 1981). Sixty five percent of *A. acidocaldarius* and 90% of *A. acidoterrestris* cell membranes contain 11-cyclohexylundecanoic and 13-cyclohexyltridecanoic acids (De Rosa *et al.*, 1971). It is believed that these closely packed ω-cyclohexane and ω-cycloheptane fatty acids contribute to the heat resistance of *Alicyclobacillus* by forming a protective coating with strong hydrophobic bonds. These hydrophobic bonds stabilise and reduce membrane permeability in extreme acidic and high temperature environments (Kannenberg *et al.,* 1984; Wisotzkey *et al.*, 1992; Jensen, 1999).

Other factors that have been associated with the heat resistance of *Alicyclobacillus* endospores include the presence of heat stable proteins and enzymes and the mineralisation by divalent cations with dipicolinic acid (DPA), especially the calcium-dipicolinate (Ca-DPA) complex (Chang & Kang, 2004; Jay *et al.,* 2005b). The structural integrity of *A. acidoterrestris* endospores under low pH conditions was shown to be affected by divalent cations as their heat resistance was associated with strong binding characteristics to calcium (Ca) and manganese (Mn). Heat resistance of *A. acidoterrestris* endospores was greatly enhanced by DPA and Ca, more so than when only Ca was present (Yamazaki *et al.,* 1997). The mineral content of mature endospores become depleted under adverse pH conditions which eventually leads to a decrease in their heat resistance (Bender & Marquis, 1985; Chang & Kang, 2004). Remineralisation with certain minerals could have a protective effect on the bacteria, in that they may increase their heat resistance by decreasing the water activity (Jay *et al.,* 2005b).

Additionally, cell age, cell numbers, protoplast dehydration and sporulation temperature could affect heat resistance. Generally, vegetative cells of thermophillic, endospore-forming bacteria are reported to be more heat resistant during their stationary growth phase than during the logarithmic phase. Large microbial populations may also provide a higher degree of heat resistance due to thermo-protective, extracellular proteins that are excreted by their cells (Jay *et al.*, 2005b). *Bacillus subtilus* strain 168 secretes between 150 - 180 membrane proteins such as *Yfnl* and *YflE* (Hirose *et al.,* 2000), whilst the extracellular protein *GroEL* have been found to promote tolerance to heat in *C. perfringens* and *C. difficile* (Heredia *et al.,* 2009). Studies to identify and determine the exact molecular role of these extracellular proteins are ongoing (Hirose *et al.,* 2000; Heredia *et al.,* 2009).

Heat resistance of microbes are also associated with the state of the endospore protoplast that is greatly influenced by the contractile cortex. The cortex either reduces the water content or maintains the state hydration (Zbigniew & Ludlow, 1993).

Alicyclobacillus endospores that are grown at maximum growth temperatures are more heat resistant than those grown at lower temperatures and they are thermally adapted with a lowered protoplast water content. A 20 ℃ r aise in sporulation temperature (from 45 °C to 65 ℃) will increase the heat resistance of *A. acidocaldarius* by eightfold and result in extremely heat resistant endospores (Palop *et al.,* 2000; Jay *et al.*, 2005b).

Alicyclobacilli have a highly resistant nature along with the ability to grow and cause spoilage in pasteurised acidic fruit products (pH < 4.6) (Pinhatti *et al*., 1997; Wisse & Parish, 1998). It has, therefore, been suggested to consider *Alicyclobacillus* as target microbes for product quality evaluation and for the design of pasteurisation processes for high-acid fruit products (Eguchi *et al*., 1999; Silva *et al*., 1999; Vieira *et al*., 2002).

Temperature, pH and soluble solid content

The reported decimal reduction values (*D*-values) (the thermal processing time at a specified temperature required to destroy 90% of the viable microbial population) of *Alicyclobacillus* in high-acid concentrated fruit products have been summarised in Table 2. There are few reports that investigated the simultaneous effect of temperature, pH and soluble solid content on the heat resistance of *Alicyclobacillus* endospores (Silva *et al.*, 1999; Maldonado *et al*., 2008; Ceviz *et al.*, 2009). Temperature, in conjunction with the pH and the soluble solid content of high-acid concentrated fruit products has a significant effect on the *D*-values. However, temperature is the parameter with the greatest influence (Silva *et al.*, 1999; Maldonado *et al*., 2008), with its effect reported to be three times higher than that of pH (Bahçeci & Acar, 2007). The *D*-values of *A. acidoterrestris* had a non-linear association with temperature, and a linear association with pH and soluble solid content (Silva *et al.,* 1999). In contrast to this, *A. acidoterrestris* endospore resistance was found to be minimally affected by the varying pH of the heating solution and significant differences between *D*-values were reported only between malt extract broth (MEB) buffers pH 3.5 and pH 4.0, at 95 ℃ (Ceviz *et al.*, 2009) and between McIlvaine buffers pH 5.0 and pH 8.0, at 92 ℃ (Murakami *et al.,* 1998). The effects of pH and soluble solid content appear to be more pronounced at lower temperatures (Pontius *et al*., 1998, Silva *et al*., 1999; Maldonado *et al*., 2008), showing slight increases in *D*-values with increasing soluble solids and pH at temperatures between 85 ℃ and 97 ℃ (Silva *et al.,* 1999). No significant effect on the heat resistance of *A. acidoterrestris* endospores was found in the presence of organic acids (malic, citric and tartaric) between pH 2.8 - 4.0 at either low or high temperatures

Table 2 Heat resistance of *Alicyclobacillus* endospores in high-acid concentrated fruit products

Concentrated juice	Soluble solids (°Brix)	pH	Temperature (°C)	*D*-value [±SD] (min)	Reference
Blackcurrant (Light)	26.10	2.50	91	3.84 [±0.49]	Silva *et al.,* 1999
Blackcurrant	58.50	2.50	91	24.10 [±2.70]	
Grape (Concord)	30.00	3.50	85	76.00	Splittstoesser *et al*., 1998
			90	18.00	
			95	2.30	
Grape (Concord)	65.00	3.50	85	276.00	
			90	127.00	
			95	12.00	
Mango	NR	4.00	80	4.00 [±1.50]	de Carvalho *et al.,* 2008
			85	25.00 [±0.10]	
			90	11.66 [±1.80]	
			95	8.33 [±2.00]	
Lemon (Clarified)	50.00	2.28	82	17.36	Maldonado *et al*., 2008
			86	18.06	
			92	7.60	
			95	6.20	
	50.00	2.80	82	25.81	
			86	22.01	
			92	15.35	
			95	11.32	
	50.00	3.50	82	33.66	
			86	68.95	
			92	16.87	
			95	12.63	
	50.00	4.00	82	21.95	
			86	35.16	
			92	23.19	
			95	9.72	
Lemon (Non-clarified)	50.00	2.45	82	15.50	
			86	14.54	
			92	8.81	
			95	8.56	
	68.00	2.28	82	15.50	
			86	14.54	
			92	8.81	
			95	8.55	
	68.00	2.80	82	50.50	
			86	31.67	
			92	39.30	
			95	22.02	
	68.00	3.50	82	38.00	
			86	95.15	
			92	59.50	
			95	17.22	
	68.00	4.00	82	27.48	
			86	58.15	
			92	85.29	
			95	23.33	

SD-Standard deviation; NR-Not reported

(91 °- 97 ℃), although the resistance was more pr onounced at temperatures below 91 ℃ (Pontius *et al.,* 1998).

High concentrations of sugars in processed fruit concentrates, purees and pulps cause an increase in the heat resistance of microbes, in part due to the decrease in the water activity (Juven *et al.,* 1978; Hui *et al.,* 2006). This resistance, however, can only be achieved in media with a very high soluble solid content, as no significant difference was found between the *D*-values of *A. acidoterrestris* endospores in apple juice, orange juice and MEB at 10 ℃Brix and 20 ℃Brix (Ceviz *et al.,* 2009). *Alicyclobacillus acidoterrestris* endospores only had an increased heat resistance in Concord grape juice concentrate at 65 ℃Brix, compared to concentr ates at 16 ℃Brix and 30 ℃Brix (Table 2) (Splittstoesser *et al.,* 1998). Similarly, the resistance of *A. acidoterrestris* endospores in concentrated lemon juice increased as the soluble solid content increased from 50 ℃Brix to 65 ℃Brix. Furthermore, it was shown that endospores were less resistant in clarified concentrates than in non-clarified concentrates (Table 2) (Maldonado *et al*., 2008). The *D*-values of *Alicyclobacillus* endospores measured in prediction models (determined in broth) are often smaller than the corresponding values in actual fruit products, indicating that the thermal resistance may well be affected by constituents present in the fruit products (Splittstoesser *et al*., 1994; Silva *et al.,* 1999; Terano *et al*., 2005; Maldonado *et al*., 2008). Therefore, to enhance the accuracy of thermal resistance studies on *Alicyclobacillus* endospores, it was suggested that water activity should be measured instead of soluble solid content as different sugars produce different water activities that could affect the *D*-values (Silva *et al.,* 1999).

From the *D*-values of *Alicyclobacillus* in Table 2 it is clear that the current industrial hot-fill-hold pasteurisation process (86 °- 96 ℃ for approximately 2 min) does not eliminate *Alicyclobacillus* endospores from high-acid concentrated fruit products, even if the raw product is contaminated at low levels (Pinhatti *et al*., 1997; Silva & Gibbs, 2004; Terano *et al*., 2005).

Natural habitats

Soil is considered to be the main source of contamination of fresh fruit during harvesting, as alicyclobacilli are soil-borne microbes (Walls & Chuyate, 2000; Bahçeci *et al.*, 2005; Parish & Goodrich, 2005). Groenewald *et al.* (2008) recovered *A. acidoterrestris* and *A. acidocaldarius* from orchard soil in the Western Cape, South Africa and showed that *A. acidoterrestris* isolated from soil outside of a fruit concentrate factory had identical RAPD-PCR banding patterns to isolates from fruit concentrates

(Groenewald *et al.,* 2009a). Between 10^4 - 10^6 cfu g^{-1} *Alicyclobacillus* were recovered from soil samples from under and around orange trees in São Paulo, Brazil (Eguchi *et al*., 1999), while 7 out of 18 orange grove soil samples from various parts of the world had approximately 3 cfu g^{-1} *Alicyclobacillus* endospores (Wisse & Parish, 1998).

A segment of the hazard analysis critical control point (HACCP) regulation (21 CFR 120) for juice forbids the use of fallen fruits (also called grounders, windfall fruit, or drops) for the use in unpasteurised fruit juice. The AIJN recommends not for fruit to be picked up from the ground or stored in direct contact with soil when used in concentrates, purees, fruit juices and nectars (FDA, 2004; Parish & Goodrich, 2005; AIJN, 2008). Ultimately, *Alicyclobacillus* are transported on the external fruit surfaces from orchards to the processing environment which signifies the importance of adequate washing procedures for the prevention of *Alicyclobacillus* contamination (Wisse & Parish, 1998; Eguchi *et al*., 1999; Bahçeci *et al.*, 2005).

D. Occurrence of *Alicyclobacillus* in the fruit processing environment

Fruit

A variety of microbes are harboured on the external surface of plant and plant products, particularly on the skin of fruits and vegetables. Healthy fruit surfaces may harbour a diverse range of microbes, either normally present or contaminated due to exposure to the environment through air, water, soil, insects and human contact during harvesting. Consequently, microbes are transported on the external fruit surfaces from orchards to the processing environment and if not removed, inactivated or controlled could contaminate the processed products (McIntyre *et al.,* 1995; Bahçeci *et al.*, 2005; Hui *et al*., 2006). The occurrence of *Alicyclobacillus* on fruit surfaces have been reported by several authors (McIntyre *et al.,* 1995; Walls & Chuyate, 1998; Groenewald *et al.,* 2009a). *Alicyclobacillus acidoterrestris* were detected in two of the 12 batches of apple samples supplied to large scale apple concentrate processing plants in Turkey (Bahçeci *et al.,* 2005). More than one third of the fruit (591 of 1 575) sampled from orange juice processing facilities in Florida was contaminated with *Alicyclobacillus,* showing that incoming fruit are a substantial means by which *Alicyclobacillus* enters the processing environment (Parish & Goodrich, 2005). *Alicyclobacillus* were also recovered from unwashed fruit surfaces at 8 out of 10 citrus processing facilities, and from washed fruit surfaces at 6 out of 9 citrus processing facilities in Florida. The estimated number of endospores on washed and unwashed fruit was calculated to be at least 6 endospores

per fruit (Wisse & Parish, 1998). Furthermore, it was suggested that fruit itself is the main source of contamination in the fruit processing environment after finding *Alicyclobacillus* counts on unwashed fruits ranged between 2 - 600 cfu kg^{-1}, which after washing ranged between < 1 - 284 cfu kg^{-1} (Eguchi *et al*., 1999). During a laboratory scale study on the effects of apple juice processing treatments on *A. acidoterrestris* endospores, the presence of endospores in the final processed product was found to be dependent on the initial level of contamination on the fruit. Thereby indicating that efficient washing at the early stages of juice processing may help reduce *Alicyclobacillus* counts in the final product (Bahçeci *et al.,* 2003).

Fruit cleaning and wash water

Adequate washing and sorting procedures are needed to reduce and prevent *Alicyclobacillus* from contaminating and cross-contaminating fruit and the processing environment (Wisse & Parish, 1998; Bahçeci *et al.*, 2005; Chen *et al.,* 2006; AIJN, 2008). Pre-processing stages of fruit concentrate production requires the use of dump tanks and flume water to unload and transport fruit into the processing environment and is a useful tool to reduce and remove soil, extraneous vegetable matter (leaves and twigs), insects and sediment from fruit surfaces prior to processing. This forms part of the first washing stages during fruit concentrate processing, consequently, the frequency with which this water is changed can influence the level of *Alicyclobacillus* contamination on the fruit (Roberts, 1994; McIntyre *et al.,* 1995; AIJN, 2008). The best practice guideline by the AIJN for the reduction and control of thermophillic, endospore-forming bacteria (*Alicyclobacillus*) in fruit juices, concentrates, purees and nectars pays particular attention to the quality of flume (transportation) and condensate (recovered) water as a source of contamination and recontamination by *Alicyclobacillus* spp. (AIJN, 2008). The probability of contamination in a fruit processing environment was confirmed when *A. acidoterrestris* isolates from wash water and pear concentrate, and *A. acidocaldarius* isolates from condensate water and pear mash, showed identical RAPD-PCR banding patterns (Groenewald *et al.,* 2009a).

Condensate water from evaporators is a by-product of the juice concentration process and proposed rules by the European Union require re-using this water for fruit washing purposes (Wisse & Parish, 1998). The warm and acidic conditions of condensate from evaporators are ideally suited to the growth of thermo-acidophillic bacteria and the occurrence of high numbers of *Alicyclobacillus* endospores in this water has been reported (Wisse & Parish, 1998; Eguchi *et al*., 1999; Chen *et al.,* 2006;

AIJN, 2008; Groenewald *et al.,* 2009a). The condensate water from 6 out of 7 citrus processing plants in Florida contained *Alicyclobacillus* endospores, ranging from non-detectable levels to 2.3 x 10^3 MPN mL^{-1} by using a three-tube most probable number (MPN) technique (Wisse & Parish, 1998). *Alicyclobacillus* were also recovered from an apple concentrate processing facility in China, with 15 and 5 strains recovered from wash water and condensate water, respectively (Chen *et al.,* 2006). *Alicyclobacillus* were recovered from condensate water at levels up to 6 x 10^3 cfu mL^{-1} and 1.5 x 10^6 MPN mL^{-1} when using a most probable number technique (Eguchi *et al*., 1999). After washing the fruit with condensate water, *Alicyclobacillus* counts increased on fruit surfaces (Eguchi *et al*., 1999). It should, therefore, be assumed that condensate water contains high levels of *Alicyclobacillus* endospores (> 1 000 cfu mL^{-1}) and should not be re-used within the processing environment, unless properly treated before re-use (AIJN, 2008).

Semi-finished and finished high-acid fruit concentrates

Alicyclobacilli are renowned for their thermal resistance in high-acid concentrated fruit products (Table 2) and that concentration and pasteurisation processes do not allow for the complete inactivation of *Alicyclobacillus* endospores, even if raw material are contaminated at low levels (Pettipher *et al*., 1997; Eiroa *et al.*, 1999; Baumgart & Menje, 2000; AIJN, 2008). Between 10 - 1 700 cfu mL^{-1} *Alicyclobacillus* were recovered from single strength juice that was supplied to the evaporator. After evaporation, the counts in concentrated juice ranged between 70 - 3 400 cfu mL^{-1}, thereby confirming that *Alicyclobacillus* levels are not altered by the concentration and pasteurisation processes (Eguchi *et al*., 1999).

Alicyclobacillus acidocaldarius was isolated from pre-pasteurised pear puree, taken from an evaporator outlet (Groenewald *et al.,* 2009a), whilst 40 *Alicyclobacillus* endospores per g frozen concentrated orange juice (65 °Brix) were recovered from an evaporator at a Florida processing plant (Wisse & Parish, 1998). Temperature conditions in evaporators are optimal to the growth of alicyclobacilli and static solids harbouring these bacteria could accumulate within the system if the equipment is not subjected to regular cleaning and sterilisation (Hays & Riester, 1952; AIJN, 2008).

The presence of *Alicyclobacillus* endospores in pasteurised, high-acid concentrated fruit products intended for retail has been reported by several authors from different parts of the world, as shown in Table 3. Additionally, it was reported that

Table 3 *Alicyclobacillus* endospores isolated from concentrated fruit juice products

Concentrated juice	Soluble solids (°Brix)	Endospores (cfu mL^{-1}) [no. positive samples]	Origin	Isolation method	Reference
Concentrated orange juice	66	70 - 3 400	São Paulo (Brazil)	Dilute 10 mL single-strength sample (12 °Brix, with sterile distilled water) in 90 mL *Bacillus acidoterrestris* (BAT) medium, heat-shock (80 °C for 10min), pour plate aliquots on BAT medium and incubate at 50 °C for 4 d (plates monitored for up to 10 d).	Eguchi *et al*., 1999
Frozen concentrated orange juice (1994,1996 crop season)	66	10 - 3 400 [23 of 28]	São Paulo (Brazil)		
Pear	32	NR	Western Cape (SA)	Enrich 1 mL heat-shocked sample (80 °C for 10 min) in yeast starch glucose (YSG) broth (pH 4.0), incubate at 45 °C for 24 h, dilute enriched aliquots with sterile distilled water, pour into YSG agar (pH 4.0) and incubate at 45 °C for 5 d.	Groenewald *et al*., 2009a
Apple, Orange	NR	NR	Parma (Italy)	Malt extract agar (MEA) (pH 4) at 50 °C for 4 - 5 d.	Previdi *et al.* 1999
Orange, Apple, Watermelon	NR	NR	Brazil, Austria, USA, Thailand	Dilute sample, isolate on YSG agar (pH 3.7) and incubate at 50 °C for 5 d.	Goto *et al*., 2006
Apple	NR	< 5 vegetative cells	USA	Concentrates diluted (1:6.5, with sterile distilled water), spread plate 0.2 mL on orange serum agar (OSA) and incubate at 44 °C for 48 h (for presence/absence method, pre-incubate samples at 44 °C for 48 h).	Pettipher *et al*., 1997
Apple	NR	NR	Washington (USA)	Strains were isolated on K agar (pH 3.7) at 43 °C for up to 5 d.	Walls & Chuyate, 1998
Concentrated orange juice	> 50	< 6.8 - 947 MPN 100 g^{-1} [14.7% of 75]	Brazil	Reconstituted single-strength juice (9 °Brix, with distilled water), heat-shock (70 °C for 20 min), enrich 10 mL juice in *Bacillus acidocaldarius* medium (BAM) broth 44 °C for 48 h, and isolate on BAM agar (pH 4.0) and incubate at 44 °C for 5 d.	Eiroa *et al*., 1999

Table 3 Continued

Concentrated juice	Soluble solids (°Brix)	Endospores (cfu mL^{-1}) [no. positive samples]	Origin	Isolation method	Reference
Frozen concentrated orange juice (1995-1996 crop season)	65	< 30, 150, 230, 230, 430 MPN g^{-1} [5 of 23]	Florida (USA)	100 mL Reconstituted single-strength juice (11 ° - 14 °Brix, with sterile water), heat-shock (90 °C for 20 min), incubate at 45 °C until turbid or off-odour is sensed (after 10 d) spread plate 0.1 mL on *Alicyclobacillus* (ALI) agar (pH 3.5) and incubate at 45 °C for 48 h.	Wisse & Parish, 1998
Pear (1995-1996 crop season)	NR	< 30 MPN g^{-1}	Florida (USA)		
Banana, Apricot	NR	< 100 [3 of 18]	Germany	Heat-shock sample (70 °C for 20 min), incubate in potato dextrose broth (PDB) (pH 3.5) at 46 °C for 3 d, streak out on potato dextrose agar (PDA) (pH 3.5) incubated at 46 °C for 2 d.	Baumgart & Menje, 2000
Apple	72	NR	China	Heat-shock 50 mL diluted sample (12 °Brix) at 80 °C for 10 min, filter through 0.45 µm membrane, place filter on K agar (pH 3.7) and incubate at 45 ° - 50 °C for 2 - 5 d.	Chen *et al*., 2006
Frozen concentrated orange juice	66	< 1 - 12 000	Costa Rica, Mexico, Florida (USA), Brazil	Dilute 10 mL frozen concentrated orange juice to single-strength with 90 mL distilled water (other concentrates are diluted according to manufacturer specifications), heat-shock samples (80 °C for 10 min), pour plate in BAM and SM medium and incubated at 50 °C for 24 - 48 h.	Pinhatti *et al.*, 1997
Concentrated orange juice	> 40	36, 60	USA		
Limeade	> 40	510	USA		
Lemonade	> 40	3 020	USA		
Mango	NR	NR [16 of 24]	South Africa	Dilute 10 mL sample in 90 mL sterile distilled water, heat-shock (80 °C for 10 min), pour-plate 1 mL aliquots in YSG agar (pH 3.7) and incubate aerobically 55 °C for 7 d (presumptive *Alicyclobacillus* cultures are maintained on PDA (pH 3.7) incubated at 55 °C).	Gouws *et al*., 2005

Table 3 Continued

Concentrated juice	Soluble solids (°Brix)	Endospores (cfu mL^{-1}) [no. positive samples]	Origin	Isolation method	Reference
Apple (2001, 2002 crop season)	70	[0 of 38]	Turkey	Dilute sample to 11.2 °Brix with sterile distilled water, heat-shock (80 °C for 10 min) and incubate at 46 °C for 24 h. Filter 100 mL enriched sample filter through 0.45 µm membrane, place filter on BAM agar (pH 4.0) and incubate at 46 °C for 3 - 7 d.	Bahçeci *et al.*,2005
Apple, Orange, Grapefruit, Mango, Peach, Blueberry, Pear	NR	NR	USA, Brazil, South America	Dilute 100 g concentrate to a final volume of 1 L with sterile distilled water. Vacuum filter through 0.22 µm nitrocellulose membrane filter, place filter on either acidified PDA (pH 3.5) or ALI agar (pH 5.6) and incubate aerobically at 50 °C for 5 d.	Durak *et al.,* 2010
Pineapple (2003 crop season)	NR	[0 of 5]	Australia	10 g Sample were tested with methods, as prescribed by the International Federation of Fruit Juice Producers with dilution, heat-shock, enrichment, and sub-culturing techniques, using BAT broth and agar (pH 4.0), followed by incubation at 45 °C for 5 d.	Jensen, 2005b
Apple (2003 crop season)	NR	NR [19 of 64] 300, < 100 cfu g^{-1} §	Australia		
Orange (2003 crop season)	NR	[61 of 85]	Australia		
Apple	50	0.020 - 0.615 mL^{-1} FCM [36% of166]	Various (Different suppliers)	Flow cytometry methods, results above 0.05 mL^{-1} rank as a positive identification to be in line with plating techniques that can detect 1 bacteria 20 mL^{-1} (0.05 bacteria mL^{-1}).	Borlinghaus & Engel, 1997

NR - Not reported; MPN - Most probable number; § - *A. acidocaldarius* and *A. acidoterrestris* counts, respectively

between 20 - 30% of the orange and apple concentrates used in Australia are likely to contain *Alicyclobacillus* species capable of producing spoilage taints (Jensen, 2005a).

E. Spoilage

Although not all *Alicyclobacillus* species are characterised as spoilage microbes (AIJN, 2008), *A. acidoterrestris, A. acidocaldarius, A. hesperidum, A. cycloheptanicus, A. acidiphilus, A. fastidiosus* and *A. pomorum* have frequently been implicated in spoilage incidents in high-acid fruit and vegetable products (Table 1) (Yamazaki *et al.*, 1996; Gocmen *et al.*, 2005; Goto *et al*., 2007). These bacteria can produce offensive smelling 'smoky', 'antiseptic' or 'disinfectant' like flavour taints (Yamazaki *et al.*, 1996; Gocmen *et al.*, 2005). Visually, spoilage is difficult to detect as it is not associated with secondary gas or acid production (flat-sour type spoilage), but may show an increase in turbidity and sediment formation (Borlinghaus & Engel, 1997; Walls & Chuyate, 1998; Lusardi *et al.,* 2000).

The chemical taint compounds have been identified as 2-methoxyphenol (guaiacol) which is accepted as the predominant taint compound and the halogenated phenolic compounds, 2,6-dichlorophenol and 2,6-dibromophenol that occur in lower concentrations (Jensen, 1999; Jensen & Whitfield, 2003; Gocmen *et al.*, 2005). Taste thresholds for guaiacol in orange and non-carbonated fruit juice were reported to be around 2 µg L^{-1} (ppb) and in apple juice 2.32 ppb (Pettipher *et al*., 1997), whilst the concentration of 2,6-dichlorophenol and 2,6-dibromophenol in spoiled mixed fruit drinks were reported to be 16 - 20 ng L^{-1} (ppt) and 2 - 4 ppt, respectively (Jensen & Whitfield, 2003). Additionally it was found that cell numbers between 10^5 - 10^6 cfu mL^{-1} *A. acidoterrestris* produced sufficient guaiacol (2 ppb) to spoil fruit beverages (Pettipher *et al*., 1997; Gocmen *et al.*, 2005).

Spoilage in fruit concentrates by *Alicyclobacillus* is not likely as the soluble solid content (> 20 °Brix) will inhibit the germination of *Alicyclobacillus* endospores (Splittstoesser *et al.*, 1994; Chang & Kang, 2004). These endospores, however, will retain their viability in juice concentrate which, upon dilution to single strength juice could multiply to numbers high enough to cause spoilage and product deterioration (Borlinghaus & Engel, 1997). Concentrated juice (> 40 °Brix) containing 10^2 cfu mL^{-1} *Alicyclobacillus* (Table 3) did not seem to be associated with spoilage (Pinhatti *et al*., 1997). Similarly, no significant growth or taint development was observed in concentrated raw material (50 °Brix) that was inoculated with 10^3 cfu mL^{-1}

A. acidoterrestris and subsequently stored for 4 weeks at 45 ℃. Upon dilution, however, a higher growth level of *A. acidoterrestris* was observed accompanied by spoilage and product deterioration (Borlinghaus & Engel, 1997).

F. Detection, identification and standardised test methods for *Alicyclobacillus* from concentrated fruit products

Detection parameters

Culture-based methods are mainly used for routine analysis of concentrated fruit juice products, as these techniques are easy to perform and reliable (Baumgart & Menje, 2000). During the past ten years many direct plating methods and agar media have been developed for the detection and quantification of *Alicyclobacillus* and may well explain the variety of isolation methods that are currently used in the fruit beverage industry world-wide (Murray *et al.,* 2007). Parameters that play an important role during the isolation of *Alicyclobacillus* from concentrated fruit juice products are growth media type and acidification, sample dilution, filtration steps, heat-shock treatments, pre-enrichment procedures and incubation time and temperatures (Table 3) (Pacheco*,* 2002; Murray *et al*., 2007; Yokota *et al.,* 2007).

Frequently used growth media for the detection of *Alicyclobacillus* from concentrated fruit juice products are *Bacillus acidocaldarius* medium (BAM) and *Bacillus acidoterrestris* thermophillic (BAT) agar (Deinhard *et al.,* 1987a; Eguchi *et al*., 1999; IFU, 2007), yeast starch-glucose agar (YSG) (Matsubara *et al*., 2002; Goto *et al*., 2006), potato dextrose agar (PDA) (Pinhatti *et al*., 1997; Baumgart & Menje, 2000; Durak *et al.,* 2010), orange serum agar (OSA) (Pettipher & Osmundson, 2000) and K agar (Walls & Chuyate, 1998; Chen *et al*., 2006). The best media for the recovery of *Alicyclobacillus* from undiluted fruit concentrates was found to be PDA (pH 3.7) and OSA (pH 5.5) after incubation at 50 ℃ for 3 - 5 d, compared to K agar , YSG agar and BAM (Witthuhn *et al.,* 2007). Media, regardless of the type are usually acidified to pH 3.5 - 5.6 by HCl or H_2SO_4, which is sterilely added after autoclaving in order to prevent agar hydrolysis. Incubation temperatures are between 37 ° - 55 ℃ an d incubation times range from 1 - 7 d (Walls & Chuyate, 1998; Pettipher & Osmundson, 2000; Chang & Kang, 2004).

Several isolation methods include a heat-shock treatment when testing concentrated raw materials, where alicyclobacilli are most likely to be present as endospores (IFU, 2007; Yokota *et al.,* 2007). The heat-shock treatment is applied to samples in order to kill vegetative cells and to obtain uniform activation and germination

of dormant *Alicyclobacillus* endospores (Yokota *et al.,* 2007). A treatment at 80 ℃ for 10 min is recommended by the Brazilian Association for Citrus Export (ABECitrus) and the International Federation of Fruit Juice Producers (IFU) (Eguchi *et al*., 1999; IFU, 2007; Murray *et al*., 2007). Other heat-shock treatments include 80 °C for 20 min (Terano *et al*., 2005), 90 ℃ for 20 min (Wisse & Parish, 1998) and 70 ℃ for 10 min (Jensen, 2000). A heat-shock treatment at 70 ℃ for 20 min is frequently used for the activation of endospores in concentrated fruit products and is proposed by the Japan Fruit Juice Association (JFJA) (Eiroa *et al*., 1999; Baumgart & Menje, 2000; Pacheco, 2002; Yokota *et al.,* 2007). While several heat-shock treatments exist, some form of a heat treatment is essential for the detection of *Alicyclobacillus* as a constant underestimation of the total viable *Alicyclobacillus* counts will be inevitable if a heat-shock treatment is not applied prior to media plating (Pinhatti *et al*., 1997). Heat-shock treatments are usually followed by pre-enrichment procedures of incubation at 40 ° - 50 ℃ for 48 h. This step allows for the detection of endospores which is usually present at very low levels in concentrated fruit juice products (Pinhatti *et al.,* 1997; Walker & Philips, 2008a). Additionally, it was found that intermittent shaking of samples that were stored at sub-optimal temperatures (30 ° - 35 ℃) prior to sampling increased the growth and probable detection rates of *A. acidoterrestris* (Walker & Philips, 2005).

Membrane filtration techniques are frequently used to collect *Alicyclobacillus* from fruit juice products as it allows for large sample volumes to be examined (Splittstoesser *et al*., 1994; Pettipher & Osmundson, 2000; Chang & Kang, 2004) and is a fast alternative to long enrichment procedures (Lee *et al.,* 2007). The use of 0.45 µm filters are specified by IFU Method 12 and the JFJA (IFU, 2007; Yokota *et al.,* 2007), whereas 0.2 µm filters are recommended by the AIJN for optimal retention of *Alicyclobacillus* endospores (AIJN, 2008).

With regards to direct plating techniques, it is suggested that spread plates are more effective than pour plates as surface colonies are larger and easier to enumerate (Pettipher *et al*., 1997; IFU, 2007). In a study by the JFJA Working Group, where YSG and BAT agar were evaluated for the recovery of *Alicyclobacillus*, no difference in growth was observed between the media during the spread plating or membrane filtration techniques. However, during the pour plating technique twice as many *A. acidoterrestris* endospores were recovered on YSG than on BAT agar (Yokota *et al.,* 2007). The effect of direct and spread plating methods on the enumeration of *A. acidoterrestris* (3 strains), *A. acidocaldarius* (2 strains) and *A. cycloheptanicus* (1 strain) endospores were evaluated on BAT (pH 4.0), *Alicyclobacillus* (ALI) (pH 4.0)

and K (pH 3.7) agars, following a heat-shock treatment at 80 ℃ for 10 min. Results indicated that the plating method had no significant difference ($P > 0.05$) on the recovery of endospores in 93.3% of the tests. However, in 4.7% of the tests significantly higher ($P \leq 0.05$) numbers of endospores were recovered by the spread plating technique when compared to the pour plating technique (Murray *et al*., 2007).

Standardised test methods

Since cultivation media are often matrix specific, the AIJN recommends the use of multiple isolation media for optimal detection (AIJN, 2008). The American Public Health Association proposes the use of K agar (normal pH 3.7) and incubation at 43 ℃ for 3 d (Walls & Chuyate, 1998; Murray *et al*., 2007). When incubated at 45 ℃, however, K agar predominantly supports the growth of *A. acidoterrestris* and limits the growth of other species such as *A. acidocaldarius* and *A. acidiphilus* (IFU, 2007).

The “unified test method for detection of thermo-acidophilic bacilli” issued by the JFJA in March 2003 (Report No. 535), recommends the use of acidified YSG agar plates (pH 3.7) incubated at 30 ℃ for 5 d to detec t *A. acidoterrestris, A. hesperidum, A. cycloheptanicus, A. acidiphilus* and *A. fastidiosus* in samples. Incubation at a higher temperature of 55 ℃ - 65 ℃ is suitable for *A. acidocaldarius*, whilst 45 ℃ allows for the growth and detection of all *Alicyclobacillus* species (Yokota *et al.,* 2007). Concentrated fruit juice samples are pre-enriched in YSG broth, diluted (50 g sample in 200 g sterilised water), heat-shocked at 70 ℃ for 10 min and filtered using a sterile 0.45 µm membrane filter, prior to transferring the filter onto the YSG agar plates (CCJC, 2010). Confirmation of *A. acidoterrestris*, the representative guaiacol-producing species is done by using the differential growth temperature method at 45 ℃ and 65 ℃ for 18 - 20 h and the peroxidase test, which is based on the principle that guaiacol reacts with peroxidase enzymes and hydrogen peroxide to form tetraguaiacol, a brown coloured complex (Jensen, 2005a; Murray *et al*., 2007; Yokota *et al.,* 2007).

The IFU Microbiology working group published the “First standard IFU-method on the detection of *Alicyclobacillus* in fruit juices” (IFU Handbook Microbiological Methods, Method No. 12, January-September 2004/Revised March 2007). For juice concentrates and other raw materials, Method 12 recommends the use of BAT agar (pH 4.0), YSG (pH 3.7) or K agar (pH 3.7) incubated at 45 ℃ for 2 - 5 d. BAT and YSG medium support the growth of all currently known species of alicyclobacilli, whilst K agar predominantly supports the growth of *A. acidoterrestris* strains. Method 12 prescribes a representative sample of 10 g (minimum quantity) with heat-shock treatment at 80 ℃

for 10 min that is followed by direct plating (optional) or enrichment procedures. A filtration step is optional and require 100 mL heat-shocked diluted sample (1:10 with demineralised/sterile distilled water) and 0.45 µm pore size membranes. Membranes are aseptically transferred onto K agar and BAT agar (or YSG agar). Non-filterable samples are recommended to undergo enrichment procedures which include incubation of the heat-shocked samples (approximately 100 mL) at 45 ℃. For confirmation, a selected colony is streaked onto K agar, YSG agar (duplicate) and neutral pH medium agar plates (plate count agar, brain heart infusion agar or tryptic soy agar). Agar plates are incubated at 45 ℃ for 2 - 5 d whilst a duplicate YSG agar plate is incubated at 65 ℃ for 2 - 5 d. *Alicyclobacillus* colonies on acidified media are examined microscopically for endospore production and no growth should be detected on media with neutral pH values. Growth on K agar and YSG plates that are incubated at 45 ℃ indicate presumptive taint producing alicyclobacilli, whilst taint producing strains are unlikely to grow on YSG plates that are incubated at 65 ℃. Additional peroxidise test is optional and can be used to confirm the ability of *Alicyclobacillus* isolates to produce guaiacol (IFU, 2007).

The Australian Fruit Juice Association (AJA) is in support of the standard IFU Method 12, however, recommends that focus of the method should be on taint producing *Alicyclobacillus* strains only and prescribes the use of confirmatory tests to discriminate non-taint producing from taint producing strains. Additionally, they recommend that the sample size of fruit concentrates is increased from 10 g to 100 g. Sterile distilled water with K agar and BAT (broth and agar) including a pre-enrichment period of 48 h are prescribed when detecting *Alicyclobacillus* in orange and apple concentrates, respectively (Jensen, 2005b). In a related study by Food Science Australia *Alicyclobacillus* were detected in fruit juice concentrates. Initial identification were done by the peroxidise test method and the differential growth temperature method at 45 ℃ and 65 ℃. Confirmation and classification of *Alicyclobacillus* species were either done by DNA sequence analysis of the 16S rRNA genes or by gas chromatography mass spectrometry, which identify and quantify the chemical taint compounds (Jensen, 2005b).

Members of the technical committee of ABECitrus also prescribe BAT agar (pH 4.0) for the isolation of thermo-acidophilic bacteria in concentrated fruit products. Samples are diluted to 12 °Brix (with sterile distilled water) where after the 10 mL single-strength samples are placed in 90 mL BAT broth, heat-shocked at 80 ℃ for 10 min, pour plated on BAT agar and incubated at 50 ℃ for 4 d (plates monitored for up to

10 d) (Eguchi *et al*.,1999). Prior to the screening tests, colonies are re-streaked onto BAT agar (pH 4.0) and nutrient agar (pH 7.0) with incubation at 50 ℃ for 48 - 72 h to confirm the inability of *Alicyclobacillus* to grow at neutral pH values. Morphological characterisation by Gram-staining and fatty acid analysis by gas chromatography to detect the major fatty acids (11-cyclohexylundecanoic and 13-cyclohexyltridecanoic acids) are used to confirm the presence of *Alicyclobacillus* in concentrated fruit juice samples (Eguchi *et al*., 1999).

South African fruit processors primarily make use of the IFU Method 12: 2004 for *Alicyclobacillus* testing (Williams, 2010). In a recent study, three isolation methods that are used in the South African fruit processing industry were compared with regards to the recovery of *A. acidoterrestris* from peach concentrate. It was concluded that IFU Method 12 was most effective (75.97% recovery), followed by a method that makes use of pour plating and acidified PDA (pH 3.7) (66.79% recovery), while the least effective method made use of membrane filtration and K agar (3.43% recovery) (Groenewald *et al.,* 2009b; Smit, 2009).

Alternative detection and identification methods

While culture-based methods are generally the simplest and least expensive methods for routine analysis of fruit concentrates, they can be time-consuming and unreliable during repeat analysis (Baumgart & Menje, 2000). Consequently, morphological and biochemical characterisation, such as fatty acid methyl ester (FAMES) analysis (Deinhard *et al.*, 1987a; Pinhatti *et al.*, 1997; Goto *et al.*, 2002) and API (50 CHB test strips) (Eiroa *et al.,* 1999; Goto *et al.*, 2006; Groenewald *et al.,* 2009a) are included as confirmatory tests. The need for rapid detection methods are essential for quality analysis of fruit concentrates since it can be costly and impractical to isolate bulk concentrates from the supply chain for 3 - 7 d for the duration of conventional microbial plating methods (Borlinghaus & Engel, 1997; Baumgart & Menje, 2000; Walker & Philips, 2008a). Rapid detection methods for *Alicyclobacillus* include the Fourier transform infrared (FT-IR) absorbance spectroscopy (Lin *et al*., 2005; Al-Qadiri *et al*., 2006), flow cytometry (Borlinghaus & Engel, 1997), polymerase chain reaction (PCR)-based methods (Luo *et al.*, 2004; Connor *et al.*, 2005) and gene probes (Vermicon AG identification technology; VIT®) (Thelen *et al*., 2003).

G. Control

Disinfectant treatment in fruit washing

Since *Alicyclobacillus* is likely to contaminate fruit via soil, the most practical control measures would be to prohibit the use of fallen fruits during concentrate production and to establish thorough fruit washing procedures for fruit entering the processing environment (Walls & Chuyate, 1998; FDA, 2004; Parish & Goodrich, 2005). Several fruit washing techniques have been adopted by the industry and include the use of approved disinfectants and chemical agents to reduce *Alicyclobacillus* levels on the surface of fruits (Hui *et al*., 2006; AIJN, 2008).

Aqueous chlorine based disinfectants at concentrations between 50 - 200 ppm are the most commonly used chemical sanitiser allowed on fruits and vegetables (Hui *et al*., 2006; Bevilacqua *et al.,* 2008a). Lee *et al.* (2004) found that the effectiveness of chlorine dioxide (ClO_2) to reduce *A. acidoterrestris* endospores on apples was related to the contact time and that a 4.8 log reduction was achieved with concentrations and contact times at 40 ppm for 4 min and 120 ppm for 1 min, respectively. In a similar study, the efficacy of ClO_2 gas against *A. acidoterrestris* endospores on apples was investigated. A 5 log reduction was achieved after a 1 h exposure time to medium and high release ClO_2 gas sachets that generated gas concentrations at 1.78 and 4.32 ppm, respectively (Lee *et al.,* 2006). A significant reduction ($P \leq 0.05$) of *A. acidoterrestris* endospores on apples following treatment with sodium hypochlorite (NaOCl), acidified sodium chlorite ($NaClO_2$) and hydrogen peroxide (H_2O_2) during 1 min of contact time was reported (Orr & Beuchat, 2000). Endospores treated with NaOCl at 500 and 1 000 ppm and $NaClO_2$ at 1 200 ppm resulted in a 0.73, 2.10 and 0.64 log reduction, respectively, whilst the most effective treatment was found to be 2% H_2O_2, which resulted in > 4.8 log reduction of *A. acidoterrestris* endospores on apple surfaces (Orr & Beuchat, 2000).

Disinfectant treatments can lose their effectiveness on fruit as the waxed hydrophobic nature of the skin and crevices on the surface attribute to the inaccessibility of disinfectants to *Alicyclobacillus* (Orr & Beuchat, 2000; Bevilacqua *et al.,* 2008a). Vigorous washing and the use of a detergent or food-grade surfactant have been suggested to enhance the access and contact time with disinfectants (Orr & Beuchat, 2000).

Control of *Alicyclobacillus* in processed fruit juice

Innovative techniques to control *Alicyclobacillus* endospores and to simultaneously prevent the loss of nutritional and sensorial quality during thermal processing applications include high hydrostatic pressure (HHP) at 207 - 216 MPa (Alpas *et al.,* 2003), high pressure homogenization (HPH) at 500 - 700 bar (Bevilacqua *et al.,* 2007) and radiation with electron and gamma beams at 300 kGy h^{-1} and 1 kGy h^{-1} (Nakauma *et al.,* 2004; Mahapatra *et al.,* 2005). The effects of organic acids on *A. acidoterrestris* endospores are still unknown due to uncertainty on the mode of action. However, Bevilacqua *et al.* (2008b) proposed the use of 100 - 500 ppm sodium benzoate, whilst Walker & Philips (2008b) found both sodium benzoate and potassium sorbate at 100 and 500 ppm to inhibit 10 and 10 000 cfu mL^{-1} *A. acidoterrestris*, respectively.

The recent demand for more natural preservation methods have led to the use of natural compounds such as bacteriocins (Walker & Philips, 2008a). Nisin at concentrations between 25 - 100 IU mL^{-1} (Komitopoulou *et al.,* 1999; Yamazaki *et al.,* 2000; Peña & Massaguer, 2006) and Enterosin at 1.25 - 2.5 ppm (Grande *et al.,* 2005) proved to inhibit *A. acidoterrestris* endospores. Furthermore, it was found that the essential oils, cinnemaldehyde (100 ppm), eugenol (100 ppm) and limonene (> 500 ppm) can inhibit *A. acidoterrestris* endospores (Bevilacqua *et al.,* 2008b). Temperature studies indicate that *Alicyclobacillus* will be inhibited at temperatures below 20 ℃, therefore, an alternative control measure would be to transport and store fruit juice products under refrigerated conditions (Jensen & Whitfield, 2003; Chang & Kang, 2004).

Cleaning and sanitation of the processing plant

Current good manufacturing practice (cGMP) and HACCP control measures are essential to control *Alicyclobacillus* from accumulating in the processing environment (FDA, 2004; AIJN, 2008; Walker & Philips, 2008a). Apart from the standard clean-in-place (CIP) treatments applied during fruit concentrate production, the use of several disinfectant treatments have proved effective against *Alicyclobacillus* contamination (Orr & Beuchat, 2000; Hui *et al*., 2006; Bevilacqua *et al.,* 2008a; b). In a study on the efficacy of chemical disinfectants in killing *A. acidoterrestris* endospores, it was found that NaOCl at concentrations > 1 000 ppm and 4% H_2O_2 to be the most effective with a 6 log endospore reduction when treated for 10 min (at 23 ± 2 ℃). At similar conditions, 1 200 ppm $NaClO_2$ resulted in a 2 log endospore reduction, whilst

substantially lower numbers of endospores (< 1 log reduction) were inactivated with 12% trisodium phosphate (Na_3PO_4) and 160 ppm Tsunami (contain peracetic acid as an active component) (Orr & Beuchat, 2000). The effectiveness of a peracetic acid and H_2O_2 based disinfectant on *Alicyclobacillus* endospores in a dry state (on metal surfaces) and in an aqueous solution was evaluated (Previdi *et al.*, 1999). It was found that the two treatment types did not differ substantially from each other and that > 1 h contact time of 0.5% disinfectant at 20 ℃ was need ed for a 5 - 6 log reduction in endospores, however, heating to 40 ℃ would reduced the contact time to 40 min. Heating allows for the contact time or concentration of the disinfectant to be reduced, thereby avoiding the risk of corrosion to the processing plant (Previdi *et al.*, 1999). In contrast, no synergistic effect was found on *A. acidoterrestris* endospore reduction when ClO_2 treatment in aqueous suspensions was followed by heat (Lee *et al.,* 2004). Additionally, water that is used for cleaning and sanitising in the processing environment have been found to contain high levels of *Alicyclobacillus* endospores (> 1 000 cfu mL^{-1}) and should undergo frequent testing as to prevent cross-contamination (McIntyre *et al.,* 1995, Wisse & Parish, 1998; AIJN, 2008).

H. Conclusions

The thermo-acidophilic nature of the genus *Alicyclobacillus* allow for their survival during the production of high-acid concentrated fruit products and an increasing occurrence of *Alicyclobacillus* in fruit concentrates, pulps and purees have been reported. Spoilage by *Alicyclobacillus* is a major concern to the food industry worldwide, as fruit concentrates are used in several fruit beverages and fruit based products. The quality of raw fruit and hygienic processing conditions are essential to reduce the risk of *Alicyclobacillus* endospores occurring in the final product. Furthermore, it is important to understand the nature of *Alicyclobacillus* within the fruit concentrate processing environment as it may prove important in terms of its potential to cause spoilage.

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CHAPTER 3

CONTAMINATION OF PEAR CONCENTRATE BY *ALICYCLOBACILLUS* FROM RECIRCULATING FLUME WATER DURING FRUIT CONCENTRATE PRODUCTION

Abstract

Alicyclobacillus and viable aerobic counts were monitored at nine different production stages of pear concentrate, with the functioning of either a recirculating or a one-pass flume water system. Significantly ($P < 0.05$) higher levels of *Alicyclobacillus* were detected in the final pasteurised product (102 °- 104 ℃ for 90 s) when the recirculating flume water system was operational. An average of 1.19 $\log_{10}$ cfu mL^{-1} vegetative cells and 1.35 $\log_{10}$ cfu mL^{-1} endospores were recovered, whereas 0 cfu mL^{-1} vegetative cells and endospores were detected when the one-pass flume water system was operational. *Alicyclobacillus* levels did not differ significantly ($P > 0.05$) in condensate water during the functioning of the two flume water systems, with 1.81 $\log_{10}$ cfu mL^{-1} vegetative cells and 1.01 $\log_{10}$ cfu mL^{-1} endospores (recirculating system) and 0.78 $\log_{10}$ cfu mL^{-1} vegetative cells and 0.42 $\log_{10}$ cfu mL^{-1} endospores (one-pass system) recovered, respectively. As a result, water treatment protocols should be established if untreated recirculating flume or condensate water is to be used in order to prevent *Alicyclobacillus* contamination and accumulation in the processing environment. There were no correlations between viable aerobic and *Alicyclobacillus* counts during the nine production stages of pear concentrate, therefore, viable aerobic counts cannot be used as an indicator for the presence of *Alicyclobacillus*.

Introduction

Alicyclobacilli are thermo-acidophilic, non-pathogenic, endospore-forming bacteria and have the ability to survive thermal processing applied during the production of high-acid concentrated fruit products (pH < 4.0) (Wisotzkey *et al.,* 1992; Silva & Gibbs, 2004). An increasing occurrence of *Alicyclobacillus* in raw materials such as fruit concentrates, syrups, pulps and purees are a concern for the fruit beverage industry (Bahçeci *et al.,* 2005; Chen *et al.,* 2006). Although the soluble solid content (> 20 °Brix) of

Steyn, C.E., Cameron, M., Brittin, G. & Witthuhn, R.C. (2011). Contamination of pear concentrate by *Alicyclobacillus* from recirculating flume water during fruit concentrate production. *World Journal of Microbiology and Biotechnology.* In press.

concentrated fruit products inhibit the germination of *Alicyclobacillus* endospores (Splittstoesser *et al.,* 1994; Chang & Kang, 2004), these endospores retain their viability which upon dilution to single strength could reach adequate conditions for growth and spoilage to occur (Borlinghaus & Engel, 1997). *Alicyclobacillus* can cause flat sour type spoilage in fruit beverages with offensive smelling taints due to the formation of guaiacol and halogenated phenolic compounds (Jensen & Whitfield, 2003).

The main source of *Alicyclobacillus* in the processing environment is soil that is carried into the processing environment on the surface of fruits, as well as, contaminated processing water (Wisse & Parish, 1998; Groenewald *et al.,* 2009). Furthermore, the recent best practice guideline by the European Fruit Juice Association (AIJN) paid particular attention to the quality of flume (transportation) and condensate (recovered) water as a source of contamination and recontamination by *Alicyclobacillus* (AIJN, 2008). The aim of this study was to monitor *Alicyclobacillus* counts at various stages of fruit concentrate manufacturing during the functioning of either a recirculating or an one-pass (not recirculated) flume water system.

Materials and methods

Sampling

Samples were collected from a hazard analysis critical control point (HACCP) accredited fruit concentrate manufacturing facility, located in the Western Cape region of South Africa. *Alicyclobacillus* contamination levels were monitored at nine different processing stages of pear concentrate that included whole fruit, mash, juice and concentrate. Whole fruit were sampled at random from: bins[1] entering the fruit processing area; the primary flume water wash[2]; after the fresh water rinse[3]; and after a chemical treatment[4] with an acidic based oxidising disinfectant (Perasan, JohnsonDiversey). Fruit mash[5] were sampled at an inline hammer mill while single strength juice[6], that underwent a hot-break treatment to enhance the flow properties of fruit mash (75 °- 90 ℃ for ± 10 min and cooled to 65 ℃), were sampled from an outlet prior to evaporation. Fruit concentrate (± 30 ℃Bri x)[7] were sampled from an outlet after forced circulation evaporation, as well as the final product[8], aseptically vacuum packaged pear concentrate after inline pasteurisation at 102 °- 104 ℃ for 90 s. Additionally, *Alicyclobacillus* levels were monitored in the condensate water[9], which is a by-product of the juice concentration process.

Over a period of two months and during the functioning of the recirculating flume water system, whole fruit and liquid samples were sampled in duplicate on four occasions. During the functioning of this system, flume water was recycled by filtering through a dewatering screen (to remove debris) and stored in a recycled water reservoir. The recirculating flume water system was replaced by a one-pass flume water system using a continuous flow of fresh potable water from the local municipality. Over a period of two months, whole fruit and liquid samples were sampled in duplicate on 10 occasions. No additional disinfectant treatment was applied to the water from either flume systems.

Enumeration

Liquid samples were placed in sterile bottles, whereas whole fruit (around 1 kg pears) were sampled in sterile bags. Samples were transported under low temperature conditions to the laboratory and were processed within 24 h after sampling. Fruit samples were washed with 50 mL sterile saline solution (SSS) (0.85% (m/v) NaCl (Merck, Cape Town, South Africa)) for 2 min and the wash water analysed (Parish & Goodrich, 2005; Groenewald *et al.,* 2009).

Viable aerobic counts were determined to establish the general hygiene level of the fruit concentrate manufacturing facility. The pour-plate technique and plate count agar (PCA) (Merck) were used for enumeration after incubation at 35 ℃ for 48 h.

Potato dextrose agar (PDA) (Merck) at pH 3.7, adjusted with 1M H_2SO_4 (Chang & Kang, 2004), were used to recover *Alicyclobacillus* from the samples and visible colonies were enumerated after incubation at 45 ℃ for 5 d (Splittstoesser *et al.,* 1994; Groenewald *et al.,* 2009). Vegetative cell growth was examined by directly spread plating the samples onto PDA (pH 3.7). The number of endospores in each sample was determined on PDA (pH 3.7) after a heat-shock treatment at 80 ℃ for 10 min to promote endospore germination and eliminate vegetative cells (Pinhatti *et al.,* 1997; Pettipher & Osmundson, 2000).

Duplicate tests were performed on all samples. Where possible, plates with between 30 and 300 colonies were selected for enumeration (Anon., 1997). Sterile PCA and PDA served as controls for viable aerobic and *Alicyclobacillus* counts, respectively. No growth was detected on the controls.

Identification

Alicyclobacillus was initially identified on PDA (pH 3.7) as round, creamy white (0.3 - 5.0 mm in diameter) colonies (Chang & Kang, 2004). Conventional microscopic analysis and Gram-staining were done on randomly selected plates to confirm *Alicyclobacillus* as rod-shaped Gram-positive to Gram-variable bacteria with/without oval shaped, sub-terminally located endospores (Wisotzkey *et al.,* 1992). Thereafter polymerase chain reactions (PCR) were done, and the primers CC16S-F (5' CGT AGT TCG GAT TGC AGG C-3') and CC16S-R (5' GTG TTG CCG ACT CTC GTG-3') were used to amplify a 134 base pair (bp) fragment (1254 to 1388 bp) of the 16S rRNA gene (Connor *et al.,* 2005).

PCR reactions were performed in a total mixture volume of 25 µL that contained 0.2 µM of each primer (Eurofins MWG Operon, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 1.25 U *Taq* DNA polymerase (Super-Therm, supplied by Southern Cross Biotechnologies), 1 x PCR reaction buffer (Super-Therm), 6 mM $MgCl_2$ (Super-Therm), 1 µL of 99% (v/v) dimethyl sulphoxide (DMSO) (Merck), 0.8 mM dNTP mix (AB gene, supplied by Southern Cross Biotechnologies) and 5 µL extracted DNA. An Eppendorf Mastercycler Personal (Eppendorf, Germany) with the following thermal cycling conditions was used for amplification: initial denaturation at 95 ℃ for 3 min; followed by 35 cycles of denaturation at 94 ℃ for 30 s; annealing at 55 ℃ for 30 s; elongation at 72 ℃ for 30 s and final elongation was performed at 72 ℃ for 2 min. PCR products (5 µL) were separated on a 1.5% (m/v) agarose gel, containing 0.02 µL mL^{-1} ethidium bromide, in 0.5 x TBE electrophoresis buffer (45 mM tris base, 45 mM boric acid and 1 mM EDTA) (Merck). The separated PCR fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat, Marne La Vallee, France).

Statistical analysis

Statistical analysis was done using Statistica™ 9 (StatSoft, Inc., Tulsa, OK, USA). Due to the highly skewed nature of the data, a generalised linear model using the Gamma distribution and log link function were used to determine the effect of different flume water systems on the level of *Alicyclobacillus* contamination during pear juice processing. A 5% ($P < 0.05$) significance level was used to determine significant effects.

Results and discussion

Viable aerobic counts

The effectiveness of processing techniques and hygiene measures were evaluated by monitoring the viable microbial load at nine different production stages of pear concentrate manufacturing, as shown in Fig. 1. The use of either of the two flume water systems resulted in an increase in viable aerobic counts on fruit from bins to flume wash, with counts increasing from 3.81 to 4.67 $\log_{10}$ cfu mL^{-1} (recirculating system) and from 4.53 to 4.80 $\log_{10}$ cfu mL^{-1} (one-pass system) (Fig. 1). The higher microbial load on incoming fruit when the one-pass system was operational could be attributed to post-harvest contamination, as well as a decrease in fruit quality and various deteriorating intrinsic growth factors towards the end of the pear season (Hui *et al.,* 2006). High viable aerobic counts were recovered from fruit mash samples that were sampled from the inline hammer mill, with 4.75 $\log_{10}$ cfu mL^{-1} (recirculating system) and 4.57 $\log_{10}$ cfu mL^{-1} (one-pass system) (Fig. 1). Due to the difficulty of sampling from the hammer mill, fruit mash samples contained large amounts of fruit skin which could explain the high level of viable aerobic counts at this point as contamination naturally occurs on the fruit surface.

Most acid tolerant potential spoilage microbes have a low heat resistance and their vegetative forms will be inactivated by pasteurisation treatments under 100 ℃ for relatively short times (Silva & Gibbs, 2004). This was evident as evaporation of single strength juice resulted in a decrease in viable aerobic counts from 4.61 to 3.54 $\log_{10}$ cfu mL^{-1} (recirculating system) and from 2.98 to 0.31 $\log_{10}$ cfu mL^{-1} (one-pass system) in pear concentrate. Following pasteurisation (102 ° - 104 ℃ for 90 s), 2.07 $\log_{10}$ cfu mL^{-1} (117 cfu mL^{-1}) (recirculating system) and 0.17 $\log_{10}$ cfu mL^{-1} (1 cfu mL^{-1}) (one-pass system) viable aerobic counts were recovered in the final product (Fig. 1). Furthermore, counts that were recovered from both the pear concentrate and the final pasteurised product differed significantly ($P < 0.05$) from each other during the functioning of the two respective flume systems.

Alicyclobacillus

PCR results from randomly selected colonies indicated a 134 bp fragment (data not shown) similar to the fragments obtained for *A. acidoterrestris, A. acidocaldarius* and *A. cycloheptanicus* (Connor *et al.,* 2005). Significantly higher levels of *Alicyclobacillus*

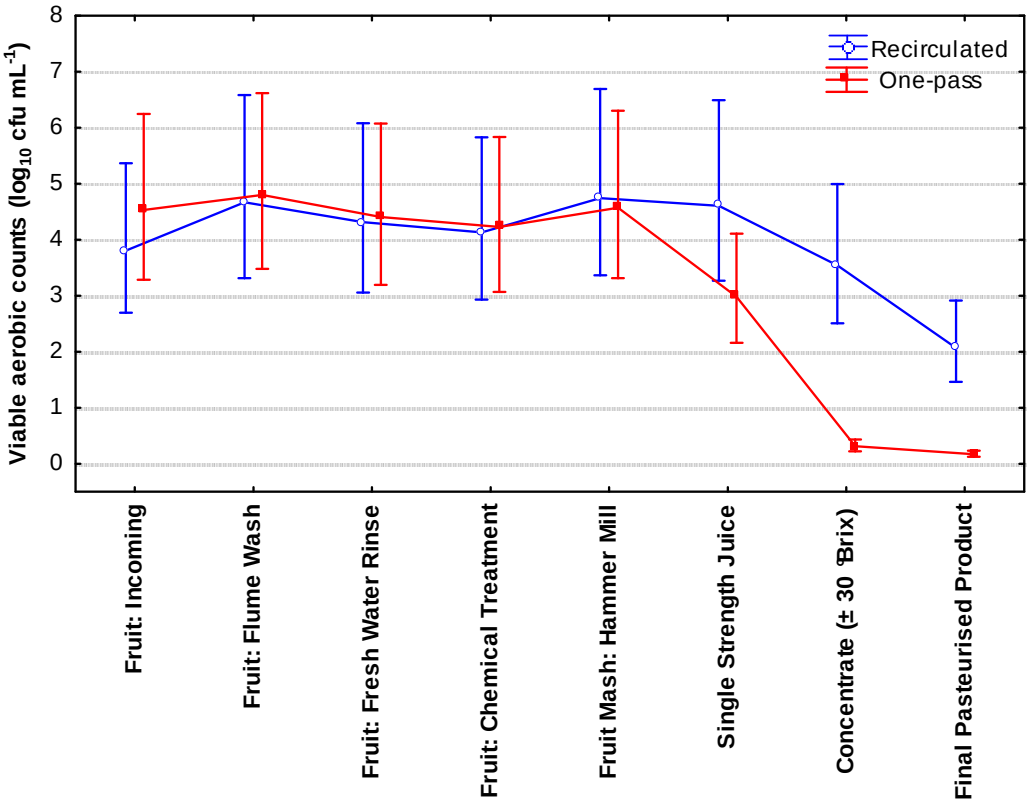

Figure 1 Viable aerobic counts at different stages during pear concentrate production (each data point represents 12 values for the recirculated and 20 values for the one-pass flume water system). Bars indicate 95% confidence intervals with a predicted average. Bars that do not overlap indicate a significant difference ($P < 0.05$).

were recovered on fruit at the fruit washing stages when the recirculating system was operational than during the one-pass system ($P < 0.05$), with 1.17 - 1.23 $\log_{10}$ cfu mL^{-1} vegetative cells and 1.01 - 1.32 $\log_{10}$ cfu mL^{-1} endospores (recirculating system), and 0.01 - 0.43 $\log_{10}$ cfu mL^{-1} vegetative cells and 0.01 - 0.08 $\log_{10}$ cfu mL^{-1} endospores (one-pass system) recovered respectively (Figs. 2 and 3). This signifies the importance of adequate washing procedures, water quality and hygiene measures for the prevention of *Alicyclobacillus* contamination and accumulation during fruit concentrate manufacturing (Parish & Goodrich, 2005; Wisse & Parish, 2006). The AIJN therefore suggests that the frequency at which flume water is changed should be influenced by the effectiveness of the final washing process to remove or reduce *Alicyclobacillus* on fruit (AIJN, 2008). In addition, when the recirculating system was operational, *Alicyclobacillus* levels on fruit increased after the chemical dose from 0.83 to 1.23 $\log_{10}$ cfu mL^{-1} (vegetative cells) and 1.10 to 1.32 $\log_{10}$ cfu mL^{-1} (endospores), whereas during the functioning of the one-pass system the vegetative cells increased from 0.01 to 0.43 $\log_{10}$ cfu mL^{-1} (Fig. 2). This suggests that the efficacy of this chemical for the current contact time should be revised.

Alicyclobacillus levels were significantly higher in fruit mash, single strength juice, concentrate and the final pasteurised product during the functioning of the recirculating system compared to when the one-pass flume water system was operational ($P < 0.05$) (Figs. 2 and 3). The increase of *Alicyclobacillus* endospores in single-strength juice from 0.79 to 1.60 $\log_{10}$ cfu mL^{-1} when the recirculating system was operational is possibly due to the activation of endospores after the hot-break treatment (75 °- 90 ℃ for ± 10 min and cooled to 65 ℃) (Fig. 3). The hi ghest *Alicyclobacillus* levels were recovered from unpasteurised pear concentrate when the recirculating flume water system was operational, with 1.66 $\log_{10}$ cfu mL^{-1} vegetative cells (Fig. 2) and 1.75 $\log_{10}$ cfu mL^{-1} endospores (Fig. 3). High levels at this point cannot solely be attributed to the concentration of juice, which would increase the amount of cells per sample volume, since similar levels of vegetative cells (1.81 $\log_{10}$ cfu mL^{-1}) and endospores (1.01 $\log_{10}$ cfu mL^{-1}) were recovered from condensate water samples (data not shown). Accumulation of alicyclobacilli in the evaporator is also a possibility given the difficulty to clean evaporators and that optimal temperature conditions in this equipment would allow for the growth of *Alicyclobacillus* (AIJN, 2008). Additional testing for biofilm formation is therefore suggested at this point. *Alicyclobacillus* levels did not build-up in single strength juice or in unpasteurised pear concentrate when the

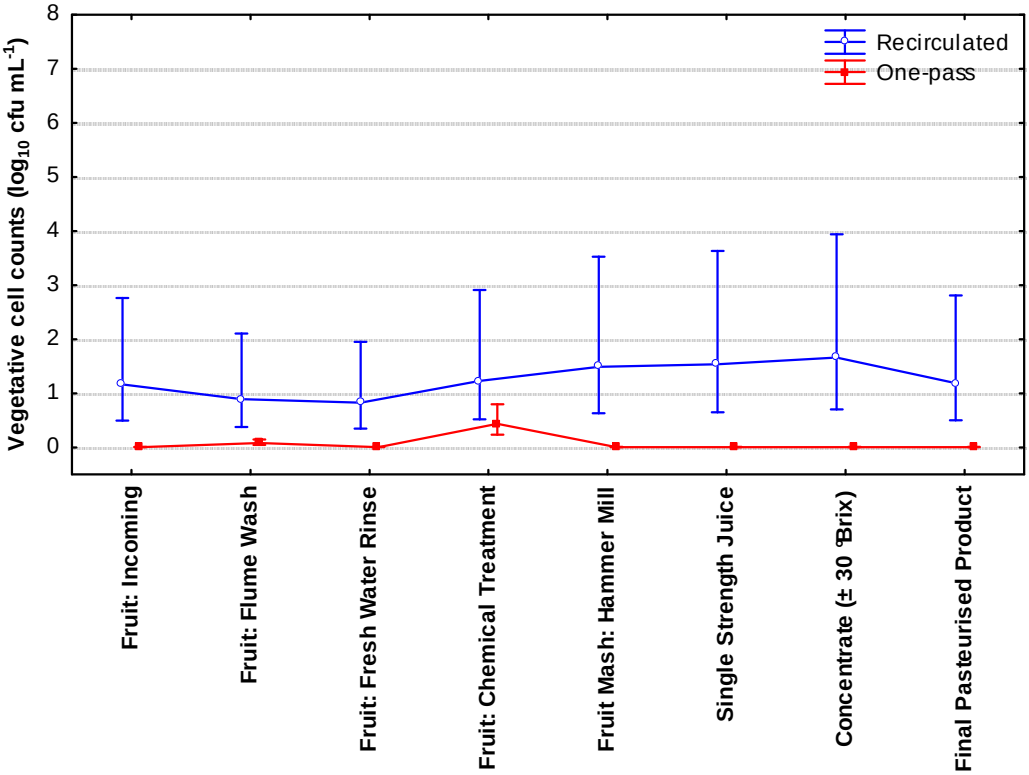

Figure 2 Vegetative *Alicyclobacillus* cell counts at different stages during pear concentrate production (each data point represents 12 values for the recirculated and 20 values for the one-pass flume water system). Bars indicate 95% confidence intervals with a predicted average. Bars that do not overlap indicate a significant difference ($P < 0.05$).

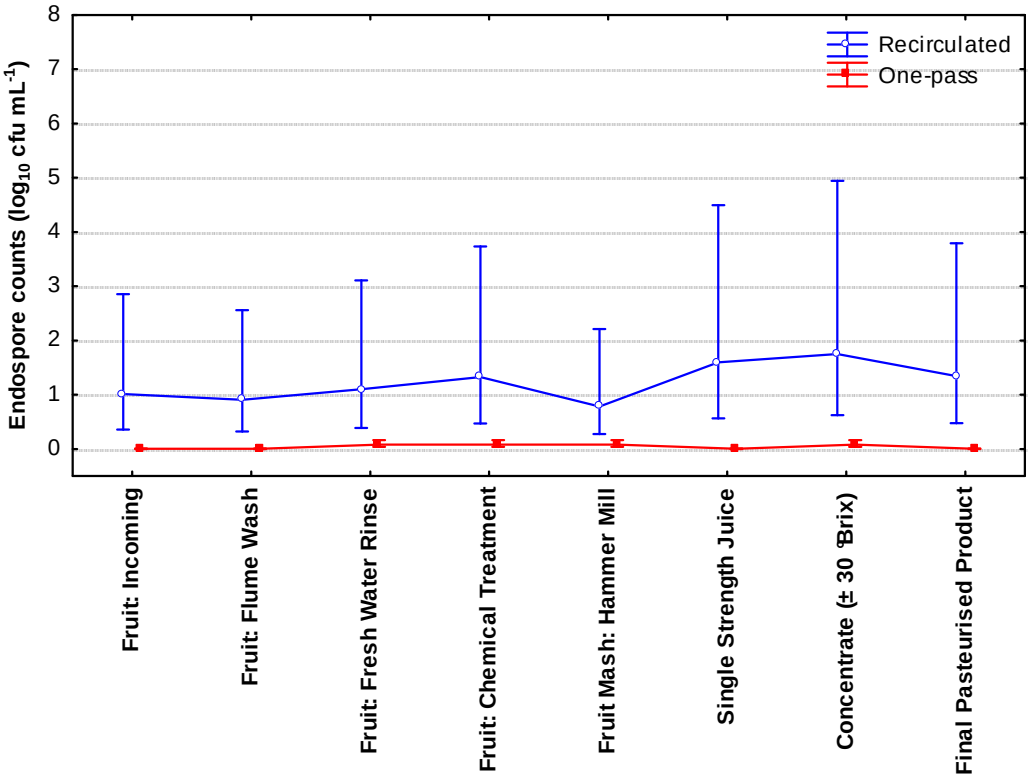

Figure 3 *Alicyclobacillus* endospore counts at different stages during pear concentrate production (each data point represents 12 values for the recirculated and 20 values for the one-pass flume water system). Bars indicate 95% confidence intervals with a predicted average. Bars that do not overlap indicate a significant difference ($P < 0.05$).

one-pass system was operational since < 0.08 $\log_{10}$ cfu mL^{-1} vegetative cells (Fig. 2) and endospores (Fig. 3) were recovered at these points.

Data suggest that there were not a complete elimination of *Alicyclobacillus* from the final product during the recirculating system, although counts reduced after pasteurisation (102 ° - 104 ℃ for 90 s) of the pea r concentrate to 1.19 $\log_{10}$ cfu mL^{-1} vegetative cells and 1.35 $\log_{10}$ cfu mL^{-1} endospores (Figs. 2 and 3). Levels of *Alicyclobacillus* that were found in concentrated orange juice (66 °Brix) from São Paulo (Brazil) ranged between 1.85 - 3.53 $\log_{10}$ cfu mL^{-1} (Eguchi *et al.,* 1999). High levels (> 2 $\log_{10}$ cfu mL^{-1}) of *Alicyclobacillus* cells were also recovered from several industrial fruit concentrates (Pinhatti *et al.,* 1997), while < 2.63 and < 1.47 $\log_{10}$ cfu g^{-1} endospores were recovered from frozen concentrated orange juice and pear concentrate, respectively, from a facility in Florida (USA) (Wisse & Parish, 1998). *Alicyclobacillus* spoilage of fruit concentrates is not likely since the soluble solid content (> 20 ℃Brix) will inhibit the germination of *Alicyclobacillus* endospores (Splittstoesser *et al.,* 1994; Chang & Kang, 2004). However, upon reconstitution to single strength, the composition of fruit beverages would be favourable for germination and growth of endospores, thus increasing the risk of spoilage (Borlinghaus & Engel, 1997; Pinhatti *et al.,* 1997). *Alicyclobacillus* levels did not accumulate in the final pasteurised product when the one-pass system was operational with 0 cfu mL^{-1} vegetative cells (Fig. 2) and endospores (Fig. 3) recovered from these samples.

Alicyclobacillus levels in the condensate water did not differ significantly during the functioning of the two flume water systems ($P > 0.05$) in that 1.81 $\log_{10}$ cfu mL^{-1} vegetative cells and 1.01 $\log_{10}$ cfu mL^{-1} endospores (recirculating system), and 0.78 $\log_{10}$ cfu mL^{-1} vegetative cells and 0.42 $\log_{10}$ cfu mL^{-1} endospores (one-pass system) were recovered respectively (data not shown). Condensate water is known to harbour *Alicyclobacillus* with up to 3.78 $\log_{10}$ cfu mL^{-1} recovered from condensate water from orange processing plants and when using the most probable number technique (MPN) with three replicate tubes per dilution, 6.18 $\log_{10}$ MPN mL^{-1} were recovered (Eguchi *et al.,* 1999). The study also found that *Alicyclobacillus* counts on fruit surfaces increased after fruit washing with condensate water (Eguchi *et al.,* 1999). Furthermore, the condensate water from 6 out of 7 citrus processing plants in Florida contained up to 3.36 $\log_{10}$ MPN mL^{-1} *Alicyclobacillus* endospores (three-tube technique) (Wisse & Parish, 1998). Therefore, unless properly treated, condensate water should not be re-used within the processing environment (AIJN, 2008).

Conclusions

Both *Alicyclobacillus* vegetative cells and endospores were recovered from samples which suggest that *Alicyclobacillus* levels will be underestimated if a heat-shock treatment is not applied prior to plating on selective media. When monitoring nine production stages of pear concentrate, it was found that *Alicyclobacillus* counts accumulated in the final product when a recirculating flume water system was operational. The highest levels of *Alicyclobacillus* were recovered from unpasteurised concentrate from the evaporator, which makes this piece of equipment a point of concern during fruit concentrate production. Improved cleaning methods, as well as the design of evaporators should be further investigated. The presence of *Alicyclobacillus* endospores in pasteurised fruit concentrates suggests that thermal processing is insufficient for the complete elimination of these bacteria and confirm reports from literature, regarding the thermal resistance of *Alicyclobacillus* endospores.

Alicyclobacillus levels did not accumulate in the final product when the one-pass flume system, during which a continuous flow of fresh potable water was used as processing water, was operational. The fact that *Alicyclobacillus* counts in the final product were related to the initial contamination levels of the raw material emphasises the importance of good plant hygiene and flume water quality. Water treatment protocols should be well established if untreated recirculated- or condensate water are required to be used as processing water. Such treatment protocols should aid in the prevention of *Alicyclobacillus* accumulation in the fruit concentrate processing environment. Furthermore, viable aerobic counts in the final pasteurised product did not correlate with that of *Alicyclobacillus* and can, therefore, not be used as an indicator for the presence of *Alicyclobacillus.*

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CHAPTER 4

PREVENTION OF THE ACCUMULATION OF *ALICYCLOBACILLUS* IN APPLE CONCENTRATES BY RESTRICTING THE CONTINUOUS PROCESS RUNNING TIME

Abstract

The aim of this study was to investigate the accumulation of vegetative cells and endospores of *Alicyclobacillus*, as well as viable aerobic counts during the continuous production of apple juice concentrate. Apples were processed for a continuous process running time of 108 h (processing rate 1.8 - 2.0 t h^{-1}) without CIP procedures in-between different batches. Samples from single strength apple juice, concentrate after evaporation (± 30 °Brix), the final product (concentrate pasteurised at 102 °- 104 °C for 90 s) and condensate water (by-product of the juice concentration process) were collected every 12 h. From 12 to 84 h of processing, vegetative *Alicyclobacillus* counts in single strength apple juice increased significantly ($P < 0.05$) from 1 to 3.15 $\log_{10}$ cfu mL^{-1}. Accumulation patterns of vegetative cells in apple concentrate and the final product were similar from 24 to 84 h of processing, with the respective counts increasing from 0.13 to 1.63 $\log_{10}$ cfu mL^{-1} and 0.01 to 1.69 $\log_{10}$ cfu mL^{-1}. The highest *Alicyclobacillus* endospore counts in single strength juice, concentrate and the final product was at 84 h of processing with 1.32, 1.59 and 1.64 $\log_{10}$ cfu mL^{-1}, respectively. It was concluded that *Alicyclobacillus* vegetative cells and endospores accumulate in fruit concentrates during a continuous process running time of 108 h. In conjunction with good manufacturing practices, fruit concentrate manufactures can minimise *Alicyclobacillus* accumulation in fruit concentrates by limiting the continuous process running time between clean-ups to under 84 h.

Introduction

Fruit concentrates form the basis of various fruit beverage products and have traditionally been regarded as microbiologically stable due to several intrinsic properties, including the high sugar concentration (65 - 80 °Brix), low pH values (pH: 2.5 - 4.0), water activity (a_w: 0.85 - 0.99), reduced oxygen and nitrogen concentrations, as well as the presence of organic acids (Pontius *et al.,* 1998; Deák, 2008). As a result,

Steyn, C.E., Cameron, M., Brittin, G. & Witthuhn, R.C. (2011). Prevention of the accumulation of *Alicyclobacillus* in apple concentrates by restricting the continuous process running time. *Journal of Applied Microbiology*, **110**, 658-665.

concentrated fruit products are considered commercially sterile and can be transported and stored unfrozen, thereby significantly reducing the costs involved.

High-acid (pH < 4.0) fruit products do, however, remain substrates for acid tolerant microbes such as lactic acid bacteria, acetic acid bacteria, yeasts and mycelial fungi (Silva & Gibbs, 2004). Due to the fact that these microbes are not heat resistant, the fruit juice industry applies a hot-fill and hold pasteurisation treatment (88 °- 96 °C for 2 min) in order to prevent spoilage (McIntyre *et al.,* 1995; Chang & Kang, 2004). It is unlikely that endospores will be destroyed by pasteurisation treatments, although it is assumed that their growth will be inhibited by the acidity of fruit products (Cerny, 1980; Silva & Gibbs, 2004).

Members of the genus *Alicyclobacillus* are thermo-acidophilic, non-pathogenic, endospore-forming microbes that have an exceptionally high resistance to thermal processing applications and are unaffected by the adverse pH conditions and soluble solid content of fruit juice products (Wisotzkey *et al.,* 1992; Palop *et al.,* 2000; Ceviz *et al.,* 2009). Although the soluble solid content of fruit concentrates exceeding 20 °Brix will inhibit endospore germination (Splittstoesser *et al.,* 1994; Chang & Kang, 2004), endospores of *Alicyclobacillus* will remain viable. Upon dilution to single strength fruit juice, these endospores could germinate and multiply to numbers high enough (> 100 cfu mL^{-1}) to cause spoilage taints and product deterioration (Borlinghaus & Engel, 1997; AIJN, 2008). In addition, it is believed that pasteurisation serves as a heat-shock treatment that could initiate endospore germination (Splittstoesser *et al*., 1994).

The first large-scale spoilage incident by *Alicyclobacillus* was reported by Cerny *et al.* (1984) who found aseptically packed apple juice in Germany to have a distinct off-flavour and slight cloudiness. Since then, spoilage incidents have been reported frequently and include a diverse range of high-acid, shelf-stable fruit and vegetable products that were either hot-filled, pasteurised, canned, ultra-heat treated or carbonated (Chang & Kang, 2004; Gouws *et al.,* 2005; Walker & Philips, 2008). The occurrence of *Alicyclobacillus* endospores in high-acid concentrated fruit products have been reported by several authors (Table 1).

The aim of this study was to investigate the accumulation of *Alicyclobacillus* during the continuous production of fruit concentrate. Endospores and vegetative cells of *Alicyclobacillus*, as well as viable aerobic counts were monitored during the manufacturing of apple juice concentrate in order to determine whether a restriction in the process running time between clean-ups could limit the occurrence of *Alicyclobacillus* in the final processed product.

Table 1 Occurrence of *Alicyclobacillus* endospores in concentrated fruit juice products

Concentrated juice	SS (°Brix)	Endospores cfu mL^{-1} [no. positive samples]	Origin	Reference
Orange	66	70 - 3 400	São Paulo (Brazil)	Eguchi *et al*., 1999
Frozen Orange	66	10 - 3 400 [23 of 28]	São Paulo (Brazil)	
Pear	32	NR	Western Cape (SA)	Groenewald *et al*., 2009
Apple, Orange	NR	NR	Parma (Italy)	Previdi *et al.,* 1999
Orange, Apple, Watermelon	NR	NR	Brazil, USA, Austria, Thailand	Goto *et al*., 2006
Apple	NR	NR	Washington (USA)	Walls & Chuyate, 1998
Orange	> 50	< 6.8 - 947 MPN 100 g^{-1} † [14.7% of 75]	Brazil	Eiroa *et al*., 1999
Frozen Orange	65	< 30, 150, 230, 230, 430 MPN g^{-1} [5 of 23]	Florida (USA)	Wisse & Parish, 1998
Pear	NR	< 30 MPN g^{-1}	Florida (USA)	
Banana, Apricot	NR	< 100 [3 of 18]	Germany	Baumgart & Menje, 2000
Frozen Orange	66	< 1 - 12 000	Costa Rica, Mexico, USA, Brazil	Pinhatti *et al.,* 1997
Orange	> 40	36, 60	Brazil, Mexico, Florida (USA)	
Limeade	> 40	510	USA	
Lemonade	> 40	3 020	USA	
Apple	50	0.020 - 0.615 ml^{-1} FCM ‡ [36% of 166]	Various origin	Borlinghaus & Engel, 1997
Mango	NR	NR [16 of 24]	South Africa	Gouws *et al*., 2005
Apple	72	NR	China	Chen *et al*., 2006
Apple	NR	NR [19 of 64]	Australia	Jensen, 2005
Orange	NR	300, < 100 CFU g^{-1} § [61 of 85]	Australia	

SS - Soluble solids, NR - Not reported; † Most probable number; ‡ Flow cytometry method (> 0.05 mL^{-1} rank as a positive identification); § Respective ca counts for *A. acidocaldarius* and *A. acidoterrestris*

Materials and methods

Sampling

Samples were collected from a hazard analysis critical control point (HACCP) accredited fruit concentrate manufacturing facility located in the Western Cape (South Africa) at four production stages of apple concentrate, including single strength apple juice after a hot-break treatment (75 °- 90 ℃ for ± 15 min and cooled to 65 ℃), apple concentrate (± 30 ℃Brix) from a forced circulation evaporator, the final apple concentrate after pasteurisation (102 °- 104 ℃ for 90 s), and the condensate water which is a by-product of the juice concentration process. Samples (ca 50 mL) were collected in sterile bottles every 12 h during a continuous process running time of 108 h (processing rate 1.8 - 2.0 t h^{-1}), with full CIP procedures in-between the experiments. Multiple batches were used per experiment, without CIP procedures in-between the batches. Duplicate tests were performed on all the samples and the experiment was repeated six times.

Enumeration

Viable aerobic counts served as general hygiene indicators of the concentrate manufacturing facility. Samples were serially diluted with sterile saline solution (0.85% (m/v) NaCl (Merck, Cape Town, South Africa)) and the pour-plate technique and plate count agar (PCA) (Merck) was used for enumeration with incubation at 35 ℃ for 48 h (Wisse & Parish, 1998).

Potato dextrose agar (PDA) (Merck) at pH 3.7, adjusted with 1M H_2SO_4 (Chang & Kang, 2004; Witthuhn *et al.,* 2007) and the spread plating method was used to recover *Alicyclobacillus* from the samples. Samples that did not receive a heat-shock treatment prior to plating were enumerated as vegetative cells, as it has been reported that *Alicyclobacillus* require a heat-shock treatment to ensure germination (Pettipher *et al.,* 1997; Yokota *et al.,* 2007). Vegetative cell growth was enumerated after incubation at 45 ℃ for 5 d (Walls & Chuyate, 1998; Groenewald *et al.,* 2009). Endospores were detected after a heat-shock treatment at 80 ℃ for 10 min prior to plating, so as to promote endospore germination and eliminate vegetative cells (Pettipher *et al.,* 1997; Pinhatti *et al*., 1997). Sterile PCA and PDA served as controls for viable aerobic and *Alicyclobacillus* counts, respectively, and no growth was detected after incubation.

Identification

Preliminary identification of *Alicyclobacillus* was done (on randomly selected plates) by conventional microscopic analysis with morphological characteristics that included rod-shaped, Gram-positive to Gram-variable microbes with/or without oval-shaped sub-terminally located endospores (Wisotzkey *et al.,* 1992; Chang & Kang, 2004). Results were confirmed by polymerase chain reactions (PCR) where the genus-specific primers, CC16S-F (5' CGT AGT TCG GAT TGC AGG C-3') and CC16S-R (5' GTG TTG CCG ACT CTC GTG-3') were used to amplify a 134 bp fragment (1254 to 1388 bp) of the 16S rRNA gene (Connor *et al.,* 2005).

PCR reactions were performed in a total mixture volume of 25 µL that contained 0.2 µM of each primer (Eurofins MWG Operon, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 1.25 U *Taq* DNA polymerase (Super-Therm, supplied by Southern Cross Biotechnologies), 1 x PCR reaction buffer (Super-Therm), 6 mM $MgCl_2$ (Super-Therm), 1 µL of 99% (v/v) dimethyl sulphoxide (DMSO) (Merck), 0.8 mM dNTP mix (AB gene, supplied by Southern Cross Biotechnologies) and 5 µL extracted DNA. The following thermal cycling conditions were used for amplification in an Eppendorf Mastercycler Personal (Eppendorf, Germany): initial denaturation at 95 ℃ for 3 min; followed by 35 cycles of denaturation at 94 ℃ for 30 s; annealing at 55 ℃ for 30 s; and elongation at 72 °C for 30 s with final elongation at 72 ℃ for 2 min. PCR products (5 µL) were separated on a 1.5% (m/v) agarose gel, containing 0.02 µL mL^{-1} ethidium bromide, in 0.5 x TBE electrophoresis buffer (45 mM tris base, 45 mM boric acid and 1 mM EDTA) (Merck). The separated PCR fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat, Marne La Vallee, France).

Statistical analysis

Statistical analysis was done using Statistica™ 9 (StatSoft, Inc., Tulsa, OK, USA). Due to the non-normal nature of the data, all variables were log transformed. A two-way ANOVA was done to investigate the effects of time and the sampling site on *Alicyclobacillus* accumulation during apple concentrate manufacturing. A 5% ($P < 0.05$) significance level was used to determine significant effects.

Results and discussion

Viable aerobic counts

Viable aerobic counts are an indication of the effectiveness of the processing techniques and hygiene measures applied during the manufacturing of apple concentrate. These counts are also used to monitor the facility's adherence to general manufacturing practices (AIJN, 2008). Changes in viable aerobic counts during a continuous process running time of 108 h are shown in Fig.1.

From 12 to 36 h of processing, counts in single strength apple juice decreased from 3.87 to 3.19 $\log_{10}$ cfu mL^{-1}. Subsequent values fluctuated between 3.13 $\log_{10}$ cfu mL^{-1} at 84 h, and 4.43 $\log_{10}$ cfu mL^{-1} at 96 h, with 3.41 $\log_{10}$ cfu mL^{-1} counts recovered at 108 h (Fig. 1). During apple concentrate manufacturing fruit skins are not removed. Furthermore, the external surface of fruits are known to harbour a variety of microbes that either occur naturally on fruit, or are collected after contact with composted soil, air, insects, human contact during harvesting or untreated processing water (McIntyre *et al.,* 1995; Parish & Goodrich, 2005). An altering pattern of aerobic counts accumulation in single strength juice during processing could, therefore, be indicative of the varying quality of raw fruit. Evaporation of single strength juice resulted in the reduction of viable aerobic counts by more than 1 log cycle (Fig. 1).

From the evaporator, viable aerobic counts in unpasteurised apple concentrate declined from 1.36 to 0.66 $\log_{10}$ cfu mL^{-1} after 24 h of processing and remained constant for the rest of the 108 h process running time. In the final pasteurised product the counts ranged from 0.18 $\log_{10}$ cfu mL^{-1} (< 2 cfu mL^{-1}) at 48 h to non-detectable levels at 60 h (Fig. 1), confirming the microbiological stability of the product. This was expected due to the thermal processing treatments (evaporation and pasteurisation at 102 °- 104 ℃ for 90 s) (Pontius *et al.,* 1998; Deák, 2008). The fact that viable aerobic counts (Fig. 1) in the final product did not correlate with *Alicyclobacillus* counts (Figs. 2 and 3) suggest that aerobic counts should not be used as an indicator for the presence of *Alicyclobacillus* in fruit concentrates.

A significant ($P < 0.05$) accumulation of viable aerobic counts in condensate water during the continuous process running time of 108 h, from 2.25 to 4.75 $\log_{10}$ cfu mL^{-1} (Fig. 1), indicate that condensate water should be a critical control point in manufacturing facilities that recover and re-use this water. Similar results were reported by Wisse & Parish (1998), who found the total microbial population in condensate water to range between non-detectable levels and 7.9×10^4 cfu mL^{-1}.

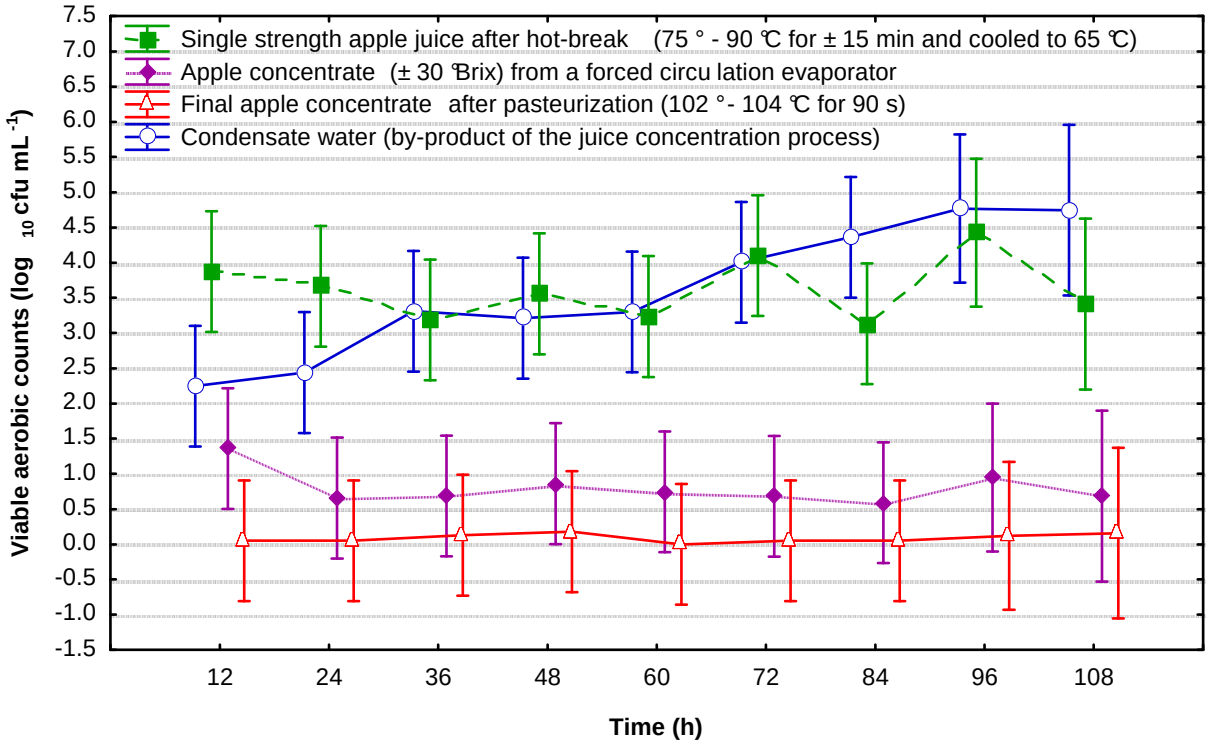

Figure 1 Viable aerobic counts during a continuous process running time (vertical bars denote 0.95 confidence intervals).

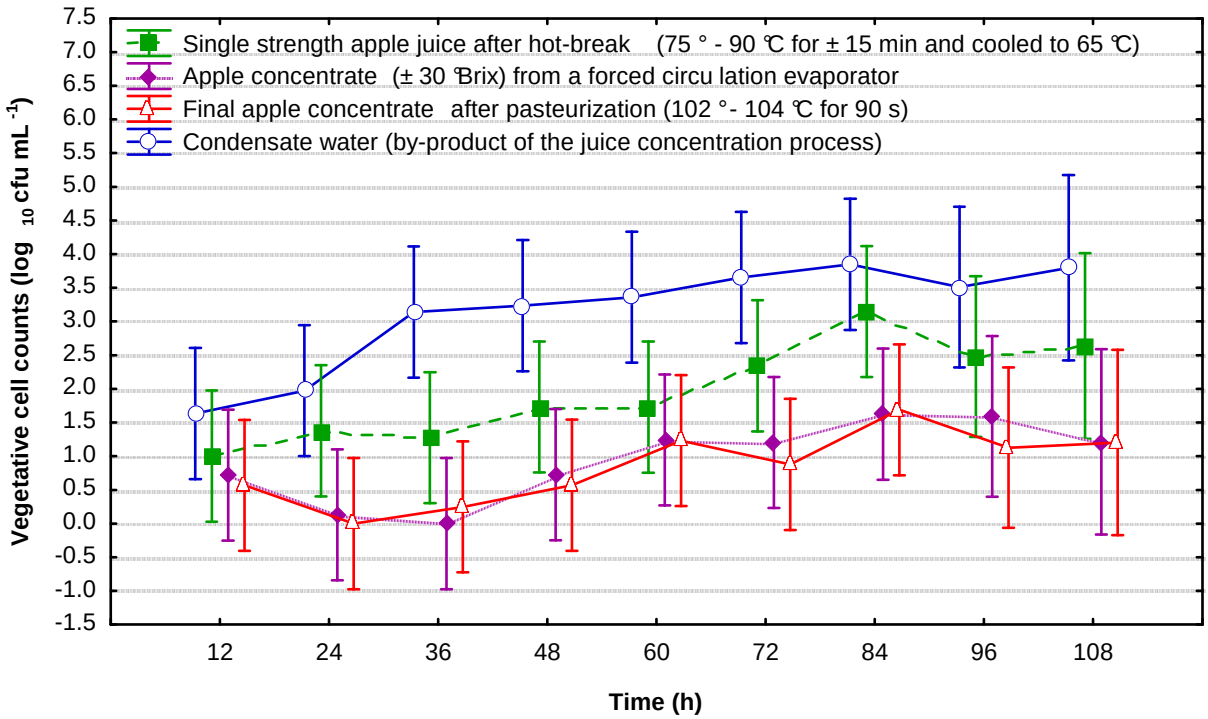

Figure 2 Vegetative *Alicyclobacillus* cell counts during a continuous process running time (vertical bars denote 0.95 confidence intervals).

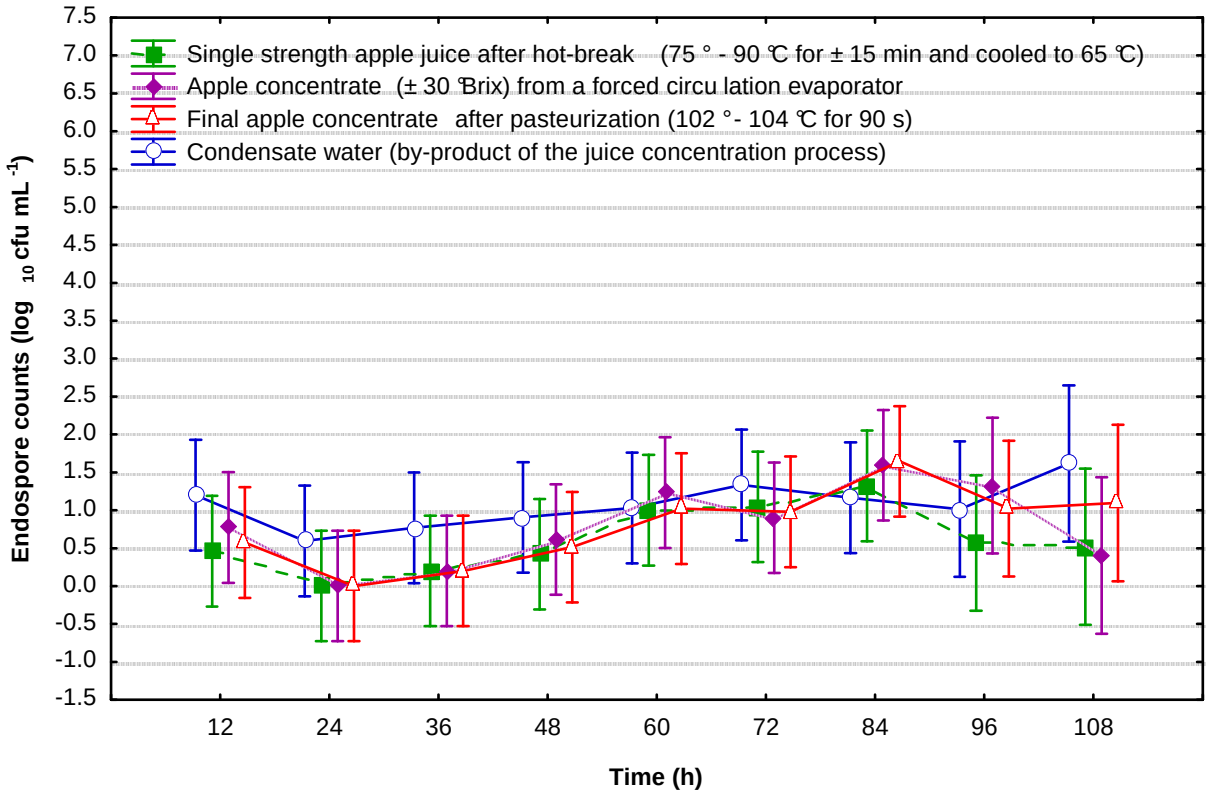

Figure 3 *Alicyclobacillus* endospore counts during a continuous process running time (vertical bars denote 0.95 confidence intervals).

Alicyclobacillus vegetative cells

PCR reactions on colonies from randomly selected plates (data not shown) indicated a 134 bp fragment similar to the fragments from *A. acidoterrestris, A. acidocaldarius,* and *A. cycloheptanicus* strains (Connor *et al.,* 2005). The build-up of vegetative *Alicyclobacillus* cells during 108 h are shown in Fig. 2.

A steady, significant ($P < 0.05$) accumulation of vegetative *Alicyclobacillus* counts in single strength apple juice ranging between 1 - 3.15 $\log_{10}$ cfu mL^{-1} was observed from 12 to 84 h of processing (Fig. 2). Thereafter the counts declined to 2.64 $\log_{10}$ cfu mL^{-1} at the end of the continuous process running time at 108 h. The main source of *Alicyclobacillus* in the fruit concentrate processing environment is thought to be from soil adhering to fruit surfaces in conjunction with poor fruit washing procedures and contaminated processing water (McIntyre *et al.,* 1995; Walls & Chuyate, 1998; AIJN, 2008). Additionally, it is believed that the presence of *Alicyclobacillus* in the final processed product could be dependent on the initial contamination level of raw fruit (Bahçeci *et al.,* 2005).

Following evaporation and pasteurisation (102 ° - 1 04 ℃ for 90 s) of single strength apple juice, a reduction in vegetative *Alicyclobacillus* cell counts was evident in apple concentrate and the final product (Fig. 2). Furthermore, the cell counts and accumulation patterns of vegetative cells in apple concentrate and the final product were very similar. In unpasteurised apple concentrate, counts reduced from 0.72 to 0.13 $\log_{10}$ cfu mL^{-1} after 24 h of processing, where after the counts increased to 1.63 $\log_{10}$ cfu mL^{-1} at 84 h and declined once more to 1.21 $\log_{10}$ cfu mL^{-1} at 108 h (Fig. 2). After 24 h of processing, counts in the final pasteurised product reduced from 0.57 $\log_{10}$ cfu mL^{-1} to non-detectable levels, increased to 1.69 $\log_{10}$ cfu mL^{-1} at 84 h and decreased once more to 1.20 $\log_{10}$ cfu mL^{-1} at 108 h (Fig. 2). Alicyclobacilli are renowned for their thermal resistance, therefore, it was expected that pasteurisation would not be sufficient for their complete elimination from the final product (Yamazaki *et al.,* 1997; Eguchi *et al*., 1999; Ceviz *et al.,* 2009). Although few reports separate vegetative and endospore alicyclobacilli counts, the results in Fig. 2 coincide with the findings of Pettipher *et al.* (1997) who recovered < 5 cfu mL^{-1} vegetative cells from apple concentrate, and Eguchi *et al*. (1999) who recovered between < 10 - 1.7 x 10^3 cfu mL^{-1} counts from single-strength orange juice. As far as we know, this is the first report to link vegetative *Alicyclobacillus* counts in fruit concentrates to a specific processing time in addition to monitor the effect of alicyclobacilli accumulation during fruit concentrate production.

Condensate water is a by-product of the juice concentration process and proposed rules by the European Union require re-using this water for fruit washing purposes (Wisse & Parish, 1998). However, the recent best practice guideline by the European Fruit Juice Association paid particular attention to water which is heated and re-used in other production steps, as this could lead to contamination and recontamination by *Alicyclobacillus* (AIJN, 2008). A significant increase ($P < 0.05$) in vegetative *Alicyclobacillus* counts were observed in condensate water during the production of apple concentrate (Fig. 2). Counts increased from 1.63 to 3.65 $\log_{10}$ cfu mL^{-1} from 12 to 72 h of processing and to 3.85 $\log_{10}$ cfu mL^{-1} at 84 h, where after the counts decreased to 3.80 $\log_{10}$ cfu mL^{-1} at 108 h. Similar *Alicyclobacillus* counts were recovered in condensate water from washing systems of orange processing facilities (São Paulo) with up to 6 x 10^3 cfu mL^{-1} and 1.5 x 10^6 MPN mL^{-1} when using the most probable number technique with three replicate tubes per dilution (Eguchi *et al*., 1999). Risks are, therefore, involved when re-using untreated condensate water, especially when the processing time exceeds 36 h (> 1 x 10^3 cfu mL^{-1}) (Fig. 2), given that alicyclobacilli could thereby be inadvertently re-introduced into the processing environment.

Alicyclobacillus endospores

The occurrence of *Alicyclobacillus* endospores in Fig. 3 signifies the importance of a heat-shock treatment during the enumeration of this microbe from fruit juice concentrates (Pinhatti *et al*., 1997; Pettipher *et al.,* 1997). *Alicyclobacillus* endospores are extremely heat resistant and current industrial hot-fill-and-hold pasteurisation processes (86 °- 96 ℃ for approximately 2 min) do not eliminate them from high-acid concentrated fruit products (Eiroa *et al.,* 1999; Silva & Gibbs, 2004; Ceviz *et al.,* 2009). As a result, very similar endospore counts and accumulation patterns were expected from single strength apple juice, apple concentrate and the final product. During the first 24 h of continuous processing, endospore counts decreased from 0.46, 0.77 and 0.57 $\log_{10}$ cfu mL^{-1} in single strength apple juice, concentrate and the final product, respectively to non-detectable levels in all samples. The highest recovery of endospores from these samples were after 84 h of continuous processing with 1.32, 1.59 and 1.64 $\log_{10}$ cfu mL^{-1}, respectively (Fig. 3). These results are comparable to *Alicyclobacillus* endospore counts that were recovered from concentrated fruit products in Table 1. Borlinghaus & Engel (1997) indicated that concentrated raw materials inoculated at high levels (1 x 10^3 cfu mL^{-1}) did not develop off-flavours during storage at

45 ℃ for 4 weeks. Upon dilution, however, higher growth levels of *Alicyclobacillus*, as well as characteristic off-flavours were observed in single strength products (Borlinghaus & Engel, 1997). It is, therefore, important to reduce *Alicyclobacillus* endospores from high-acid concentrated fruit products, a fact that was emphasised by Silva *et al*. (1999) who suggested using *A. acidoterrestris* endospores as the target for the pasteurisation processes. A steady accumulation of endospores from 1.20 to 1.62 $\log_{10}$ cfu mL^{-1} were observed in the condensate water during a continuous process running time from 12 to 108 h (Fig. 3). This is noteworthy for operations that are required to re-use untreated water for fruit washing procedures (AIJN, 2008).

Conclusions

Results clearly indicate that vegetative cells and endospores of *Alicyclobacillus* accumulate during the continuous processing of apple juice concentrate. Furthermore, the presence of *Alicyclobacillus* endospores in pasteurised concentrates (± 30 °Brix) suggests that pasteurisation at 102 ° - 104 ℃ for 90 s is insufficient for the complete elimination of this microbe. To our knowledge this is the first study to separately monitor the accumulation of *Alicyclobacillus* vegetative cells and endospores in fruit concentrate during a continuous process running time. In conjunction with good manufacturing practices, fruit concentrate manufactures can minimise *Alicyclobacillus* cells from accumulating in fruit concentrates by limiting the processing time in-between clean-ups. A continuous processing time of less than 84 h is suggested. Furthermore, if condensate water is to be used as processing water, water treatment protocols should be established in order to prevent the cross-contamination of *Alicyclobacillus* in fruit concentrates and the processing environment.

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CHAPTER 5

EFFECT OF FRUIT VARIETY ON THE OCCURRENCE OF *ALICYCLOBACILLUS* IN FRUIT CONCENTRATES

Abstract

The contamination of concentrated fruit products by *Alicyclobacillus* is of great concern to the fruit beverage industry. Research on alicyclobacilli in fruit concentrates has predominantly focused on the contamination of apple and citrus fruit concentrates. In this study, the effect of fruit skin type, specifically hairy-skin (stone fruits, peach and apricot) and smooth-skin (pome fruits, apple and pear), on the occurrence of *Alicyclobacillus* in concentrates were examined. The presence of vegetative *Alicyclobacillus* cells and endospores were determined using acidified PDA (pH 3.7) with incubation at 45 ℃ for 5 d. Identification w as done based on morphology and PCR amplification using the genus-specific primers, CC16S-F and CC16S-R. The occurrence of vegetative *Alicyclobacillus* cells (average %) in concentrate samples were the highest in apple (50%), followed by apricot (40%), peach (15%) and the lowest in pear (10%). The occurrence of *Alicyclobacillus* endospores in concentrate samples were again the highest in apple (50%), followed by pear (25%) and apricot (20%), with peach having the least amount of endospores (10%). The occurrence of *Alicyclobacillus* vegetative cells and endospores in concentrates from hairy-skin, stone fruit varieties (peach and apricot) did not differ significantly ($P > 0.05$) from smooth-skin, pome fruit varieties (apple and pear). It was concluded that fruit washing steps prior to processing was more important for the control of *Alicyclobacillus* than the type of fruit skin.

Introduction

There is continuous pressure on the industry to improve the quality of concentrated fruit products in order for reconstituted fruit beverages to compete with fruit beverages that are made from fresh fruits (Hegenbart, 1994; Ross, 2007). *Alicyclobacillus* became a major concern for the beverage industry since many high-acid, concentrated food products such as fruit concentrates, pulps and purees have been found to be

contaminated with *Alicyclobacillus* (Table 1) (Pinhatti *et al*., 1997; Eguchi *et al*., 1999; Chang & Kang, 2004; Jensen, 2005; Chen *et al.,* 2006; Durak *et al.,* 2010).

Members of the genus *Alicyclobacillus* are endospore-forming, thermo-acidophilic microbes that have the ability to grow in high-acid environments (pH < 4.0) (Walls & Chuyate, 1998; Chen *et al.,* 2006). They survive thermal processing temperatures that are applied during the production of fruit concentrates, such as the hot-break treatment (75 °- 90 ℃ for ± 10 min and cooled to 65 ℃) to enhance the flow properties of fruit mash, evaporation and conventional pasteurisation (86 °- 96 ℃ for ± 2 min) (Pontius *et al.,* 1998; Silva & Gibbs, 1999). The temperatures required to inactivate *Alicyclobacillus* endospores during fruit concentrate manufacturing could be detrimental to the nutritional and organoleptic qualities of fruit beverages (Walls & Chuyate, 1998; Palop *et al.,* 2000). Under favourable conditions, *Alicyclobacillus* endospores present in the concentrates may germinate and cause flat-sour type spoilage with offensive smelling chemical taint compounds, of which guaiacol (2-methoxyphenol) is considered to be the predominant taint compound (Borlinghaus & Engel, 1997; Chang & Kang, 2004).

Soil that adheres to unwashed or poorly washed fruit is the main source of *Alicyclobacillus* in the processing environment and fruit concentrates (Eguchi *et al*., 1999; Groenewald *et al.,* 2009). More than one third of the fruit (591 of 1575) sampled from orange juice processing facilities in Florida, USA was found to be contaminated with *Alicyclobacillus* (Parish & Goodrich, 2005). *Alicyclobacillus* counts on unwashed fruit from citrus processing facilities in São Paulo, Brazil ranged between 2 - 600 cfu kg^{-1}, which after washing ranged between < 1 - 284 cfu kg^{-1} (Eguchi *et al*., 1999). *Alicyclobacillus* was also recovered from unwashed fruit surfaces at 8 out of 10 citrus processing facilities, and from washed fruit surfaces at 6 out of 9 citrus processing facilities in Florida. The estimated number of endospores on washed and unwashed fruit was calculated to be at least 6 endospores per fruit (Wisse & Parish, 1998).

Previous studies on *Alicyclobacillus* predominantly focused on isolation-, enumeration-, identification- and characterisation methods, as well as the potential of alicyclobacilli to cause spoilage in high-acid fruit products (Table 1) (Pettipher *et al*., 1997; Pinhatti *et al.,* 1997; Wisse & Parish, 1998; Baumgart & Menje, 2000; Gouws *et al*., 2005; Groenewald *et al*., 2009; Durak *et al.,* 2010). Recent studies have also focused on the occurrence of *Alicyclobacillus* in the fruit processing environment and in concentrated fruit products, with the focus mainly on citrus and apple varieties (Table 1)

Table 1 Occurrence of *Alicyclobacillus* endospores in high-acid concentrated fruit juice products

Concentrated juice	**Soluble solids (°Brix)**	**Endospores (cfu mL^{-1}) [no. positive samples]**	**Origin**	**Reference**
Orange	66	70 - 3 400	São Paulo (Brazil)	Eguchi *et al*., 1999
Frozen Orange	66	10 - 3 400 [23 of 28]	São Paulo (Brazil)	
Pear	32	NR	Western Cape (SA)	Groenewald *et al*., 2009
Apple	NR	NR	Washington (USA)	Walls & Chuyate, 1998
Orange	> 50	< 6.8 - 947 MPN 100 g^{-1} * [14.7% of 75]	Brazil	Eiroa *et al*., 1999
Apple	70	NR [0 of 38]	Turkey	Bahçeci *et al.,*2005
Mango	NR	NR [16 of 24]	South Africa	Gouws *et al*., 2005
Apple	NR	< 5 vegetative cells	USA	Pettipher *et al*., 1997
Frozen Orange	65	< 30, 150, 230, 230, 430 MPN g^{-1} [5 of 23]	Florida (USA)	Wisse & Parish, 1998
Pear	NR	< 30 MPN g^{-1}	Florida (USA)	
Apple, Orange, Grapefruit, Mango, Peach, Blueberry, Pear	NR	NR	USA, Brazil, South America	Durak *et al.,* 2010
Frozen Orange	66	< 1 - 12 000	Costa Rica, Mexico, USA, Brazil	Pinhatti *et al.,* 1997
Orange	> 40	36, 60	Brazil, Mexico, Florida (USA)	
Limeade	> 40	510	USA	
Lemonade	> 40	3 020	USA	
Apple	50	0.020 - 0.615 mL^{-1} FCM # [36% of 166]	Various origin	Borlinghaus & Engel, 1997
Apple	72	NR	China	Chen *et al*., 2006
Banana, Apricot	NR	< 100 [3 of 18]	Germany	Baumgart & Menje, 2000
Apple	NR	NR [19 of 64]	Australia	Jensen, 2005
Orange	NR	300, < 100 cfu g^{-1} [61 of 85]	Australia	

NR - Not reported; * Most probable number; # Flow cytometry method (> 0.05 mL^{-1} rank as a positive identification)

(Borlinghaus & Engel, 1997; Eguchi *et al*., 1999; Eiroa *et al*., 1999; Chen *et al*., 2006; Jensen, 2005; Durak *et al.,* 2010).

The aim of this study was to determine whether the skin type of different fruit varieties, specifically smooth-skin and hairy-skin fruit, had a significant effect on *Alicyclobacillus* levels in the fruit concentrates. To our knowledge this is the first study to consider the effect of fruit skin types and different fruit concentrate varieties on the occurrence of *Alicyclobacillus*.

Materials and methods

Sampling and enumeration

Pasteurised (102 ° - 104 ℃ for 90 s) and aseptical ly packaged fruit concentrate samples (20 for each fruit variety) from peach, apricot, apple and pear concentrates were sourced at random over 2 seasons (2009 and 2010) from a HACCP accredited fruit concentrate manufacturing facility located in the Western Cape, South Africa. The manufacturing process of all fruit concentrates were the same, except for peach and apricot that included a de-stoning step. The stone fruit, peach and apricot concentrates were selected to represent hairy-skin fruit varieties, whilst pome fruit, apple and pear concentrates represented smooth-skin fruit varieties.

Fruit concentrate samples were analysed in triplicate for the occurrence of *Alicyclobacillus*. The spread plate method with acidified potato dextrose agar (PDA) (Merck, Cape Town, South Africa) at pH 3.7 (1M H_2SO_4) and incubation at 45 ℃ for 5 d was used to determine the presence of both *Alicyclobacillus* vegetative cells and endospores (Chang & Kang, 2004; Durak *et al.,* 2010). For the detection of *Alicyclobacillus* endospores, samples received a heat-shock treatment at 80 ℃ for 10 min prior to spread plating on PDA (Pettipher *et al*., 1997; Pinhatti *et al*., 1997). A third of the concentrate samples (6 from each fruit variety) were examined in duplicate for viable aerobic counts to monitor the facility's adherence to general manufacturing practices (AIJN, 2008). The pour plate technique with plate count agar (PCA) (Merck) and incubation at 35 ℃ for 48 h was used (Wisse & Parish, 1998). Sterile PCA and PDA served as controls for viable aerobic and *Alicyclobacillus* counts, respectively, and no growth was detected after incubation.

Identification

Alicyclobacillus colonies (randomly selected) were streaked on PDA (pH 3.7) plates. After incubation, colonies were examined microscopically for endospore formation and morphological characteristics, including rod-shaped, Gram-positive to Gram-variable bacteria (Chang & Kang, 2004). *Alicyclobacillus* identification were confirmed by polymerase chain reactions (PCR) where the genus-specific primers, CC16S-F (5' CGT AGT TCG GAT TGC AGG C-3') and CC16S-R (5' GTG TTG CCG ACT CTC GTG-3') were used to amplify a 134 bp fragment of the 16S rRNA gene (Connor *et al.,* 2005).

PCR reactions were performed in a total mixture volume of 25 µL that contained 0.2 µM of each primer (Eurofins MWG Operon, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 1.25 U *Taq* DNA polymerase (Super-Therm, supplied by Southern Cross Biotechnologies), 1 x PCR reaction buffer (Super-Therm), 6 mM $MgCl_2$ (Super-Therm), 1 µL of 99% (v/v) dimethyl sulphoxide (DMSO) (Merck), 0.8 mM dNTP mix (AB gene, supplied by Southern Cross Biotechnologies) and 5 µL DNA template. The following thermal cycling conditions were used for amplification in an Eppendorf Mastercycler Personal (Eppendorf, Germany): initial denaturation at 95 ℃ for 3 min; followed by 35 cyc les of denaturation at 94 ℃ for 30 s; annealing at 55 ℃ for 30 s; and elongation at 72 ° C for 30 s with final elongation at 72 ℃ for 2 min. PCR products (5 µL) were separate d on a 1.5% (m/v) agarose gel, containing 0.02 µL mL^{-1} ethidium bromide, in 0.5 x TBE electrophoresis buffer (45 mM tris, 45 mM boric acid and 1 mM EDTA) (Merck). The separated PCR fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat, Marne La Vallee, France).

Statistical analysis

Statistical analysis was done using Statistica™ 9 (StatSoft, Inc., Tulsa, OK, USA). Non-parametric bootstrap with Bonferroni multiple testing corrections were used to investigate the effect of different fruit concentrate varieties and the occurrence of *Alicyclobacillus.* A 5% ($P < 0.05$) significance level was used to determine significant effects.

Results and discussion

No viable aerobic growth was detected on PCA plates. This was expected as the production of fruit concentrates includes a pasteurisation treatment at 102 °- 104 °C for 90 s. The pH of the concentrate samples measured between pH 3.0 - 4.1 (apple 3.3 - 3.5; pear 3.7 - 4.1; peach 3.8 - 4.0; apricot 3.0 - 3.4) and the soluble solid content was > 30 °Brix (according to manufacturing specifications). Inherent physiochemical characteristics of fruit concentrates such as a low pH (3.5 - 4.0) and a high sugar concentration are strong inhibitors of the growth of most mesophilic, food-spoilage microbes (Pontius *et al.*, 1998; Chang & Kang, 2004; Silva & Gibbs, 2004). However, the pH values of all the fruit concentrate samples were within the optimum pH growth range of *Alicyclobacillus* (pH 2.0 - 6.0) (Walls & Chuyate, 1998; Chang & Kang, 2004).

Since soil is the main source of alicyclobacilli on fruit (Walls & Chuyate, 2000; Bahçeci *et al.*, 2005) and because soil could easily become trapped in the hair/fur on peach and apricot skins and cause contamination, it was expected that concentrates from hairy-skin fruit varieties would have a higher percentage occurrence of *Alicyclobacillus* than concentrates from smooth-skin fruit varieties. The percentage occurrence of vegetative *Alicyclobacillus* cells in the fruit concentrate samples, shown in Fig. 1, indicate that apple concentrates had the highest percentage occurrence with 50% (average %) of the samples being contaminated. Apple concentrates was followed by apricot (40%), peach (15%) and pear (10%) concentrates. The occurrence of vegetative cells in apple concentrates (50%) was significantly higher ($P < 0.05$) than in peach (15%) and pear (10%) concentrates, although not significantly higher ($P > 0.05$) than in apricot concentrates (40%). The effect seasonal variation can be disregarded as the fruit concentrate samples were sourced over two seasons. Contrary to what was expected, concentrates from stone, hairy-skin fruit (peach and apricot) did not have a higher percentage occurrence of vegetative alicyclobacilli than concentrates from pome, smooth-skin fruit (apple and pear). Furthermore, apple and pear concentrates, both smooth-skin fruit varieties, differed significantly regarding the occurrence of vegetative cells, whilst apricot, a hairy-skin fruit variety, did not differ significantly from apple (Fig. 1).

The percentage occurrence of *Alicyclobacillus* endospores in the fruit concentrate samples are shown in Fig. 2. These results were consistent with vegetative *Alicyclobacillus* cells (Fig. 1) as apple concentrate samples over the two seasons again

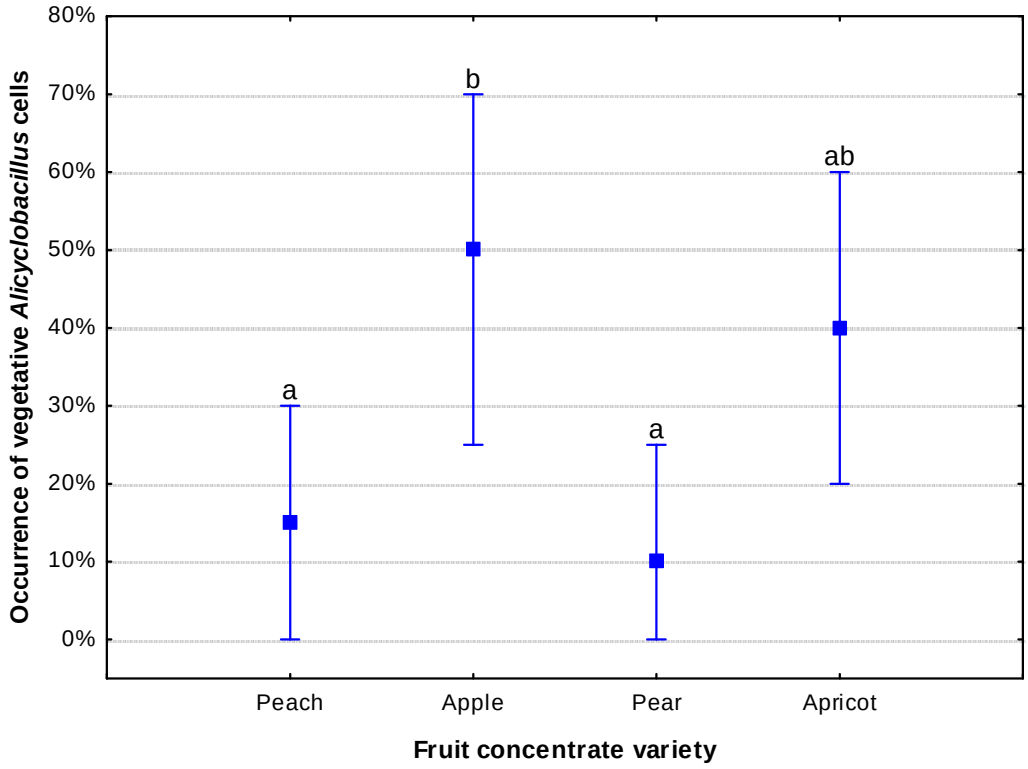

Figure 1 The percentage occurrence of vegetative *Alicyclobacillus* cells in fruit concentrates. Vertical bars denote 0.95 bootstrap confidence intervals (bars with the same letter are not significantly different ($P > 0.05$)).

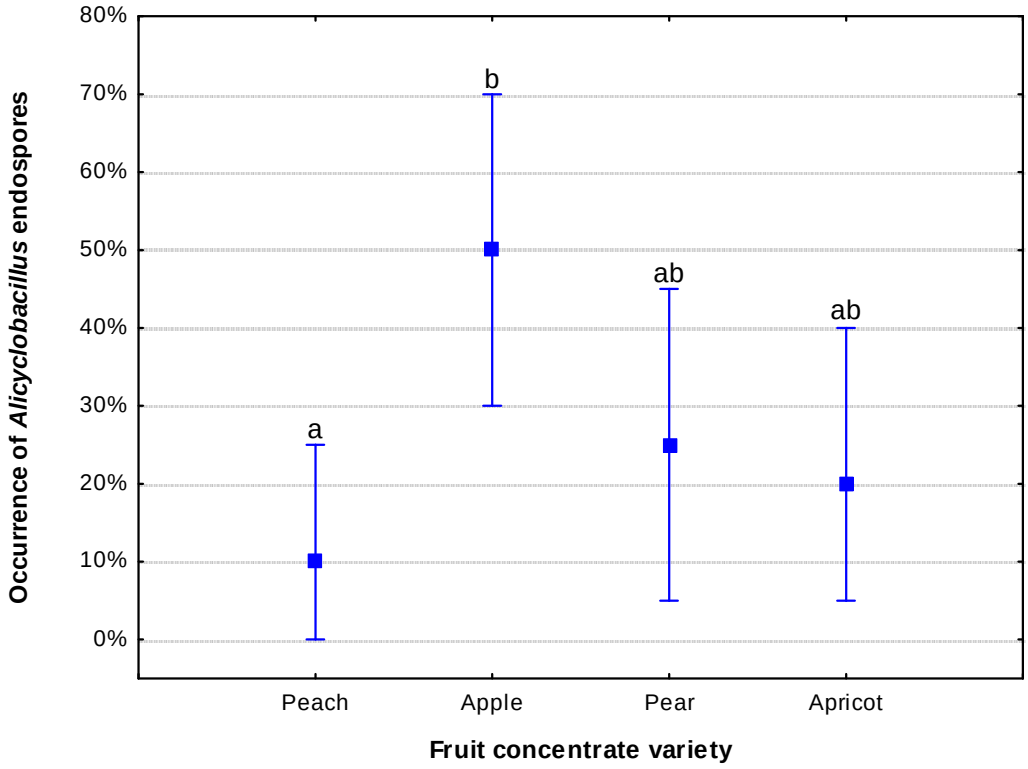

Figure 2 The percentage occurrence of *Alicyclobacillus* endospores in fruit concentrates. Vertical bars denote 0.95 bootstrap confidence intervals (bars with the same letter are not significantly different ($P > 0.05$)).

had the highest percentage occurrence of endospores, with 50% of the samples contaminated. Apple concentrates was followed by pear (25%) and apricot (20%) concentrates, with peach concentrates having the least percentage occurrence of endospores (10%). The percentage occurrence in apple (50%) was significantly higher ($P < 0.05$) than in peach (10%), but not significantly higher ($P > 0.05$) than in pear (25%) and apricot (20%) concentrates (Fig. 2). No significant difference ($P > 0.05$) was found between the occurrence of *Alicyclobacillus* endospores in peach and apricot concentrates, both hairy-skin fruit varieties, or between apple and pear concentrates, both smooth-skin fruit varieties. We expected the concentrates from hairy-skin, fruit varieties to have the highest percentage occurrence of endospores, however, data showed that peach and apricot had the lowest occurrence, whilst smooth-skin fruit (apple and pear) had the highest occurrence of endospores. There were also no significant differences ($P > 0.05$) between the percentage occurrence of endospores in apple, pear and apricot concentrates (Fig. 2).

Apple concentrates had the highest occurrence of *Alicyclobacillus* with 50% of the samples contaminated with both vegetative cells and endospores (Figs. 1 and 2). This is a concern as apple juice contains high concentrations of tyrosine (4.1 ppm), vanillic acid (10.07 ± 1.13 ppm) and ferulic acid (peeled fruit 122 ± 1 ppm; skin 149 ± 13 ppm) (Piacquadio *et al.*, 2000; Chang & Kang, 2004) compared to pear juice with tyrosine (1 ± 0 ppm) and ferulic acid (peeled fruit 12 ± 1 ppm; skin 15 ± 1 ppm) (Van Gorsel *et al*., 1992; Leontowicz *et al*., 2002), peach juice with tyrosine (9.1 ppm) and ferulic acid (peeled fruit 42 ± 5 ppm; skin 49 ± 6 ppm) (Van Gorsel *et al*., 1992; Leontowicz *et al*., 2002), apricot juice with ferulic acid (4 - 12 ppm) (Möller & Herrmann, 1983) and orange juice with tyrosine (3.4 - 13.5 ppm), vanillic acid (4.6 ± 0.7 ppm) and ferulic acid (117.8 ± 52.2ppm) (Chang & Kang, 2004; Swatsitang *et al*., 2010). Tyrosine, vanillic acid and ferulic acid are all possible precursor components for the formation of guaiacol by *Alicyclobacillus* spp. (Chang & Kang, 2004). A combination of factors will, however, influence the formation of guaiacol, including the *Alicyclobacillus* strain present, the contamination level, sufficient oxygen, the storage temperature of the final processed product, the presence of organic acids, pH and the sugar concentration of the final product (Borlinghaus & Engel, 1997; Pontius *et al.,* 1998; Chang & Kang, 2004).

Alicyclobacillus acidoterrestris was detected on two of the 12 batches of fruit supplied to apple processing facilities in Turkey, and its occurrence in the final processed product was found to be dependent on the initial level of contamination on

the fruit (Bahçeci *et al.,* 2003; 2005). Several control measures have been implemented by the industry to prevent *Alicyclobacillus* from entering and contaminating the processing environment. HACCP Regulation 21 CFR 120 prohibits the use of fallen fruits (grounders, windfall fruit, or drops) and the European Fruit Juice Association (AIJN) recommends for fruit not to be stored in direct contact with soil when intended for the production of concentrates, purees, juices and nectars (Walls & Chuyate, 1998; FDA, 2004; Parish & Goodrich, 2005; AIJN, 2008). Furthermore, washing techniques of fruit prior to processing include the use of approved disinfectants and chemical agents to reduce *Alicyclobacillus* levels on fruit skins (AIJN, 2008). It was found that disinfectant treatments during fruit washing procedures can reduce in their effectiveness on apples as the waxed hydrophobic nature of the skin and irregularities on the fruit (skin defects) attribute to the inaccessibility of disinfectants to alicyclobacilli (Orr & Beuchat, 2000). Rigorous washing and the use of detergents or food-grade surfactants were suggested to access protected areas on fruit skins and to enhance the contact time with disinfectants (Orr & Beuchat, 2000). Introducing extra cleaning steps such as in-line brushes or scrubbers could help to reduce *Alicyclobacillus* counts on fruit prior to processing.

Conclusions

This study found no significant difference in the occurrence of *Alicyclobacillus* vegetative cells and endospores between fruit concentrates from hairy-skin, stone fruit and smooth-skin, pome fruit ($P > 0.05$). The highest occurrence of alicyclobacilli was found in apple concentrates with an average of 50% of the samples contaminated with both vegetative cells and endospores. This was the first study, to our knowledge, that considered the effect of fruit skin type and different fruit concentrate varieties on the occurrence of *Alicyclobacillus*.

Fruit skin type did not influence the occurrence of alicyclobacilli in fruit concentrates, although further research with additional swab tests of fruit skins are suggested to confirm these findings. Fruit washing steps prior to processing are more important for the control of *Alicyclobacillus* than the type of of fruit skin being processed. Good manufacturing practice (GMP) and HACCP regulations are essential for the control of *Alicyclobacillus* in the processing environment and to prevent contamination of fruit juice and semi-prepared beverage ingredients.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The occurrence of *Alicyclobacillus* in high-acid, concentrated fruit products is a major concern to the food and beverage industry as alicyclobacilli favour an acidic environment (pH < 4.0), are unaffected by the high soluble solid content (> 20 °Brix) and have an exceptional ability to survive conventional pasteurisation treatments (86 ° - 96 °C for ± 2 min) of concentrated fruit pr oducts (Yamazaki *et al.*, 1996; Murakami *et al.,* 1998; Pontius *et al.,* 1998; Silva & Gibbs, 2004). Products that are reconstituted from fruit concentrates are at risk of spoilage by *Alicyclobacillus*, although a combination of factors will attribute towards creating an environment for spoilage to occur, including the specific constituents that are present in the reconstituted product (pH, oxygen content and soluble solid content), processing temperatures, the *Alicyclobacillus* species present, as well as the contamination level (Borlinghaus & Engel, 1997; Pinhatti *et al*., 1997; Walker & Philips, 2008).

The aim of this study was to assess the distribution and extent of contamination of *Alicyclobacillus* within the fruit concentrate processing environment. Additionally, the effect of fruit skin type and current manufacturing practices were monitored on the occurrence of *Alicyclobacillus* in fruit concentrates. These practices included the recirculation (recycling) of flume water and continuous process running times.

The results presented in this research confirmed that *Alicyclobacillus* are widely distributed within the fruit concentrate processing environment. As expected, the main source of *Alicyclobacillus* was found to be the raw fruit entering the processing environment (Walls & Chuyate, 1998; Wisse & Parish, 1998; Eguchi *et al*., 1999; Bahçeci *et al.,* 2005; Parish & Goodrich, 2005; Groenewald *et al.,* 2009). Results from this study also confirm that fruit sorting and adequate fruit washing procedures are critical at the early stages of processing, as a reduction in the initial *Alicyclobacillus* levels on fruit could limit or prevent contamination in the final processed fruit concentrate (Bahçeci *et al.*, 2003; 2005; Chen *et al.,* 2006; AIJN, 2008).

High levels of *Alicyclobacillus* were found in unpasteurised fruit concentrate and condensate water from the evaporator. Previous studies that isolated *Alicyclobacillus* from pre-pasteurised concentrate from the evaporator have recognised that the warm and acidic conditions in this equipment are optimal for the growth of alicyclobacilli (Wisse & Parish, 1998; Chen *et al.,* 2006; AIJN, 2008; Groenewald *et al.,* 2009).

Regular cleaning and sterilisation procedures of the evaporator are crucial control measures for preventing alicyclobacilli from accumulating within the system and cross-contaminating concentrates during manufacturing (Hays & Riester, 1952; AIJN, 2008). With the intention of conserving water, fruit concentrate manufacturers are often required to re-use condensate water, a by-product of the juice concentration process, for fruit washing procedures (Wisse & Parish, 1998). Results from this study indicated that up to 3.85 and 1.62 $\log_{10}$ cfu mL^{-1} *Alicyclobacillus* vegetative cells and endospores, respectively were found in condensate water. These levels of contamination were in agreement with the findings of Wisse & Parish (1998) and Eguchi *et al*. (1999). This study recommends for water treatment protocols to be established before untreated condensate water is re-used for fruit washing. These protocols should aid in the prevention of *Alicyclobacillus* contamination in the processing environment.

Results from this research indicated a presence of up to 3 $\log_{10}$ cfu mL^{-1} *Alicyclobacillus* endospores in the final concentrated fruit product. This indicates that conventional hot-fill-hold pasteurisation (86 °- 9 6 ℃ for ± 2 min) is not effective for the elimination of *Alicyclobacillus* endospores from high-acid concentrated fruit products and confirm reports of the thermal resistance of *Alicyclobacillus* endospores (Silva & Gibbs, 2004; Terano *et al*., 2005; Maldonado *et al*., 2008; Ceviz *et al.*, 2009). Many high-acid, concentrated food products such as fruit concentrates, pulps and purees have been found to be contaminated with similar levels of *Alicyclobacillus* endospores, including > 2 $\log_{10}$ cfu mL^{-1} (Pinhatti *et al*., 1997), < 1.47 - 2.63 $\log_{10}$ cfu g^{-1} (Wisse and Parish 1998) and 1.85 - 3.53 $\log_{10}$ cfu mL^{-1} (Eguchi et al. 1999). Reconstituted fruit beverage products form fruit concentrates that are contaminated with alicyclobacilli are at risk of developing spoilage taints. The growth of *A. acidoterrestris* were found to be elevated under favourable conditions, which at levels between 5 - 6 $\log_{10}$ cfu mL^{-1} produced sufficient guaiacol to cause spoilage and product deterioration (Borlinghaus & Engel, 1997; Pettipher *et al*., 1997).

Recommendations

Current manufacturing practices such as the recirculation (recycling) of flume water and continuous process running times can lead to the contamination and accumulation of *Alicyclobacillus* in fruit concentrates and the processing environment. Significantly higher levels ($P < 0.05$) were recovered from fruit concentrate processing stages during the functioning of a recirculating flume system compared to when a one-pass flume

system was functioning. Waste-water treatment protocols should be established if flume is recycled, in order to facilitate water conservation and prevent *Alicyclobacillus* from accumulating in the processing environment. When fruit concentrate manufacturing facilities are processing at full capacity, vegetative cells and endospores of *Alicyclobacillus* can accumulate in fruit concentrates during continuous processing running times. A restriction of the continuous process running time to under 84 h in between CIP procedures will minimise *Alicyclobacillus* accumulation. Fruit washing steps prior to processing are more important for the control of alicyclobacilli than the type of fruit skin, as their occurrence (vegetative cells and endospores) did not differ significantly ($P > 0.05$) between concentrates from hairy-skin, stone- and smooth-skin, pome fruit varieties. Furthermore, the quality of raw fruit entering the processing environment should be analysed regularly and the use of fallen fruits (grounders, windfall fruit or drops) should be prohibited as advised by hazard analysis critical control point (HACCP) regulations (FDA, 2004) and European Fruit Juice Association (AIJN) guidelines (AIJN, 2008).

The results presented in this thesis indicates that it is crucial for *Alicyclobacillus* to be controlled at specific manufacturing stages in the processing environment, especially as the current processing techniques are insufficient for their complete elimination from fruit concentrates. Guidelines from the AIJN (AIJN, 2008), the International Federation of Fruit Juice Producers (IFU, 2007) and the Food and Drug Administration (FDA, 2004), in conjunction with good manufacturing practices (GMP) and HACCP control measures are essential to control *Alicyclobacillus* during the production of juices, juice concentrates, purees and nectars. It is equally important for the employees of concentrate manufacturing facilities to be trained in quality control procedures as this will aid in the enforcement of control measures, to reduce *Alicyclobacillus* in concentrated fruit products and prevent future spoilage incidents of reconstituted products.

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