TAXONOMY OF SPECIES OF *ALICYCLOBACILLUS* FROM SOUTH AFRICAN ORCHARDS AND FRUIT CONCENTRATE MANUFACTURING ENVIRONMENTS AND THE PREVENTION OF FRUIT JUICE CONTAMINATION

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

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ABSTRACT

Species of *Alicyclobacillus* are acid-tolerant and heat-resistant bacteria that cause spoilage of heat-treated fruit juices stored at room temperature. During the past decade, *Alicyclobacillus* spp. have become a major cause of spoilage in pasteurised fruit juices leading to significant economic losses world-wide. Spoilage has been reported in apple, pear, orange, peach, mango and white grape juice, as well as in fruit juice blends, fruit juice containing drinks and tomato products, such as tomato juice and canned tomatoes. Spoilage is characterised by a medicinal smell and guaiacol production. These endospore-formers have been shown to survive pasteurisation conditions of 95 ℃ for 2 min, grow at temperatures between 25° and 60 ℃ and a pH range of 2.5 to 6.0. Knowledge of this organism is limited, both locally and internationally and the route of contamination to the final product is not well established.

In this study the fruit concentrate processing environment was investigated as a potential source and route of contamination for the final product. Species of *Alicyclobacillus* were isolated from orchard soil, various stages during processing and from fruit juice and concentrates. The isolates were identified based on morpholological, biochemical and physiological properties. Identification to species level was done by 16S ribosomal RNA gene sequencing and strain differentiation by RAPD-PCR. Results indicate that species of *A. acidoterrestris* and *Alicyclobacillus acidocaldarius* were found in orchard soil and throughout the processing environment. This is the first report on the isolation of these species from orchard soil, vinegar flies and the fruit processing environment. The 16 isolates identified as *A. acidoterrestris* grouped into four clusters based on RAPD-PCR banding patterns, suggesting that they belong to at least four genotypic groups. Isolates from the fruit concentrate, wash water and soil located outside of the fruit processing plant grouped into one cluster. Concluded from these results, *A. acidoterrestris* found in the wash water and soil outside of the factory could act as a potential reservoir of organisms for the contamination of the final fruit concentrate. Thus good manufacturing practices play an essential role in controlling incidence of spoilage caused by these bacteria.

Fruit juices can be treated using ultraviolet (UV-C) light with a wavelength of 254 nm, which has a germicidal effect against micro-organisms. *Alicyclobacillus acidoterrestris* spores were inoculated into tap water, used wash water from a fruit processing plant and grape juice concentrate. Ultraviolet dosage levels (J L^{-1}) of 0, 61, 122, 183, 244, 305 and 367 were applied using a novel UV-C turbulent flow system.

The UV treatment method was shown to reliably achieve in excess of a 4 $\log_{10}$ reduction (99.99%) per 0.5 kJ L^{-1} of UV-C dosage in all the liquids inoculated with *A. acidoterrestris*. The applied novel UV technology could serve as an alternative to thermal treatments of fruit juices for the inactivation of *Alicyclobacillus* spores or in the treatment of contaminated processing wash water.

Finally, the thermal inactivation at 95 °C for two strains of *A. acidoterrestris* isolated from contaminated fruit juice concentrates were investigated in a 0.1% (m/v) peptone buffer solution (pH 7.04) and grape juice (pH 4.02, 15.5 °Brix). The thermal inactivation of *A. acidoterrestris* spores followed first-order kinetics, suggesting that as the microbial population is exposed to a specific high temperature, the spores inactivated at a constant rate. D-values determined in the buffer solution were calculated to be 1.92 min and 2.29 min, while in grape juice D-values were found to be 2.25 min and 2.58 min for the two strains tested. From this study it is clear that the D-value is dependant on the strain tested, but also on the soluble solids of the solution the cells are suspended in. The results indicated that the spores of *A. acidoterrestris* isolated from South African fruit juice concentrate may survive after the pasteurisation treatment commonly applied during manufacturing.

UITTREKSEL

Spesies van *Alicyclobacillus* is suur-tolerante en hittebestande bakterieë wat bederf veroorsaak in hitte-behandelde vrugtesappe wat teen kamertemperatuur gestoor word. Gedurende die afgelope dekade het *Alicyclobacillus* spp. ‘n belangrike oorsaak van bederf in gepasteuriseerde vrugtesappe geword en beduidende ekonomiese verliese wêreldwyd veroorsaak. Bederf is aangeteken in appel-, peer-, lemoen-, perske-, mango- en witdruiwesap, sowel as in vrugtesapversnitte, vrugtesapbevattende drankies en in tamatieprodukte soos tamatiesap en ingemaakte tamaties. Bederf word gekenmerk deur ’n medisinale reuk en guaiacol produksie. Daar is gevind dat hierdie endospoorvormers pasteurisasie teen 95 °C vir 2 min kan oorleef en kan groei by temperature tussen 25° en 60 °C en ‘n pH van 2.5 to 6.0. Plaaslik sowel as internasionaal is kennis van hierdie organisme beperk en die roete van kontaminasie van produkte is nog nie goed vasgestel nie.

In hierdie studie is die vrugtekonsentraat-verwerkingsmilieu ondersoek as ‘n moontlike bron en roete van kontaminasie van die finale produk. Spesies van *Alicyclobacillus* is vanuit vrugteboordgrond, verskeie verwerkingstadia en van vrugtesap en vrugtesapkonsentraat geïsoleer. Die isolate is op grond van morfologiese, biochemiese en fisiologiese eienskappe geïdentifiseer. Identifikasie tot spesiesvlak is deur 16S rDNS sekwensering gedoen en stam differensiasie deur RAPD-PKR. Resultate het aangetoon dat *A. acidoterrestris* en *A. acidocaldarius* in vrugteboordgrond sowel as in alle stadia van die verwerkingsmilieu voorkom. Dit is die eerste verslag van die isolering van hierdie spesies uit die Suid-Afrikaanse vrugteverwerkingsmilieu, vrugteboordgrond en asynvlieë. Die 16 isolate, geïdentifiseer as *A. acidoterrestris* en in vier groepe geplaas op grond van hul RAPD-PKR bandpatrone, dui aan dat hulle aan minstens vier genotipiese groepe behoort. Isolate afkomstig van die vrugtekonsentraat, waswater en die grond buitekant die vrugteverwerkingsaanleg het een groep gevorm. Uit hierdie resultate kan afgelei word dat *A. acidoterrestris*, wat in die waswater en grond buite die aanleg voorkom, as ‘n moontlike bron van organismes vir die kontaminering van die finale vrugtekonsentraat kan dien. Goeie vervaardigingspraktyke speel dus ‘n noodsaaklike rol in die beheer van bederf veroorsaak deur hierdie bakterieë.

Vrugtesappe kan behandel word met ultravioletlig (UV-C) met ‘n golflengte van 254 nm wat ‘n dodende effek op mikro-organismes het. Kraanwater, gebruikte waswater van ‘n vrugtesapvervaardigingsaanleg en druiwesapkonsentraat is met

A. acidoterrestris spore geïnokuleer. Ultraviolet toedieningsvlakke (J L^{-1}) van 0, 61, 122, 183, 244, 305 en 367 is aangewend met behulp van ‘n nuwe UV-C drukvloei stelsel. Daar is aangetoon dat die UV-behandelingsmetode ‘n betroubare vermindering (99.99%) van meer as 4 $\log_{10}$ per 0.5 kJ L^{-1} van ‘n UV-C dosis gee in al die vloeistowwe wat geïnokuleer is met *A. acidoterrestris*. Die toegepaste nuwe UV-tegnologie kan gebruik word as ‘n alternatief tot die hittebehandeling van vrugtesap vir die deaktivering van *Alicyclobacillus* spore of in die behandeling van gekontamineerde waswater.

Ten slotte is hitte-deaktivering teen 95 °C van twee stamme van *A. acidoterrestris*, geïsoleer uit gekontamineerde vrugtesapkonsentraat, in ‘n 0.1% (m/v) peptoonbufferoplossing (pH 7.04) en druiwesap (pH 4.02, 15.5 °Brix), ondersoek. Die hitte-deaktivering van *A. acidoterrestris* spore het eerste-orde kinetika gevolg, wat aandui dat die mikrobe-populasie teen ‘n konstante tempo afsterf, wanneer blootgestel aan ‘n spesifieke hoë temperatuur. Die D-waardes in die bufferoplossing is bereken as 1.92 min en 2.29 min, terwyl daar gevind is dat die D-waardes in druiwesap 2.25 min en 2.58 min is vir die twee betrokke stamme. Vanuit hierdie studie is dit duidelik dat die D-waardes afhang van die betrokke stam, maar ook van die oplosbare vaste stowwe van die oplossing waarin die selle opgelos is. Die resultate dui daarop dat die spore van *A. acidoterrestris*, wat geïsoleer is uit Suid-Afrikaanse vrugtesapkonsentraat, die pasteurisasiebehandeling wat algemeen tydens vervaardiging toegepas word, kan oorleef.

Aangesien die toepassing van strenger hittebehandeling om spore van *A. acidoterrestris* te deaktiveer onaanvaarbare organoleptiese veranderinge in die produk tot gevolg het, word dit aanbeveel dat die risiko van bederf verminder behoort te word deur die gebruik van goeie vervaardigingspraktyke gedurende vrugteverwerking.

CONTENTS

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

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CHAPTER 1

INTRODUCTION

Fruit concentrate has traditionally been regarded as resistant to spoilage by deteriogenic micro-organisms due to its physical and chemical characteristics. These characteristics include a low pH of between 3.5 to 4.0, low water activity, high sugar concentration (typically around 66 °Brix), high viscosity and reduced aeration capacity and dissolved oxygen (Jay, 1998; Palop *et al*., 2000). The addition of a hot-fill and hold pasteurisation process as used in the fruit beverage industry, where the product is held at 86° to 96 °C for approximately 2 min, is also sufficient to destroy most non-spore-forming micro-organisms (Palop *et al*., 2000; Chang & Kang, 2004).

Spoilage of commercially available pasteurised fruit juice was first reported by Cerny *et al.* (1984) who found shelf-stable, aseptically packaged apple juice to have an off-flavour. Following this report, an increasing number of spoilage incidents arose and almost all of these were caused by the spore-forming, thermo-acidophilic bacteria, *Alicyclobacillus acidoterrestris*. The fruit juice industry now acknowledges *A. acidoterrestris* as a major quality control target for pasteurisation (Yamazaki *et al*., 1996; Pettipher *et al*., 1997, Silva & Gibbs, 2004; Walker & Phillips, 2008; Bevilacque *et al*., 2008).

Spoilage caused by this bacterium is difficult to detect visually. The spoiled juice appears normal, or might have a light sediment with no gas formation. Often, the only evidence of spoilage is apparent as a medicinal/phenolic off-flavour (Walls & Chuyate, 1998; Jensen, 1999). The chemicals responsible for this off-odour were identified as guaiacol (2-methoxyphenol) and other halophenols such as 2,6-dichlorophenol (2,6-DCP) and 2,6-dibromophenol (2,6-DBP). Guaiacol can be detected by smell in fruit juices at 2 ppb and was detected in orange and apple juices in the presence of around 5 log CFU mL^{-1} of *A. acidoterrestris* cells (Gocmen *et al*., 2005). Spoilage caused by *A. acidoterrestris* has to date been reported in apple, pear, orange, peach, mango and white grape juice, with shelf-stable apple juice most frequently being spoiled (Borlinghaus & Engel, 1997; Chang & Kang, 2004; Walker & Phillips, 2008). More diverse products such as shelf-stable iced tea containing berry juice, the ingredients of rose hip and hibiscus teas (Duong & Jensen, 2000), a carbonated fruit drink (Pettipher, 2000) and diced canned tomatoes (Chang & Kang, 2004) have also seen incidences of spoilage caused by *A. acidoterrestris*. A 2005 survey by European Fruit Juice

Association (AIJN) found that 45% of respondents had experienced spoilage problems by *Alicyclobacillus* spp. in the preceding 3 years. Of these incidents 52% were described as being either intermediate or major incidents. Apple concentrate was the most commonly contaminated product (Gerber Juice Company Ltd., 2006).

The spores of *A. acidoterrestris* can survive the typical pasteurisation regimes applied during juice manufacturing which provides the heat-shock treatment that may stimulate spore germination and outgrowth (Splittstoesser *et al*., 1994; Eiroa *et al*., 1999; Orr & Beuchat, 2000). This heat resistance was observed by Splittstoesser *et al*. (1994) who reported D-values for *A. acidoterrestris* spores of 23 min at 90 °C and 2.4 to 2.8 min at 95 °C, suggesting that spores survive the typical juice pasteurisation process that consists of holding at 88° to 96 °C for 30 s to 2 min. The ability of *A. acidoterrestris* cells to grow at the low pH (3-3.5) typically found in fruit juice then results in subsequent spoilage of the juice (Splittstoesser *et al*., 1998; Eiora *et al*., 1999; Gouws *et al*., 2005).

To date, the genus *Alicyclobacillus* includes 19 species of which only *A. acidoterrestris* has consistently been associated with product spoilage. Five other species, including *A. acidiphilus*, *A. pomorum*, *A. hesperidum*, *A. cycloheptanicus* and *A. acidocaldarius* have been implicated as potential spoilage bacteria due to their isolation from spoiled products and/or their ability to produce taint chemicals. Fruit juice contamination is thought to be the result of unwashed or poorly washed raw fruit that is processed, as well as contaminated water used during the production of fruit juices (Pontius *et al*., 1998; Orr & Beuchat, 2000; McIntyre *et al*., 1995, Groenewald *et al*., 2009).

Due to the ability of *A. acidoterrestris* to survive commercial pasteurisation regimes and the adverse effect on the organoleptic and nutritional properties of the juice, there is a need for an alternative to thermal pasteurisation.

The use of ultraviolet (UV) light in food processing is one of a number of non-thermal technologies being used as a substitute for thermal processing. UV treatment has one major advantage in that it does not result in adverse side-effects (such as nutrient and flavour loss) on fruit juice that are associated with heat treatments. In addition, UV treatment has been shown to be less energy-intensive and, therefore, more cost-effective and environmentally friendly than conventional pasteurisation (Kouchma, 2009).

The aim of this study was to investigate orchard soil and the fruit concentrate processing environment as a potential source and route of contamination for the final

product. The effectiveness of UV radiation for reducing alicylobacilli counts in water and fruit juice concentrates, as well as the effect of temperature on two wild type strains of *A. acidoterrestris* in a buffered solution and single strength grape juice were investigated.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

Fruit concentrate has traditionally been regarded as resistant to spoilage by deteriogenic micro-organisms due to its physical and chemical characteristics. These characteristics include a low pH of between 3.5 to 4.0, low water activity, high sugar concentration (typically around 66 °Brix), high viscosity, reduced aeration capacity and reduced dissolved oxygen (Jay, 1998a; Palop *et al*., 2000). The addition of a hot-fill and hold pasteurisation process as used in the fruit beverage industry, where the product is held at 88° to 96 °C for approximately 2 min before packaging, is also sufficient to destroy most non-spore-forming micro-organisms (Palop *et al*., 2000; Chang & Kang, 2004).

Microbial contamination of fruit juice concentrate can occur due to the presence of bacteria, mycelial fungi and yeasts. Only a restricted portion of these micro-organisms have the ability to grow in the reconstituted juice and cause product spoilage (Walker & Phillips, 2008). Deteriogenic bacterial species associated with processed fruit juices include micro-organisms from the genera *Bacillus, Clostridium, Lactobacillus,* and *Leuconostoc* (Murdock & Hatcher, 1975). *Streptococcus* and *Pediococcus* species are found in lower frequency in processed fruit juice. These micro-organisms may promote the deterioration of food products by degrading their compounds (such as carbohydrates, proteins and vitamins) to produce undesirable odour and off-flavour, colouration, pH and texture changes. Spoilage by *Lactobacillus* and *Leuconostoc* species is due to an off-odour produced by the lactic acid bacteria (LAB) during growth in reconstituted juice. The chemical mainly responsible for the undesirable flavour and off-odour, described as similar to “acid butter or milk”, has been identified to be diacetyl. As LAB do not form spores and are unable to survive the commercial pasteurisation process, spoilage has been attributed to post-pasteurisation contamination of the fruit juice product (Shearer *et al*., 2002).

The high thermal resistance of spores of certain bacterial species can result in them being viable after the high temperature treatments associated with the pasteurisation process. Most bacterial spores, however, will fail to germinate at a pH

below 4.1, limiting spoilage to a small number of organisms that are able to grow at pH 3.8 or lower such as *Bacillus coagulans* and *Clostridium pasteurianum* (Pontius *et al*., 1998). The occurrence of the *B. coagulans* spores have been reported in products based on tomato (juice or paste) and canned fruit (Thomson, 1981). This thermophile is capable of growth at pH 4 and causes a flat-sour type of spoilage by producing off-flavour and souring of the product. These spores are of low heat resistance compared with those of the obligate thermophiles, with only the occasional report of contamination in low acid canned foods. Spoilage by *Clostridium pasteurianum* (grows at pH 3.8 to 5.0) is a result of butyric acid type fermentation in acidic foods, such as tomato juice, fruits, fruit juices or in medium acidic foods such as corn, peas, and spinach with swelling of the container due to the production of CO_2 and H_2. *Clostridium pasteurianum* is rarely encountered and spores of this organism are of low heat resistance and, therefore, susceptible to destruction by commercial pasteurisation processes (Hsu & Beuchat, 1986).

The first instance of spoilage reported to be caused by members of the genus *Alicyclobacillus* occurred in 1982 in aseptically packaged apple juice in Germany. The impact of these spore-forming thermo-acidophilic bacteria in fruit juices was not acknowledged until more than a decade later when numerous reports of *A. acidoterrestris* spoilage were reported (Walls, 1994; Yamazaki *et al*., 1996; Pettipher *et al*., 1997; Walls & Chuyate, 1998; Pettipher, 2000). Spoilage caused by *A. acidoterrestris* has to date been reported in apple, pear, orange, peach, mango and white grape juice, as well as in fruit juice blends, fruit juice containing drinks and tomato products, such as tomato juice and canned tomatoes (Borlinghaus & Engel, 1997; Chang & Kang, 2004; Gouws *et al*., 2005). Due to their thermo-acidophilic properties and their occurrence in several spoiled pasteurised products, Silva *et al*. (1999) recommend *A. acidoterrestris* spores as a target for pasteurisation of high acidic food products. The fruit juice industry now acknowledges *A. acidoterrestris* as a major quality control target for pasteurisation and for which effective control measures needs to be implemented to minimise spoilage (Silva & Gibbs, 2004).

B. DISCOVERY AND HISTORY

In 1967, well before the genus *Alicyclobacillus* was established, Uchino & Doi (1967) isolated thermo-acidophilic spore-forming bacteria from hot springs in the Tohoku district of Japan. Water temperatures at these hot springs can reach 75° to 80 °C, while

the pH is typically between 2 and 3. The isolates were provisionally classified as strains of *B. coagulans* due to a morphological resemblance and their ability to grow at 55 °C, but not at 37 °C. Similar organisms were isolated by Darland & Brock (1971) and De Rosa *et al*. (1971) from aqueous and terrestrial thermal acid environments in the United States of America and in Italy, respectively. Of the fourteen isolates isolated by Darland & Brock (1971) none grew at 40 °C, all at 45° to 65 °C, six at 70 °C and none at 75 °C. Most isolates grew at pH 2, all at pH 3 to 5, eight at pH 6 and none at a pH higher than 6.4. Being Gram-variable spore-forming rods the isolates were placed in the genus *Bacillus*. However, the DNA composition of the isolates was approximately 62% mol Guanine + Cytosine (%mol G + C) compared with that of *Bacillus*, which is between 45 and 50 %mol G+ C. As a result of the difference in DNA composition, as well as their acidophilic and strictly aerobic nature, a new species *Bacillus acidocaldarius* was proposed (Darland & Brock, 1971). Research carried out by De Rosa *et al.* (1971) at around the same date established that the membrane fatty acids of *B. acidocaldarius* comprised up to 65% 11-cyclohexylundecanoic and 13-cyclohexyltridecanoic acids with no unsaturated fatty acids.

Hippchen *et al.* (1981) isolated thermo-acidophilic bacteria from soils that were neither acidic nor hot. Soil samples from neutral environments were pasteurised and then either streaked directly onto acidic media or subjected to an enrichment step before incubation at 50 °C, at a pH between 3 and 4. Isolates from the soil samples subjected to the enrichment step yielded bacteria with ω-cyclohexane fatty acids, demonstrating the existence of thermo-acidophilic bacilli similar to *B. acidocaldarius* in neutral soils. Subsequently similar bacteria were isolated in 1982 from spoiled apple juice produced in Germany (Cerny *et al*., 1984). These bacilli were able to grow at a temperature range of 26° to 55 °C and at pH between 2.5 and 6.0, while their spores showed extremely high thermal resistance (D_{90} of 15 min at pH 3.5). The bacteria were able to survive the industrial pasteurisation applied to fruit juice and germinate under the low pH conditions of apple juice (Cerny *et al*., 1984). After extensive research on the isolates of Hippchen *et al.* (1981) and Cerny *et al.* (1984), Deinhard *et al.* (1987a) proposed the new species *Bacillus acidoterrestris*. Characteristics which distinguished *B. acidoterrestris* from *B. acidocaldarius* were: a %mol G + C content of the DNA that is 7% lower than *B. acidocaldarius*, low DNA-DNA homology between the two species as shown by DNA-DNA hybridisation studies, and a slightly lower optimum growth temperature. Differences in their use of carbon-sources were also used to separate the

two species with *B. acidocaldarius* being able to utilise erythritol, sorbitol, and xylitol (Cerny *et al*., 1984).

Poralla & König (1983) described a third thermo-acidophilic bacillus which differed from *B. acidocaldarius* and *B. acidoterrestris* in that it contained mainly ω-cycloheptane fatty acids in its membrane. This microorganism was named *Bacillus cycloheptanicus* (Deinhard *et al*., 1987b) and also differed from *B. acidocaldarius* and *B. acidoterrestris* by having an obligate nutrient requirement for methionine. A narrow growth temperature range of between 35° and 53 °C in comparison with the temperature range for *B. acidocaldarius*, which was approximately between 45° and 70 °C was another distinguishing feature. Further taxonomic investigations on the comparative sequence analyses of the 16S ribosomal RNA (rRNA) genes of these three species indicated that the sequences of *B. acidocaldarius* and *B. acidoterrestris* were very similar (98.8%), but that of *B. cycloheptanicus* showed lower homology to *B. acidocaldarius* (93.2%) and *B. acidoterrestris* (92.7%). Comparing the levels of similarity between the 16S rRNA genes of these three species and *Bacillus subtilis* (84.3 – 85.3%), *B. coagulans* (85.0 – 85.2%) and *B. stearothermophilus* (86.2 – 86.8%) indicated that the three ω-alicyclic fatty acid containing bacteria were closely related, but markedly different from other *Bacillus* species. Based on these results, Wisotzkey *et al*. (1992) proposed that these three bacilli should be reclassified into a new genus, *Alicyclobacillus* gen. nov., in the family *Bacillaceae*.

Over a decade after the first reported incidence of spoilage caused by *A. acidoterrestris,* a second report of spoilage caused by these bacteria occurred during two very hot European summers in 1994 and 1995 (Splittstoesser *et al.,* 1994). Two strains of acidophilic bacilli were isolated from apple juice and from a hot-filled apple-cranberry juice. The juices presented off-odour, with no gas formation and slight turbidity. The isolates were later identified as belonging to *A. acidoterrestris* (Splittstoesser *et al*., 1998). McIntyre *et al.* (1995) recovered acidophilic sporulated bacilli from juices reconstituted from concentrate and pasteurised by the hot-fill and hold system. Again deterioration was detected based on the presence of off-odour and visible growth. Prevedi *et al.* (1995) characterised strains of acido-thermophilic sporulated bacteria isolated from non-deteriorated orange juice from Italy, which presented similar characteristics to that of *A. acidoterrestris.* The following year Yamazaki *et al.* (1996) isolated strains of *A. acidoterrestris* from samples of deteriorated acidic and isotonic beverages in Japan. Webster *et al.* (1996), suggested after isolating *A. acidoterrestris* from non-deteriorated samples of fruit juices and canned tomato that

acidic foods must have their quality evaluated in relation to the presence of *A. acidoterrestris.* Wisse & Parish (1998) were the first to isolate *A. acidoterrestris* from the fruit processing environment, emphasising the vulnerability of fruit products to contamination by these bacteria. The high occurrence of *A. acidoterrestris* in fruit juice and its ability to survive the commercial pasteurisation process leading to spoilage led Silva *et al*. (1999) to recommend *A. acidoterrestris* spores as a target for pasteurisation of high acidic food products.

The description of the genus *Alicyclobacillus* was amended in 2003 with the isolation of *Alicyclobacillus pomorum*, which did not contain the most distinctive characteristics of *Alicyclobacillus spp.*, namely the presence of ω-alicyclic fatty acids (Goto *et al*., 2003). Based on 16S rRNA gene sequence, DNA re-association studies and the presence of ω-alicyclic fatty acids and the isoprenoid quinone menaquinone-7 (MK-7) in their cell membrane, *Sulfobacillus thermosulfidoaxians* subsp. *thermotolerans* and *Sulfobacillus disulfidooxidans* were reclassified as *Alicyclobacillus tolerans* and *Alicyclobacillus disulfidooxidans,* respectively (Karavaiko *et al*., 2005). The genus *Alicyclobacillus* currently comprises 19 recognised species, 2 genomic species and 4 sub-species, namely *A. acidiphilus*, *A. acidocaldarius* subsp. *acidocaldarius*, *A. acidocaldarius* subsp. *rittmannii*, *A. acidoterrestris*, *A. contaminans*, *A. cycloheptanicus*, *A. disulfidooxidans*, *A. fastidiosus*, *A. herbarius*, *A. hesperidum* subsp. *hesperidum*, *A. hesperidum* subsp. *aigle, A. kakegawensis*, *A. macrosporangiidus*, *A. pohliae*, *A. pomorum*, *A. sacchari*, *A. sendaiensis*, *A. shizuokensis*, *A. tolerans; A. vulcanalis*; *A. ferrooxydans*; *Alicyclobacillus* genomic species 1 and *Alicyclobacillus* genomic species 2 (Albuquerque *et al*., 2000; Goto *et al*., 2002a,b; Bevilacque *et al*., 2008; Jiang *et al*., 2008) (Table 1).

C. CHARACTERISTICS

Phylogenetically, the alicyclobacilli are members of the *Clostridium-Bacillus* subdivision of Gram-positive eubacteria, also known as the *Firmicutes*. The genus *Alicyclobacillus* is most closely related to the genus *Bacillus* and comprises thermophilic–acidophilic spore-forming bacteria (Table 1). The % mol G + C of the chromosomal DNA ranges from 48.6 to 63.0 as determined by the thermal denaturation. The value is nearer to 62% for *A. acidocaldarius* and *Alicyclobacillus* genomic species 1, and near to 55% for the other species of *Alicyclobacillus* (Goto *et al*., 2007; Jiang *et al*., 2008; Walker & Phillips, 2008). The %mol G+C of *A. acidoterrestris* varies between 51.5 and 53.3%

depending on the strain, , with the type strain, *A. acidoterrestris* DSM 3922^T , having a DNA %mol G+C of 51.5% (Tsuruoka *et al*., 2003; Bevilacque *et al*., 2008; Walker & Phillips, 2008).

Growth factors, such as vitamins and organic sources of nitrogen, may or may not be required. The size of the colonies is dependant on the growth medium, reaching 2 to 5 mm on acidified yeast starch glucose (YSG) media at optimum growth temperatures (Goto *et al*., 2007; Walker & Phillips, 2008). Depending on the growth media and strain, colonies can be non-pigmented, creamy white, yellow, translucent to opaque in colour, becoming slightly darker with age. Their sheen is slightly glossy. The colony morphology can differ slightly depending on strain, but in general round colonies are formed. Some old cultures may appear contaminated due to their heterogeneous morphology (Wisotskey *et al*., 1992; Chang & Kang, 2004; Goto *et al*., 2007; Bevilacque *et al*., 2008).

Vegetative cells of the *Alicyclobacillus* genus are coccoids or rods, 0.3 to 0.8 µm wide by 1 to 4.5 µm long. These bacteria display aerobic growth, even though they can survive micro-aerobic conditions. A very low level of oxygen such as 0.1% dissolved oxygen can permit growth. When oxygen is depleted growth stops and the vegetative cells will sporulate (Goto *et al*., 2007; Jiang *et al*., 2008). *Alicyclobacillus pohliae* is, however, the exception to this as it is a facultative anaerobe (Bevilacque *et al*., 2008). Motility is always weak and only present under certain conditions while three species and one strain namely, *A. disulfidooxidans*, *A. ferrooxydans*, *A. fastidiosus* and *A. acidocaldarius* subsp. *rittmanni* appear to be strictly non-motile (Wisotskey *et al*., 1992; Walls & Chuyate, 1998; Jiang *et al*., 2008).

Alicyclobacilli cells stain Gram-positive in the early stages of cultivation, and become Gram-negative or Gram-variable at the end of cultivation. *Alicyclobacillus sendaiensis,* however, always stains Gram-negative (Goto *et al*., 2006). Under environmental and nutritional adverse conditions spores are formed that are approximately 0.7 to 1 µm wide and 3 to 5 µm long. The location of these spores can be terminal, subterminal or central, and can be oval, ellipsoidal or round in shape depending on the species (Chang & Kang, 2004; Goto *et al*., 2006). The swelling of cells due to spore formation may or may not occur, depending on species and strains with a greater number of strains showing swelling (Goto *et al*., 2006; Walker & Phillips, 2008).

The bacteria of the genus *Alicyclobacillus* are strictly acidophilic and can grow

Table 1 The isolated and described species and subspecies of the genus *Alicyclobacillus*

Species	Source	ω-cyclohexane/ ω-heptane fatty acids	Reference
A. acidiphilus	"Off flavour" orange juice	ω-Cyclohexane	Matsubara *et al*., 2002
A. acidocaldarius subsp. *acidocaldarius*	Soil	ω-Cyclohexane	Darland & Brock 1971; Wisotzkey *et al*., 1992
A. acidocaldarius subsp. *rittmannii*	Crater of Mount Rittmann	ω-Cyclohexane	Nicolaus *et al*., 1998
A. acidoterrestris	Soil	ω-Cyclohexane	Deinhard *et al*., 1987a; Wisotzkey *et al*., 1992
A. contaminans	Fruit juice	None	Goto *et al*., 2007
A. cycloheptanicus	Acid soil	ω-Cycloheptane	Deinhard *et al*., 1987b; Wisotzkey *et al*., 1992
A. disulfidooxidans	Waste water sludge	ω-Cyclohexane	Dufresne *et al*., 1996, Karavaiko *et al*., 2005
A. fastidiosus	Apple juice	ω-Cyclohexane	Goto *et al*., 2007
A. herbarius	Dried hibiscus flowers	ω-Cycloheptane	Goto *et al*., 2002b
A. hesperidum subsp *hesperidum*	Sulpher-conaining soils	ω-Cyclohexane	Albuquerque *et al*., 2000
A. hesperidum subsp. *aigle*	Sulpher-conaining soils	ω-Cyclohexane	Goto *et al*., 2006
A. kakegawensis	Soil	ω-Cycloheptane	Goto *et al*., 2007
Alicyclobacillus genomic species 2	Soil near a geyser	ω-Cycloheptane	Goto *et al*., 2002a
Alicyclobacillus genomic species 1	Solfataric soils	ω-Cycloheptane	Albuquerque *et al*., 2000
A. macrosporangiidus	Soil	None	Goto *et al*., 2007
A. pohliae	Geothermal soil	None	Imperio *et al*., 2008
A. pomorum	Mixed fruit juice	None	Goto *et al*., 2003,
A. sacchari	Sugar	ω-Cyclohexane	Goto *et al*., 2007
A. sendaiensis	Soil	ω-Cyclohexane	Tsuruoka *et al*., 2003
A. shizuokensis	Soil	ω-Cycloheptane	Goto *et al*., 2007
A. tolerans	Oxidisable lead-zinc ores	ω-Cyclohexane	Karavaiko *et al*., 2005
A. vulcanalis	Geothermal pool	ω-Cyclohexane	Simbahan *et al*., 2004
A. ferrooxydans	Sulpher-conaining soils	None	Jiang *et al*., 2008

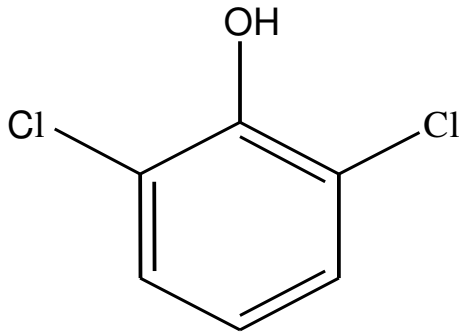
between pH 2 and 6.5 with *A. tolerans* and *A. disulfidooxidans* able to grow at a pH of below 1.5. Optimum pH for growth lies between pH 3 to 5.5 with the exception of *A. tolerans* and *A. disulfidooxidans* which have an optimum growth pH of between 1.5 and 2. Alicyclobacilli are thermophillic bacteria with growth temperatures between 17° and 70 °C and optimum growth temperatures between 35° and 65 °C, with the exceptions of *A. tolerans*, *A. disulfidooxidans* and *A. ferrooxydans* which grow best under mesophilic conditions. Sugars are effectively metabolised resulting in acid production, but no gas is produced. The sugars that can be metabolised tend to form groupings according to species, although there is considerable variation within species (Chang & Kang, 2004; Goto *et al*., 2007). As in *Bacillus,* the major respiratory lipoquinone of *Alicyclobacillus* is meaquinone-7 (MK-7), although some strains have a quinone with shorter chains such as MK-3. The relative amount of these quinones present is dependant on the strain and the conditions of cultivation (Deinhard *et al*., 1987; Goto *et al*., 2007).

The genus name *Alicyclobacillus* refers to a distinct trait, the presence of aliphatic, cyclic fatty acids in the cytoplasmic membrane. These ω-alicyclic acids contain terminal cyclohexyl or cycloheptyl rings (Fig. 1). Members of the *Alicyclobacilllus* genus that contain predominantly ω-cyclohexane fatty acids are *A. acidocaldarius* subsp. *acidocaldarius* (Darland & Brock, 1971; Wisotzkey *et al*., 1992), *A. acidocaldarius* subsp. *rittmannii* (Nicolaus *et al*., 1998), *A. acidoterrestris* (Hippchen *et al*., 1981; Deinhard *et al*., 1987a; Wisotzkey *et al*., 1992; Walls & Chuyate, 1998), *A. hespiridum* (Albuquerque *et al*., 2000), *A. acidiphilus* (Matsubara *et al*., 2002), *A. sendaiensis* (Tsuruoka *et al*., 2003), *A. vulcanalis* (Simbahan *et al*., 2004), *A. disulfdooxidans* (Dufresne *et al*., 1996; Karavaiko *et al*., 2005), *A. tolerans* (Karavaiko *et al*., 2005), *A. fastidiosus* (Goto *et al*., 2007) and *A. sacchari* (Goto *et al*., 2007). Four species of *Alicyclobacillus,* namely *A. cycloheptanicus* (Deinhard *et al*., 1987b; Wisotzkey *et al*., 1992), *A. herbarius* (Goto *et al*., 2002b), *A. kakegawensis* (Goto *et al*., 2007) and *A. shizuokensis* (Goto *et al*., 2007) possess predominantly ω-cycloheptane fatty acids. Experiments with *A. acidocaldarius* mutants unable to synthesize cyclohexyl fatty acids demonstrated the importance of these lipids for growth at temperatures above 50 °C and below pH 4. *Alicyclobacillus acidocaldarius* mutants exhibited poorer growth in low pH and high temperature conditions, while their sensitivity to heat shock and ethanol was increased compared to wild type organisms (Krischke & Poralla, 1990). Studies on artificial membranes by Kannenberg *et al.* (1984) showed that the addition of cyclic fatty acids lowered the transition temperature of membranes, but also had a

condensing effect, leading to a higher impermeability for low molecular weight substances up to 20 ℃ above the transition temperature. A greater proportion of ω-cyclohexane fatty acids in the membrane lead to increased acyl chain density enabling a denser packing of the lipids in the membrane core. This increases the structural stabilisation of the membrane, lowers membrane fluidity and reduced permeability, thereby protecting the bacteria against acidic conditions and high temperatures.

The presence of ω-cyclohexyl fatty acids are not essential in protecting alicylcobacilli from high temperatures and low pH. Goto *et al.* (2003) isolated thermo-acidophillic bacteria from mixed fruit juice which was classified as members of *Alicyclobacillus* based on 16S rRNA and *gyr*B gene sequence analysis despite the lack of ω-cyclohexyl fatty acids in their cell membrane. This bacterium, *A. pomorum,* with four other species of *Alicyclobacillus* lack ω-cyclohexyl fatty acids. These are namely: *A. contaminans* (Goto *et al*., 2007), *A. macrosporangiidus* (Goto *et al*., 2007), *A. pohliae* (Imperio *et al*., 2008) and *A. ferrooxydans* (Jiang *et al*., 2008). Instead these 4 species of *Alicyclobacillus* possess iso-and anteiso-branched fatty acids (Goto *et al*., 2007).
Another adaptation to extreme environments is the presence of hopanoids in the cells of a number of species of *Alicyclobacillus* (Poralla *et al*., 1980; Hippchen *et al*., 1981; Chang & Kang, 2004). The hopane ring is structurally similar to cholesterol and it is speculated that hopanes are phylogenetic precursors and structural equivalents of sterols (Poralla *et al*., 1980; Hippchen *et al*., 1981). Hopane glycolipids have a condensing effect on the membrane, decreasing the mobility of the acyl chains of the lipids and thereby stabilising the membrane. At low pH the condensing action hinders the passive diffusion of protons through the membrane, facilitating the establishment of an approximately neutral cytoplasmic pH (Poralla *et al*., 1980). In a study by Krischke & Poralla (1990) mutant cells containing only branched-chain fatty acids have significantly higher hopanoid content when compared to cells containing ω-cyclohexane fatty acids. The low membrane viscosity induced by the branched-chain fatty acids is thus compensated for by the presence of a higher concentration of hopanoids, leading to a more stable membrane.

The spores of *Alicyclobacillus* species are more resistant in high acid conditions than the spores of many *Bacillus* species (Yamasaki *et al*., 1997). At a low pH, fully-formed bacilli spores are easily demineralised, which decreases the heat resistance of the spores. Re-mineralisation of spores with divalent cations, such as calcium or manganese contributes to the stabilisation of spores against heat (Bender & Marquis,



B

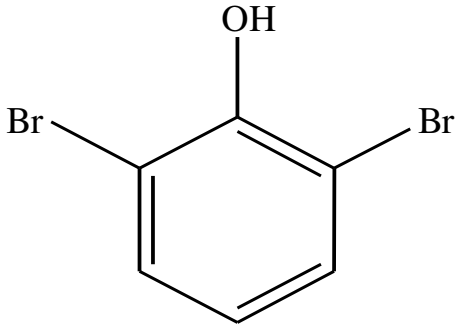


Figure 1 Chemical structures of 2,6-dichlorophenol (2,6-DCP) (A) and 2,6-dibromophenol (2,6-DBP) (B) (Jensen, 1999).

1985). In particular Ca^{2+} plays an important role in stabilising the heat resistance bychelating with dipicolinic acid (DPA) to form Ca-DPA which stabilises spores and contributes to heat resistance (Yamazaki *et al*., 1997). The ability of *A. acidoterrestris* spores to bind Ca^{2+} and Mn^{2+} strongly at a low pH and thereby keeping Ca-DPA levels constant contributes to their heat resistance (Yamazaki *et al*., 1997).

The similarity of the 16S rRNA gene sequence within the genus *Alicyclobacillus* is reported to be over 92%. Within closely related species, especially belonging to the *A. acidocaldarius* group, the similarity is over 98%. Extensive research on the genotype of *Alicyclobacillus* spp. using 16S rRNA gene analysis, *gyr*B gene analysis, DNA-DNA hybridisation studies and comparison with the phenotype, suggest there is no obvious correlation between phenotype and genotype in the alicyclobacilli (Goto *et al.,* 2002a, b; 2007; 2008). Results suggest that species within the genus *Alicyclobacillus* are comprised of numerous strains with heterogeneous traits. Other bacterial genera with similar species composition include the LAB and the acetic acid bacteria.

D. *ALICYCLOBACILLUS ACIDOTERRESTRIS*

Of the 19 species recognised as belonging to the genus *Alicyclobacillus*, *A. acidoterrestris* is the species that has the biggest impact on the food industry as it is widespread and has the potential to spoil food products (Sinigaglia *et al*., 2003; Walker & Phillips, 2005) (Table 2). When grown on laboratory media *A. acidoterrestris* forms distinctive, translucent, cream coloured colonies, round with flat interiors and raised centres that become darker and more opaque and umbonate with age (Deinhard *et al*., 1987a; Walls & Chuyate, 1998; Jensen, 1999).

Reported growth temperature ranges for *A. acidoterrestris* are 42° to 53 °C (Pontius *et al*., 1998; Walls & Chuyate, 1998), 26° to 50 °C (Borlinghaus & Engel, 1997) and 25° to 60 °C (Yamazaki *et al*., 1996). The optimum growth temperature is 40° to 45 °C (Bevilacque *et al*., 2008). The pH growth range extends from 2.0 to 6.0, with an optimum of 4 to 4.5 (Bevilacque *et al*., 2008). Recently some strains have been isolated from soils that are capable of growth at a pH of between 2 and 7 (Bevilacque *et al*., 2008). Germination of spores and growth has been reported in apple (pH 2.5; 11.4 °Brix), tomato (pH 4.0; 7.0 °Brix), white grape juice (pH 2.8 to 3.4; 7.8 to 10.8 °Brix),

Thermal resistance

Bacterial spore and vegetative cell heat resistance is measured as the decimal reduction time (D-value). The D-value is the time required to destroy 90% of the bacteria at a given temperature and is equal to the time (in minutes) required for the survival curve to traverse one log cycle at a given temperature (Jay, 1998b). The z-value of an organism is the temperature that is required for the thermal destruction curve to move one log cycle. While the D-value gives an indication of the time needed at a certain temperature to kill an organism, the z-value relates the survival of an organism to different temperatures. The z-value enables one to calculate a thermal process of equivalency, if one D-value and the z-value is known. It is a valuable tool when attempting to alter commercial processing conditions to either decrease the time needed to achieve product safety and stability, or decrease the temperature to enhance product quality. Thus when process time is decreased, the z-value is used to determine the new target processing temperature. If a lower temperature is desired to improve product flavour, the z-value also provides the increased time needed to achieve the same product safety and stability (Parish, 2006).

A wide range of D and z-values have been reported (Bahçeci & Acar, 2007) for the heat resistance spores of *A. acidoterrestris*, although the experimental conditions protocols varies while the taxonomy of this group is still unclear. A review of D and z-values determined in different fruit products and buffers are presented in Tables 3 and 4. The D_{90} values determined for different strains of *A. acidoterrestris* in apple juice (pH 3.2-6.8, 11.4 °-12.2 °Brix), apple nectar with ascorbic acid (pH 2.95, 14.0 °Brix), apple nectar without ascorbic acid (pH 2.97, 14.0 °Brix), a clear apple drink, grape juice (pH 3.3, 15.8 °Brix and pH 3.5, 16 °-30 °Brix), orange juice (pH 3.15-3.92, 9.0 °Brix), an orange drink, grapefruit juice (pH 3.42) and mango pulp (pH 4.0) range from 5.95 to 23.1 min. D_{95}-values determined in apple juice (pH 3.5-3.51, 11.4 °Brix), grape juice (pH 3.3, 15.8 °Brix and pH 3.5, 16 ° and 30 °Brix), orange juice (pH 3.15 to 4.1, 5.3 °-9.0 °Brix), berry juice, a fruit drink (pH 3.5, 4.8 °Brix), a fruit nectar (pH 3.5, 6.1 °Brix), cupuaçu extract (pH 3.6, 11.3 °Brix), grapefruit juice (pH 3.42) and mango pulp (pH 4.0) range from 1.0 to 8.7 min (Table 3) (Murakami *et al*., 1998; Pontius *et al*., 1998; Komitopoulou *et al*., 1999; Silva *et al*., 1999; Yamazaki *et al*., 2000; Bahçeci & Acar, 2007; Maldonado *et al*., 2008). The z-values range from 6.9° to 21.27 °C in fruit products and from 5.9° to 10.0 °C in buffers (Table 4) (Palop *et al*., 2000). Differences between the D-values reported in literature may be attributed to differences in strains,

Table 2 Description of the species *Alicyclobacillus acidoterrestris* (adapted from Bevilacqua *et al.*, 2008)

General description	Gram-positive spore-former, motile, rod-shaped (0.6–0.8 × 2.9–4.3 µm), catalase positive. C+G mol% is 51.5–53.3%.	Karavaiko *et al.*, 2005
A_w-soluble solids	Growth occurred for a_w values >0.984, with an optimal value of 0.992 (Soluble solids, 12.5 °Brix). Soluble solids >18 °Brix inhibited spore germination; spores showed to retain their viability in juice concentrates (70 °Brix)	Sinigaglia *et al.*, 2003
pH	acidophilic, able to grow at pH 2.0–6.0 (pH opt, 4.0–4.5). Strains, able to survive and grow at pH 2.0–7.0, isolated from soil	Bevilacqua *et al.*, 2006
Temperature	T_{opt} is 40°–45 °C, within a range of 35°–60 °C. Strains from soil were able to grow at 25–30 °C	Bevilacqua *et al.*, 2006
NaCl	Growth was observed at 5% (m/v) NaCl; some strains showed a moderate grow in Malt Extract broth + 7–8% (m/v) NaCl	Bevilacqua *et al.*, 2006
Acid production	From glucose, erythritol, ribose, D-xylose, D-fructose, rhamnose, inositol, mannitol, sorbitol, xylitol, β-gentiobiose	Karavaiko *et al.*, 2005
Fatty acid composition[1]	ω-cyclohexane-C17:0, 67.4%; ω-cyclohexane-C19:0, 24.6%; 15:0 iso, traces; 15:0 anteiso, traces; 16:0, 2.5%; 17:0 iso, 1.2%; 17:0 anteiso, 4.2% (A. *acidoterrestris* DSM 3922)	Tsuruoka *et al.*, 2003
Growth in juices[2]	Germination of spores and growth could occur in apple (pH 2.5; 11.4 °Brix), tomato (pH 4.0; 7.0 °Brix), white grape (pH 2.8–3.4; 7.8–10.8 °Brix), grapefruit (pH 3.1; 10.4 °Brix), orange (pH 3.6; 11.4 °Brix) and pineapple juices (pH 3.3; 13.4 °Brix) and shelf-stable iced tea. Growth did not occur in red grape juices (pH 2.3.–3.8 ; 9.1–12.2 °Brix) and Cupuaçu (pH 3.6; 11.3 °Brix).	Silva & Gibbs, 2001

[1] The concentration of each fatty acids is reported as percentage (w/w) on the total amount of fatty acids of the membrane.

[2] For each juice pH and soluble solids (°Brix) are reported in brackets, as they could be the limiting factors for the germination of alicyclobacilli spores.

sporulation temperature, nutrient composition and pH of the heating medium, water activity, presence or absence of divalent cations and antimicrobial compounds (Bahçeci & Acar, 2007).

Temperature was the factor with the most impact on the D-value of *A. acidoterrestris* spores, followed by soluble solids and lastly pH. D-value decreased with increasing temperature and decreasing soluble solids and pH (Pontius *et al*., 1998; Splittstoesser *et al*., 1998; Silva *et al*., 1999; Bahçeci & Acar, 2007; Maldonado *et al*., 2008). Bahçeci and Acar (2007), found that *A. acidoterrestris* spores followed first-order kinetics, suggesting that as the microbial population was heated at a specific temperature, the spores inactivated at a constant rate.

Temperature also affects the role that parameters such as pH and soluble solids play in the overall effect on D-values. While influence of pH on D-value was observed at the lowest temperature studied, the magnitude of the effect was not clear at higher temperatures (Pontius *et al*., 1998; Silva *et al*., 1999; Komitopoulou *et al.,* 1999; Bahçeci & Acar, 2007). Pontius *et al.* (1998) found that the D-values of *A. acidoterrestris* spores were significantly influenced by pH in the range of 2.5 to 6.9 at 91 °C, but not at 97 °C. A similar decrease in D-values was shown when the pH was changed from 4 to 3 in grapefruit juice by Komitopoulou *et al.* (1999). The effect of a lower pH on D-values was more apparent at 80 °C than 95 °C. The trend of a more pronounced effect of pH and acid on the heat resistance at lower temperatures may be explained by the greater spore-acid contact time at lower temperatures (Pontius *et al*., 1998). In contrast to the findings of these authors, Murakami *et al*. (1998) found no significant differences between D-values in McIlvaine buffer for A*. acidoterrestris* AB-1 spores at pH values from 3.0 to 8.0 at given temperatures between 88° and 95 °C. Palop *et al.* (2000) also found that the pH had no influence on the heat resistance of *A. acidocaldarius* spores for temperatures of 110 °C, 115 °C, 120 °C and 125 °C.

The type of organic acid (malic, citric or tartaric acid) did not have a significant effect on the heat resistance of *A*. *acidoterrestris* spores at either the high or low temperature range. Although not found to be statistically significant at the temperatures tested, the type of acid had a more profound effect on the heat resistance at 91° than at 97 °C. At lower temperatures, the inactivation rates in malic acid were higher (lower D-value) than in tartaric acid, while inactivation rates of citric acid were intermediate (Pontius *et al*., 1998; Silva *et al*., 1999).

The D-value decreased with decreasing soluble solids, exhibiting a linear relationship between soluble solids and D-values (Pontius *et al*., 1998; Silva *et al*.,

1999; Splittstoesser *et al*., 1998; Bahçeci & Acar, 2007; Maldonado *et al*., 2008). When increasing the soluble solids from 26.1 to 58.5 °Brix in blackcurrant concentrate the D_{91}-value of *A. acidoterrestris* NCIMB13137 spores increased from 3.8 to 24.1 min (Silva *et al.,* 1999). Splittsoesser *et al*. (1998) investigated the effect of soluble solids on D-values for *A. acidoterrestris* WAC spores in grape juice. D-values increased from 11 min in 16 °Brix to 127 min in 65 °Brix at 90 °C. At 95 °C D-values increased from 1.9 min in 16 °Brix grape juice to 12 min in 65 °Brix (Table 3).

There are other constituents in the fruit product that greatly affect the heat resistance. When model systems have been developed to test the effect of soluble solids on *A. acidoterrestris* spores the predicted D-values were, in most cases, lower than those determined in fruit products (Silva *et al*., 1999; Bahçeci & Acar, 2007). Therefore, different components present in fruits might increase the heat resistance of *A. acidoterrestris* spores.

Alicyclobacillus acidoterrestris shows considerable differences in heat resistance among strains (Murakami *et al*., 1998; Pontius *et al*., 1998; Eiora *et al*., 1999; Bahçeci & Acar, 2007). In McIlvaine buffer at pH 4 and 90 °C strain AB-1 (Murakami *et al*., 1998) showed approximately twice the heat resistance of strain DSM 2498 (Bahçeci & Acar, 2007). In a study by Eiroa *et al.* (1998) *A. acidoterrestris* strains DSM 2498, 145, 046 and 070 showed differing heat resistance in orange juice (pH 3.15, 9.0 °Brix). D-values varied from 60.8 to 94.5 min at 85 °C, 10.0 to 20.6 min at 90 °C and 2.5 to 8.7 min at 95 °C. Of three strains identified as *A. acidoterrestris* (VF, WAC and IP) by Pontius *et al*. (1998), two strains, VF and WAC had similar heat resistance, while strain IP was less heat resistant. At pH 3.1 at 97 °C, the D-values for VF and WAC were 54.3 and 53.2 min, respectively while the D-values for IP was 32.6 min. The difference in the D-values between IP and strains VF and WAC was found to correlate with their ability to grow at higher temperatures. Strains WAC and VF grew faster at 55 °C than strain IP. This relationship between tolerance to higher growth temperatures and greater heat resistance has been reported for several *Bacillus* species (Warth, 1978; Pontius *et al*., 1998). The heat resistance of *A. acidoterrestris* spores is much higher than that found for spores of most *Bacillus* species (Silva & Gibbs, 2004). Results indicated that the spores of all *A. acidoterrestris* strains generally survive in fruit juices and nectars after the commercial pasteurisation treatment commonly applied in the food industry to render the product commercially sterile (Splittstoesser *et al*., 1994; Splittstoesser *et al*.,

Table 3 Thermal inactivation kinetic parameters of *Alicyclobacillus acidoterrestris* spores in different heating media

Heating medium	Strain	pH	Soluble solids (˚Brix)	[a]T (˚C)	D-value	z-value	Reference
Apple juice	[b]nr	3.2	nr	90	15	nr	Cerny *et al*., 1984
	VF	3.5	11.4	85 90 95	56 23 2.8	7.7	Splittstoesser *et al*., 1994
	Z CRA 7182	3.5	nr	80 90 95	41.15 7.38 2.3	12.2	Komitopoulou *et al*., 1999
	DSM2498	3.68	12.2	90 93 96 100	11.1 4.2 2.1 0.7	8.5	Bahçeci & Acar, 2007
Apple nectar without ascorbic acid	DSM2498	2.97	14.0	90 93 96 100	11.1 4.2 2.1 0.7	8.5	Bahçeci & Acar, 2007
Apple nectar with ascorbic acid (250 mg/L)	DSM2498	2.95	14.0	90 93 96 100	14.4 6.7 3.3 1.2	9.2	Bahçeci & Acar, 2007
Clear apple drink	AB-5	nr	nr	90	20.8	nr	Yamazaki *et al*., 2000
Berry juice	nr	nr	nr	81.8 91.1 95	11.0 3.8 1.0	7.2	McIntyre *et al*., 1995
Cupuaçu extract	NCIMB 13137	3.6	11.3	85 91 95 97	17.5 5.35 2.82 0.57	9.0	Silva *et al*., 1999
Concord grape juice	WAC	3.5	16	85 90 95	53 11 1.9	6.9	Splittstoesser *et al*., 1998
	WAC	3.5	30	85 90 95	76 18 2.3	6.6	Splittstoesser *et al*., 1998
	WAC	3.5	65	85 90 95	276 127 12	7.4	Splittstoesser *et al*., 1998
Grape juice	WAC	3.3	15.8	85 90 95	57 16 2.4	7.2	Splittstoesser *et al*., 1994
Light blackcurrant concentrate	NCIMB 13137	2.5	26.1	91	3.84	nr	Silva *et al*., 1999
Blackcurrant concentrate	NCIMB 13137	2.5	58.5	91	24.1	nr	Silva *et al*., 1999

Table 3 Continued

Heating medium	Strain	pH	SS (˚Brix)	[a]T (˚C)	D-value	z-value(˚C)	Reference
Orange juice	[b]nr	4.1	5.3	95	5.3	9.5	Baumgart *et al*., 1997
	Z CRA 7182	3.9	nr	80 90 95	54.3 10.3 3.59	12.9	Komitopoulou *et al*., 1999
	46	3.15	9	85 90 95	60.8 10.0 2.5	7.2	Eiora *et al*., 1999
	70			85 90 95	67.3 15.6 8.7	11.3	Eiora *et al*., 1999
	145			85 90 95	94.5 20.6 3.8	7.2	Eiora *et al*., 1999
	DSM 2498			85 90 95	50.0 16.9 2.7	7.9	Eiora *et al*., 1999
	NCIMB 13137	3.5	11.7	85 91	65.6 11.9	7.8	Silva *et al*., 1999
Orange drink	AB-5	nr	nr	90	23.1	nr	Yamazaki *et al*., 2000
Fruit drink	nr	3.5	4.8	95	5.2	10.8	Baumgart *et al*., 1997
Fruit nectar	nr	3.5	6.1	95	5.1	9.6	Baumgart *et al*., 1997
Grapefruit juice	Z CRA 7182	3.42	nr	80 90 95	37.87 5.95 1.85	11.6	Komitopoulou *et al*., 1999
Mango pulp	DSM2498	4.0	nr	80 85 90 95	40 25 11.66 8.33	21.27	De Carvalho *et al*., 2008
Non-clarified lemon juice	nr	2.28	68	82 86 92 95	15.50 14.54 8.81 8.55	nr	Maldonado *et al*., 2008
Non-clarified lemon juice concentrate	nr	2.80	68	82 86 92 95	50.50 31.67 39.30 22.02	nr	Maldonado *et al*., 2008
	nr	3.5	68	82 86 92 95	38.00 95.15 59.50 17.22	nr	Maldonado *et al*., 2008
	nr	4.00	68	82 86 92 95	27.48 58.15 85.29 23.33	nr	Maldonado *et al*., 2008
Non-clarified lemon juice	nr	2.45	50	82 86 92 95	15.50 14.54 8.81 8.56	nr	Maldonado *et al*., 2008

Table 3 Continued 2

Heating medium	Strain	pH	SS (˚Brix)	[a]T (˚C)	D-value	z-value(˚C)	Reference
Non-clarified lemon juice	[b]nr	2.45	9.8	82 86 92 95	16.72 11.32 10.58 9.98	nr	Maldonado *et al*., 2008
		2.45	6.2	82 95	17.82 9.44	nr	Maldonado *et al*., 2008
Clarified lemon juice	nr	2.28	50	82 86 92 95	17.36 18.06 7.60 6.2	nr	Maldonado *et al*., 2008
	nr	2.80	50	82 86 92 95	25.81 22.01 15.35 11.32	nr	Maldonado *et al*., 2008
	nr	3.50	50	82 86 92 95	33.66 69.95 16.87 12.63	nr	Maldonado *et al*., 2008
	nr	4.00	50	82 86 92 95	21.95 35.16 23.19 9.72	nr	Maldonado *et al*., 2008
	nr	3.50	9.8	82 86 92 95	11.23 10.54 9.47 8.55	nr	Maldonado *et al*., 2008
	nr	3.50	6.2	82 95	13.21 9.38	nr	Maldonado *et al*., 2008
Buffers representing a model fruit juice system acidified with: Malic acid	VF	2.8	nr	94	12.3	nr	Pontius *et al*., 1998
	VF	3.1	nr	91 97	31.3 7.9	10.0	
	VF	3.4	nr	88 94 100	81.2 16.6 0.8	5.9	
	VF	3.7	nr	91 97	54.3 8.8	7.7	
	VF	4.0	nr	94	20.7	nr	
Citric acid	VF	3.1	nr	91 97	46.1 8.2	8.5	Pontius *et al*., 1998
	VF	3.7	nr	91 97	57.9 10.8	8.2	
Tartaric acid	VF	3.1	nr	91 97	49.1 8.4	7.8	
	VF	3.7	nr	91 97	69.5 10.0	7.1	

Table 3 Continued 3

Heating medium	Strain	pH	SS (˚Brix)	[a]T (˚C)	D-value	z-value(˚C)	Reference
Buffers representing a model fruit juice system acidified with: Malic acid	WAC	3.1	[b]nr	91 97	40.5 8.0	8.5	Pontius *et al*., 1998
	WAC	3.7	nr	91 97	53.2 9.0	7.7	
Buffers representing a model fruit juice system acidified with: Malic acid	IP	3.1	nr	91 97	20.3 3.6	8.0	Pontius *et al*., 1998
	IP	3.7	nr	91 97	32.6 3.8	6.5	
Citrate buffer: 20 mM	AB-1	6.0	nr	90	13.6	nr	Murakami *et al*., 1998
100 mM	AB-1	6.0	nr	90	14.4	nr	
Phosphate buffer: 20 mM	AB-1	6.0	nr	90	12.9	nr	Murakami *et al*., 1998
100 mM	AB-1	6.0	nr	90	12.3	nr	
McIlvaine buffer	AB-1	3.0	nr	88 90 92 95	24.1 14.8 6.2 2.7	nr	Murakami *et al*., 1998
	AB-1	4.0	nr	88 90 92 95	25.9 16.1 6.1 2.8	nr	
	AB-1	5.0	nr	88 90 92 95	29.1 16.6 7.1 2.7	nr	
	AB-1	6.0	nr	88 90 92 95	25.9 16.8 6.8 2.3	nr	
	AB-1	7.0	nr	88 90 92 95	24.7 15.7 6.7 2.2	nr	
	AB-1	8.0	nr	88 90 92 95	25.7 16.1 5.7 2.3	nr	
McIlvaine citrate-phosphate buffer	DSM2498	3.0	nr	90 93 96 100	6.0 2.8 1.1 0.4	8.2	Bahçeci & Acar, 2007
	DSM2498	3.5	nr	90 93 96 100	6.5 3.2 1.3 0.4	8.4	

Table 3 Continued 4

Heating medium	Strain	pH	SS (˚Brix)	[a]T (˚C)	D-value	z-value(˚C)	Reference
McIlvaine citrate-phosphate buffer	DSM2498	4.0	[b]nr	90 93 96 100	7.3 3.8 1.7 0.5	8.5	Bahçeci & Acar, 2007
Bam broth	DSM2492	3.0	nr	50	18.86	8.5	Alpas *et al*., 2003

[a]T = temperature
[b]nr = not reported

Table 4 Thermal inactivation kinetic parameters of *Alicyclobacillus acidocaldarius* spores in different heating media

Heating medium	Strain	pH	Soluble solids (˚Brix)	[a]T (˚C)	D-value	z-value	Reference
McIlvaine buffer	STCC 5137	7	[b]nr	110 115 120 125	2.6 0.54 0.097 0.014	6.7	Palop *et al*., 2000
	STCC 5137	4	nr	110 115 120 125	2.6 0.99 0.11 0.035	7.5	Palop *et al*., 2000
Distilled water	STCC 5137	nr	nr	110 115 120 125	3.7 0.48 0.11 0.024	6.7	Palop *et al*., 2000
Orange juice	STCC 5137	nr	nr	110 115 120 125	3.9 0.61 0.087 0.027	6.8	Palop *et al*., 2000

[a]T = temperature
[b]nr = not reported

1998; Eiora *et al*., 1999; Komitopoulou *et al*., 1999; Silva *et al*., 1999; Yamazaki *et al*., 2000; Bahçeci & Acar, 2007; De Carvalho *et al*., 2008).

Food spoilage

Alicyclobacillus acidoterrestris is now recognised by the beverage industry as the most important target species in the genus *Alicyclobacillus,* as it is routinely isolated from spoilt fruit beverages and is responsible for the production of the taint compound guaiacol. *Alicyclobacillus acidiphilus*, *A. herbarius*, and *A. hesperidum* subsp. *aigle* also produce guaiacol, but are infrequently isolated and are rarely associated with spoilage (Bevilacque *et al*., 2008; Goto *et al*., 2006). The spores of *A. acidoterrestris* can survive the typical pasteurisation regimes applied during juice manufacturing. This pasteurisation process during manufacturing provides the heat-shock treatment that may stimulate spore germination and outgrowth. In single strength juice these bacteria find a favorable environment for germination and growth, that under certain conditions can lead to product deterioration (Jensen, 1999; Chang & Kang, 2004).

Spoilage caused by *A. acidoterrestris* has to date been reported in apple, pear, orange, peach, mango and white grape juice, with shelf-stable apple juice most frequently being spoiled (Borlinghaus & Engel, 1997; Chang & Kang, 2004; Walker & Phillips, 2008). More diverse products such as shelf-stable iced tea containing berry juice, the ingredients of rose hip and hibiscus teas (Duong & Jensen, 2000), a carbonated fruit drink (Pettipher, 2000) and diced canned tomatoes (Chang & Kang, 2004) have also seen incidences of spoilage caused by *A. acidoterrestris*. A survey by the National Food Processors Association (NFPA) in the USA in 1998 reported that just over half of the manufacturers who responded to the survey (35% of the 60% who responded) reported incidence of spoilage of juice products by thermo-acidophilic bacteria suspected to be *A. acidoterrestris* (Walls & Chuyate, 1998).

Spoilage seemed to occur in the warmer seasons when large volumes of hot-filled products were allowed to cool naturally, suggesting that slow cooling of hot-filled products may allow bacterial growth (Pinhattiet *et al*., 1997). Over the last decade more varied products have been found to be susceptible to contamination and spoilage by *A. acidoterrestris,* while newer and better isolation techniques have led to an increase in the number of reported spoilage incidents (Walker & Phillips, 2008). It is important to note that incidence of *A. acidoterrestris* in fruit juice is not directly associated with deterioration. Detection of *A. acidoterrestris* in non-deteriorated fruit juices (Previdi *et*

al., 1997; Cerny *et al*., 1999; Bahçeci *et al*., 2005; Walker & Phillips, 2008) suggests deterioration to be incidental, requiring adequate conditions for its development.

The visual detection of spoilage of fruit juice products by *A. acidoterrestris* is difficult as the organism does not produce gas during growth. The spoiled juice appears normal with little or no change in pH. Occasionally, turbidity and/or white sediment may form at the bottom of the container. Members of the *Alicyclobacillus* genus causes some clarified fruit juices to have a light sediment, cloudiness or haze. However, the main spoilage characteristic is an off-flavour or odour caused by guaiacol (2-methoxyphenol) (Yamazaki *et al*., 1996; Borlinghaus & Engel, 1997; Walls & Chuyate, 1998; Jensen, 1999). This odour has been described as medicinal, sweet, chemical and "medical office" like (Siegmund & Pöllinger-Zierler, 2007). Other taint chemicals, such as the halophenols, 2,6-dichlorophenol (2,6-DCP) (Fig. 1A) and 2,6-dibromophenol (2,6-DBP) (Fig. 1B) can also be produced in fruit juice by *A. acidoterrestris* (Yamazaki *et al*., 1996; Komitopoulou *et al*., 1999; Jensen & Whitfield, 2003). These phenolic compounds are well known within the food industry to instill 'disinfectant'-like taints in food. The odour of the taint has also been described as smoky and pungent (Siegmund & Pöllinger-Zierler, 2007). Although *A. acidoterrestris* has been implicated in the production of these taints, it is important to note that trace quantities of these halophenols can also easily be formed in the presence of some sanitisers (Saxby, 1996).

Guaiacol is produced via the degradation of vanillic acid by *A. acidoterrestris* (Fig. 2) (Jensen, 2000). Vanillic acid can be present in fruit juices because of contamination, but is also naturally derived from the plant polymer lignin. The synthetic pathway for the production of guaiacol from ferulic acid is presented in Fig. 2. Ferulic acid, a major component of lignin, is converted to vanillin or 4-vinyl-guaiacol by decarboxylation. 4-vinyl-guaiacol is then oxidised to vanillin, which is then further oxidised to vanillic acid. After a final decarboxylation step, the vanillic acid is converted to the taint chemical guaiacol (Pometto *et al*., 1981; Huang *et al*., 1993). Another precursor for the formation of guaiacol is which is found in apple juice at a concentration of approximately 4.1 $\mu L.mL^{-1}$ and in orange juice at higher a concentration of up to 13.5 $\mu L.mL^{-1}$ (Chang & Kang, 2004).

The best estimate threshold (BET) for recognition of guaiacol in fruit juice was found to be approximately 2 parts per billion (ppb) (Pettipher *et al*., 1997; Orr *et al*., 2000). This equates to the presence of about 5 log CFU mL^{-1} of *A. acidoterrestris* cells in orange or apple juices (Borlingghaus & Engel, 1997; Brown, 2000). Guaiacol production by *A. acidoterrestris* depends on the concentration of the organism present,

the type of strain present, the storage temperature, the oxygen concentration in the fruit beverage product, the use of heat shock which encourages germination of the spores, and finally the concentration of precursors to guaiacol, such as vanillin in the fruit juice (Pettipher *et al*., 1997; Chang & Kang, 2004, Bahçeci *et al*., 2005; Goto *et al*., 2008).

Spoilage can occur even if low numbers of *A. acidoterrestris* are initially present in fruit juice as it is during growth of the vegetative cell that guaiacol is produced. The concentration of *A. acidoterrestris* cells and spores does not necessary correlate with guaiacol levels in spoilt fruit juice. In a study by Orr *et al*. (2000), the number of *A. acidoterrestris* cells and spores remained constant in inoculated apple juice stored over 61 d at either 21° or 37 °C. However, levels of guaiacol varied over this period of time. In contrast, Goto *et al.* (2007) reported that production of guaiacol follows the growth curve, gradually accumulating and is ultimately degraded.

Evidence on the influence oxygen levels in the growth media has on the growth and rate of guaiacol production of *A. acidoterrestris* remains contradictory. Jensen & Whitfield (2003) noted that taint was produced more quickly in containers with a large headspace, while Walker and Philips (2005) observed increased growth rates of *A. acidoterrestris* in containers with 25% (v/v) headspace as opposed to those with no headspace. However, Cerny *et al.* (1999) found that the presence or absence of a headspace in the packaging system did not significantly influence the growth of *A. acidoterrestris* DSM 2498. In a study by Siegmund and Pöllinger-Zierler (2007) both taint compounds guaiacol and 2,6-DPB were produced in greater amounts by *A. acidoterrestris* DSM 2498 when oxygen was in limited supply even though the growth rate was slower.

All strains of *A. acidoterrestris* that have been tested for the ability to produce taint compounds have been able to produce guaiacol and either/both 2,6-DPB and 2,6-DCP, dependent on the strain (Gocmen *et al*., 2005; Goto *et al*., 2007; Goto *et al*., 2008). A study by Goto *et al*. (2007) on *Alicyclobacillus* strains showed a great variability in their guaiacol production. Strains were inoculated into YSG liquid media containing 1 ppm vanillin and cultured at optimum temperature for 72 h. After cultivation, guaiacol concentration in the culture broth was analysed by GC-MS. *Alicyclobacillus acidoterrestris* B2065 produced the least guaiacol (40 ppb), while strain DSM 3924 was capable of producing the most (500 ppb). The type strain ATCC 49025^{T} produced 218 ppb of guaiacol. Studies using GC-olfactory and GC-MS on two strains of *Alicyclobacillus* UFL-CA8 and UFL-CA11, presumptively identified as *A. acidoterrestris* showed them to be capable of larger guaiacol production than *A. acidoterrestris* ATCC

49025^T (Gocmen *et al*., 2005). UFL-CA8 and UFL-CA11 were isolated from juice containing drinks that developed a medicinal off-aroma within 2 week storage at room temperature (Gocmen *et al*., 2005). Research carried out on 36 *A. acidoterrestris* strains isolated from fruit orchard soils throughout Japan also showed variability in their ability to produce guaiacol. The majority of strains, including *A. acidoterrestris* ATCC 49025^T, produced more than 20 ppm of guaiacol following the 10 h incubation period in Va-YSG media. However, one strain 122-1 was capable of about 40 ppm of guaiacol within 5 h, while strain 31-1 scarcely produced any guaiacol within 24 h. After 48 h similar levels of guaiacol were produced by all isolated strains (Goto *et al*., 2008). In the same study the 16S rRNA gene-based phylogenetic relatedness between the strains of *A. acidoterrestris*, *A. acidophilus*, *A. hesperidum* subsp. *aigle* and *A. contaminans* all isolated from Japanese fruit orchard soil were compared. It was found that the guaiacol production is conserved among certain *Alicyclobacillus* species, but no correlation was observed between levels of guaiacol production and 16S rRNA gene-based phylogenetic relatedness (Goto *et al*., 2008).

Temperature also has an effect on taint formation. Guaiacol was detected more quickly at 37 ℃, compared to 21 ℃ in inoculated apple juice. This suggests that guaiacol formation is limited at room temperatures (20–25 ℃) at which *A. acidoterrestris* grow poorly (Orr *et al*., 2000; Bahçeci *et al*., 2005). Bahçeci *et al.* (2005) also found that the amount of guaiacol formed in apple juice was dependant on the initial concentration of vanillin present in the apple juice. In apple juice samples inoculated with 10 mg.L^{-1} of vanillin and around 1 x 10^5 CFU mL^{-1} of *A. acidoterrestris* spores, vanillin was totally consumed within 24 h. The concentration of guaiacol exceeded 8 mg.L^{-1} and remained relatively stable at the end of incubation. In controls lacking vanillin, no detectable amount of guaiacol was formed despite growth of *A. acidoterrestris* in the apple juice. Heat shock treatments which lead to the activation and growth of spores, affect the production of guaiacol as it is dependant on active vegetative cells being present in fruit juice (Splittstoesser *et al*., 1998; Jensen, 2000; Chang & Kang, 2004). Walls and Chuyate (2000) found that a heatshock treatment of 80 ℃ for 10 min resulted in the highest guaiacol concentration.

As awareness arose of *A. acidoterrestris* as a potential spoilage organism, concerns were raised regarding its pathogenicity. Fortunately, there is no evidence that *A. acidoterrestris* pose a human health risk. Neither the organism nor its metabolites have been associated with any form of ill health and *A. acidoterrestris* is considered a non-pathogen (Borlinghaus & Engel, 1997). When mice were injected with alicylcobacilli

spores or guinea pigs fed inoculated fruit juices containing 5 x 10^6 CFU mL^{-1} *A. acidoterrestris*, none exhibited any illness symptoms and no animal died (Walls & Chuyate, 2000). The risk of secondary growth of other pathogens such as *Clostridium botulinum* is also not of concern, as growth of *A. acidoterrestris* in fruit juice does not affect its pH (Brown, 2000). Juice spoilage by *Alicyclobacillus* spp. has a major economical impact on the fruit juice industry, but there is no health risk involved in consuming fruit juice containing this bacterium or its spores (Borlinghaus & Engel, 1997; Walls & Chuyate, 2000).

E. CONTROL METHODS FOR *ALICYCLOBACILLUS ACIDOTERRESTRIS*

Pasteurisation treatments on fruit juice are used to kill potential pathogens and increase shelf life. The U.S. Food and Drug Administration requires all fruit juice sold in the United States to be either pasteurised or subjected to an equivalent process to achieve a mandated 5-log pathogen reduction in the juice (US FDA, 2000). Flash pasteurisation is most commonly used, a method of high temperature short time processing which employs rapid heating and cooling steps. Typically fruit juice is heated to around 88° to 96 °C for 30 s to 2 min and then rapidly cooled (Choi & Nielsen, 2005). The problem *A. acidoterrestris* pose to the fruit juice industry is the ability of its spores to survive thermal pasteurisation and hot-fill hold processes (Splittstoesser *et al*., 1994; Eiroa *et al*., 1999; Orr & Beuchat, 2000). In fact, pasteurisation serves as a heat treatment that stimulates the germination of the spores. Excessive heat treatments are not feasible due to changes in the organoleptic and nutritional properties of the juice, resulting in non-enzymatic browning and losses of vitamins and flavour compounds (Lado & Yousef, 2002; Rivas *et al.,* 2006). New techniques have been proposed in order to reduce the loss of sensorial and nutritional quality due to a thermal treatment.

Nisin and other bacteriocins

Bacteriocins are small peptides that show bactericidal activity against certain bacteria (Yamazaki *et al*., 2000). They are potent antimicrobial substances that are produced by a large and diverse number of bacterial species. Most food grade bacteriocins are produced by lactic acid bacteria and bacteriocins used in the food industry include nisin, enterocin and bovacin (Kim, 1993). Nisin is the most widely used of the bacteriocins. It is produced by fermentation of a modified milk medium by strains of *Lactococcus lactis*

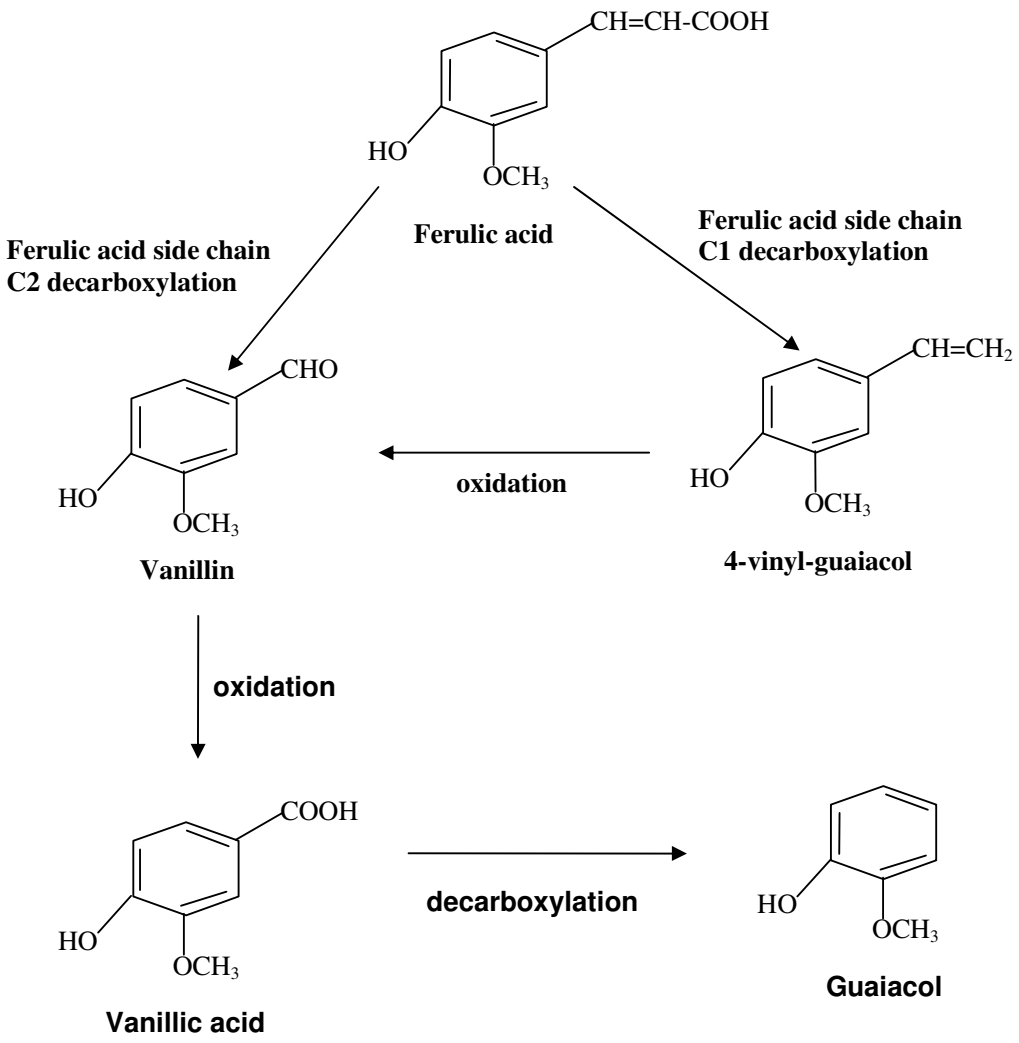


Figure 2 Simplified schematic representation of the formation of guaiacol from ferulic acid as the precursor present in food products (Chang & Kang 2004).

subsp. *lactis*. It has been demonstrated that nisin exhibits a wide range of inhibitoryeffects against Gram-positive spore-formers and pathogens, while it shows little or no inhibitory effects against Gram-negative bacteria or fungi (Stevens *et al*., 1991). Nisin functions by interacting with the phospholipids in the cytoplasmic membrane of bacteria, thus disrupting membrane function. It also prevents the outgrowth of spores by inhibiting the swelling process of germination. Nisin is most stable at pH 3 and maintains more than 70% antibacterial activity at pH 4 when autoclaved at 115 °C (Davies *et al*., 1998). The safety and efficacy of nisin as a food preservative have resulted in its widespread use (Hansen, 1994).

Komitopoulou *et al*. (1999) reported that nisin could effectively be used for the control of *A. acidoterrestris* in fruit juices and fruit juice containing products. In this study the sensitivity of *A. acidoterrestris* to nisin was determined in three fruit juices (apple, orange and grapefruit) at 25° and 44 °C. At 25 °C, 5 international units ($IU.mL^{-1}$) of nisin were able to prevent outgrowth of *A. acidoterrestris* spores in all thee fruit juices tested. At 44 °C the same level of inhibition of spores was seen in grapefruit juice, but 100 $IU.mL^{-1}$ of nisin were the minimum inhibitory concentration (MIC) required in apple and orange juice. Vegetative cell were less sensitive to nisin but when a mixed inoculum of spores and vegetative cells was incubated at 44 °C, 100 $IU.mL^{-1}$ was sufficient to prevent growth in all three juices. The addition of 50 $IU.mL^{-1}$ nisin to apple juice increased the heat sensitivity of the spores at temperatures of 80°, 90° and 95 °C, with the greatest reduction in D-value of around 40% at 80 °C.

In a similar study by Yamasaki *et al*. (2000), the MIC of nisin on seven strains of *A. acidoterrestris* at a pH of 3.4 and 4.2 on modified yeast peptone glucose agar (mYPGA) plates was investigated. The sensitivity of *A. acidoterrestris* to nisin varied among the strains tested. At pH 3.4 and 4.2 the MIC values for the vegetative cells among the strains was 1.56 to 25 $IU.mL^{-1}$ and 25 to 100 $IU.mL^{-1}$, respectively. In contrast, the spores were more sensitive to nisin than the vegetative cells, and the inhibitory levels of nisin for the spores among the strains were <0.78 to 12.5 $IU.mL^{-1}$ and 25 to100 $IU.mL^{-1}$ at pH 3.4 and 4.2, respectively. The most resistant strain, *A. acidoterrestris* AB-5 required 12.5 and 100 $IU.mL^{-1}$ for the inhibition of spore outgrowth at pH 3.4 and 4.2, respectively. The greater effectiveness of nisin at low concentrations (50 – 100 $IU.mL^{-1}$) on spores compared to cells suggests that nisin acts at the stage of pre-germinant swelling and its effect was sporostatic rather than sporicidal (Yamazaki *et al*., 2000).

When *A. acidoterrestris* AB-5 was added to orange and mixed fruit drinks no outgrowth occurred in the presence of 25 and 50 IU.mL^{-1} nisin, respectively after 12 days storage. The addition of 200 IU.mL^{-1} or 5 ppm active nisin to clear apple juice or orange drink reduced the thermal resistance of *A. acidoterrestris* AB-5 spores by 71% and 76%, respectively. The decrease in heat resistance of the bacterial spores showed a linear relationship with increasing nisin concentration in both acidic drinks. The MIC for *A. acidoterrestris* AB-5 spores at 40 ℃ was 25 IU.mL^{-1} nisin for orange drink and 50 IU.mL^{-1} nisin for mixed fruit drinks. However 600 IU.mL^{-1} nisin was unable to inhibit growth in a clear apple drink (Yamazaki *et al.,* 2000). This inhibitory action which decreased the effectiveness of nisin in the clear apple drink, may be due to the binding of nisin to some apple particles, although nisin would also be absorbed onto some particles in orange or mixed fruit drinks. As nisin is heat stable the beneficial effects of its inclusion prior to pasteurisation would be twofold: to enhance the effect of the heat process, and residual nisin would prevent outgrowth of surviving spores.

Other bacteriocins such enterocin AS-48, extracted from *Enterococcus faecalis* A-48-32 (Grande *et al*., 2005), warnericin, purified from *Staphylococcus warneri* (Minakawa *et al*., 2005) and bovacin HC5, from *Streptococcus bovis* (Carvalho *et al*., 2008) have shown antimicrobial activity against strains of *A. acidoterrestris.* Enterocin AS-48 was active against *A. acidocaldarius* CECT 4328 and three strains of *A. acidoterrestris* (LMG 16906, DSM 2498 and DSM 3922). At a concentration of 2.5 µg mL^{-1} and at 37 ℃, enterocin AS-48 was able to reduce vegetative cells of *A. acidoterrestris* LMG 16906 and DSM 2498 to below the detection limit after 24 h of incubation in *Alicyclobacillus acidocaldarius* medium (AAM). The same results were observed when strain DSM 2498 was inoculated in freshly made orange (pH 3.86) and apple (pH 3.55) juices containing 2.5 µg mL^{-1} enterocin AS-48. Vegetative cells and spores of strain DSM 2498 were also inoculated into five commercial fruit juices (orange, apple, pineapple, peach and grapefruit) containing 2.5 µg mL^{-1} enterocin AS-48 and incubated at 37 ℃, 15 ℃ and 4 ℃. No viable cells were observed 15 min after inoculation and growth was inhibited for up to 90 days in orange and pineapple juices and up to 60 days in apple, peach and grapefruit juices at 37 ℃. At 15 ℃ and 4 ℃ no viable cells were detected for the whole incubation period in all the fruit juices. Electron microscopy examination of *A. acidoterrestris* DSM 2498 cells and spores treated with enterocin AS-48 revealed cell wall damage, leakage of cytoplasmic contents and cell disorganization as well as spore degradation (Grande *et al*., 2005).

In a study by Carvalho *et al*. (2008) the antimicrobial activity of bovicin HC5

against *A. acidoterrestris* DSM 2498 was investigated. Concentrations of 40 to 160 AU mL^{-1} bovicin HC5 was able to completely inhibit growth in AAM broth (pH 4.0) at 40 °C for as long as 15 days. A bactericidal and sporicidal effect was observed when mango pulp (pH, 4.0-7.0) inoculated with *A. acidoterrestris* DSM 2498 cells or spores at a level of 10^5-10^6 CFU mL^{-1} was treated with 80 to 100 AU mL^{-1} bovicin HC5. The minimum inhibitory concentration (MIC) of bovicin HC5 was determined to be 5 and 2.5 AU mL^{-1} for vegetative cells and spores, respectively. The D-values of *A. acidoterrestris* DSM 2498 spores in mango pulp (pH 4.0) decreased between 77 and 95% at 80° to 95 °C when bovicin was added at a concentration of 80 AU mL^{-1}. The z-value decreased by 48.7% (Carvalho *et al*., 2008). The commercial use of enterocin AS-48 and bovicin HC5 is, however, limited due to the cost of extraction and purification.

F. ULTRAVIOLET RADIATION

The use of ultraviolet (UV) light in food processing is one of a number of non-thermal technologies being used as a substitute for thermal processing. Other non-thermal processing technologies include pulsed electric fields, high-pressure processing and ultrasound. These substitute technologies can be used to process food products so that they do not contain spoilage or pathogenic micro-organisms and enzymes that may decrease the nutritional and sensory characteristics of foods (Butz & Tauscher, 2002; Koutchma, 2009). The advantages associated with UV-C radiation used as a non-thermal method is that no known toxic or significant non-toxic by-products are formed during the treatment, certain organic contaminants can be removed and the treatment requires very little energy when compared to thermal pasteurisation. Fruit juices that undergo thermal pasteurisation or sterilisation tend to change colour and lose some of its aroma and vitamins during the process of heating (Choi & Nielsen, 2005). This is unlike juices that are treated with UV radiation, which tend to maintain their aroma and colour (Tran & Farid, 2004). The U.S. Food and Drug Administration has allowed UV-C radiation to be used as an alternative to pasteurisation for the elimination of pathogens from fruit juices (US FDA, 2000).

UV wavelengths of between 220 and 300 nm are considered germicidal against micro-organisms that include bacteria, viruses, protozoa, moulds, yeasts, and algae (Sizer & Balasubramaniam, 1999; Bintsis *et al*., 2000). Fungi are more resistant to UV penetration than bacteria due to the lower proportion of thymine and cytosine bases in

their DNA. Their thicker cell wall may also present a greater resistance to UV light (Tran & Farid, 2004).

The highest germicidal effect is obtained between 250 and 270 nm, decreasing as the wavelength is increased, and above 300 nm the germicidal effect is annulled. Therefore, a wavelength of 254 nm (UV-C, generated by low pressure mercury lamps) is used for disinfection of surfaces, water and some liquid food products such as fruit juice (Guerrero-Beltrán & Barbosa-Cánovas, 2004).

When UV light is applied to organisms, the DNA absorbs photons of UV light generating cyclobutane-type dimers between adjacent pyrimidines, primarily thymines but also between cytosine and thymine. These pyrimidine dimers fuse the double-stranded DNA molecule, thereby disrupting cell function (Giese & Darby, 2000). The formation of these cyclobutyl pyrimidine dimers results in DNA transcription and replication being blocked, which inhibits cellular functions and leads to cell death. The amount of dimers formed is directly proportional to the amount of UV-C absorbed. The radiation may also cause DNA mutations in the injured organism (Giese & Darby, 2000).

Spores are significantly more resistant than are their corresponding vegetative cells to 254 nm UV-C radiation (Nicholson *et al*., 2000). To date, the best understood spore resistance mechanism involves the resistance of *Bacillus subtilis* spores to 254 nm UV-C. *Bacillus subtilis* spores are approximately 10 to 20 times more resistant to UV-C than vegetative *B. subtilis* cells (Slieman & Nicholson, 2000). The UV-C resistance of spores is due to two interrelating mechanisms. Firstly, DNA in spores irradiated with UV-C radiation accumulates the unique thymine dimer, 5-thyminyl-5,6-dihydrothymine, which is known as spore photoproduct. Secondly, spores possess at least two major DNA repair pathways for accurate repair of spore photoproduct during spore germination.

This is the general nucleotide excision repair system (encoded by genes designated *uvr*) and photoreactivation. Nucleotide excision repair, often referred to as dark repair, is widely distributed and conserved through evolution. This repair process involves the action of more than a dozen proteins that coordinate the removal of DNA damage (Zimmer & Slawson, 2002). Aside from dark repair, many organisms repair damage through a process called photoreactivation which uses a single enzyme called spore photoproduct lyase (encoded in part by the *splB* gene) to reverse UV-induced damage to DNA. Photoreactivation is a light-dependent process that requires specific wavelengths of light ranging from 300 to 500 nm to complete the repair process (Slieman & Nicholson, 2000).

In food processing, UV disinfection of water has been used in brewing (McCarty & Scanion, 1993), soft drink processing (Gibbs, 2000), and in the cheese-making processes (Honer, 1988). UV has also been used in sterilising sugar syrup (Stother, 1999). However, the use of UV for disinfection of liquid food, such as juices has not been widely studied, while different legislation in different countries will only allow certain products to be treated. In Germany UV-C radiation is only allowed to treat the surfaces of fruit and vegetables, water and hard cheese (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Koutchma *et al*., 2004; Koutchma *et al*., 2007).

The efficacy of the microbial reduction in fruit juices by UV-C light at 254 nm depends on a number of factors. These include the type of organisms (including strains present in the liquid), the specific stage of microbial growth, the growth media used, the inoculum level, the % UV transmittance of the liquid (opaqueness), fluid dynamic parameters and suspended particles in the liquid. It is known that the penetration depth of UV-C light through the surface of liquids is very short, with the exception of clear water (Shama, 1999). The penetration of UV light into juices is about 1 mm for absorption of 90% of the light (Sizer & Balasubramaniam, 1999). The penetration effect of UV-C radiation depends on the type of liquid, its UV-C absorptivity, soluble solids and suspended matter in the liquid.

If the organism is suspended in rich growth media, such as peptones and sera, a shielding effect occurs. Rich media may also lead to an increase in ribosomes present within the cell and this also shields the DNA from UV light (Tran & Farid, 2004). Greater amounts of suspended particles lower the intensity of penetration of the UV-C light in the liquid. Particles can absorb, scatter and block UV light due to aggregation of bacteria to the surface of the particles (Christenen & Linden, 2001). This phenomenon is well documented for unfiltered water, however, the effect is not well documented for UV processing of juices (Shama 1999; Bintsis *et al*., 2000). The low UV transmittance due to high suspended and soluble solids necessitates a turbulent flow during liquid food processing and this is a legal requirement by the Food and Drug Administration (US FDA, 2000; Keyser *et al*., 2007).

The growth phase and stage at which the organisms is at prior to UV treatment is also important, as cells in the exponential phase are more susceptible to treatment than cells in the stationary phase which tend be more resistant (Tran & Farid, 2004; Gruetzmacher & Bradley Jnr, 1999). Finally, the efficacy of the treatment is also greatly dependant on temperature. Dimer formation between adjacent thymine nucleotides in single-stranded DNA is increased at temperatures below 25 °C. At low temperatures,

the natural state of single stranded DNA is a stacked structure that positively increases dimerisation. At higher temperatures, a slightly higher UV dose is required for the same effect (Severin *et al*., 1983).

Because of the extensive variety of organisms, including strains, the dose levels required for disinfection can vary according to the final requirement for each food product. In order to obtain a microbiologically safe food product all parts of the fluid should be exposed to a minimum 400 $J.m^{-2}$ of UV light at 254 nm to ensure an adequate reduction of 5 log cycles of a surrogated micro-organism (Hoyer *et al*., 1998).

The effect that UV radiation has on the organoleptic properties of fruit juices, particularly on orange juices has been studied in recent years. The process of conventional pasteurisation significantly inactivates pectin methylesterase. This enzyme de-esterifies pectin preventing the orange juice from becoming cloudy. UV treatment of juices is usually performed at about 25 °C, and UV light has no effect on the activity of the pectin methylesterase enzyme. Therefore, UV treated juices have a tendency to become cloudy. Vitamin C degradation was also observed in orange juice that were UV treated and this reduction was in the same order as the degradation of vitamin C in thermally treated fruit juice. The reason for this remains unclear (Tran & Farid, 2004).

The use of UV radiation also has the advantage over thermal pasteurisation that no expensive equipment is required and the maintenance and running costs of a UV light system is far less than that for a pasteurisation system. The UV light system also provides ease of operation as the UV dosage is dependant on flow rate which can be adjusted according to the manufacturers requirements in terms of the volumes or the amount of product treated. UV light does not only have to be used to treat the product, but may also be used to disinfect the water in cleaning the processing equipment, which may be a key factor in eliminating process contamination.

G CONCLUSIONS

Incidents of spoilage by *A. acidoterrestris* of a variety of pasteurised fruit juices and fruit juice products have increased notably in recent years (Yamazaki *et al*., 1996; Pettipher *et al*., 1997; Silva & Gibbs, 2004; Walker & Phillips, 2008; Bevilacque *et al*., 2008). Due to their thermo-acidophilic properties and their occurrence in several spoiled pasteurised products, *A. acidoterrestris* spores has been recommend as a target organism for pasteurisation of high acidic food products (Silva *et al*., 1999). Currently the source and route of contamination of the fruit juice remains unclear. However as

members of the genus *Alicyclobacillus* are soil-borne organisms, it is thought that contaminated fresh fruit introduced during processing without proper cleaning leads to contamination and subsequent spoilage (McIntyre *et al*., 1995; Pontius *et al*., 1998; Splittstoesser *et al*., 1998; Orr & Beuchat, 2000). For the development of prevention strategies of contamination the source and route of contamination needs to be clearly established.

The ability of *A. acidoterrestris* spores to survive thermal pasteurisation and hot-fill and hold processes used during fruit processing and fruit juice production necessitates the development of alternative processing techniques to pasteurisation (Splittstoesser *et al*., 1994; Eiroa *et al*., 1999; Orr & Beuchat, 2000). The use of UV light as a germicidal medium is one such promising technology. Advantages associated with UV-C radiation used as a non-thermal method is that no known toxic or significant non-toxic by-products are formed during the treatment, certain organic contaminants can be removed and the treatment requires very little energy when compared to thermal pasteurisation. However, the use of UV for the disinfection of liquid foods, such as juices has not been widely studied and its efficacy against spores of *A. acidoterrestris* is unknown. Another drawback is that UV-C radiation has little effects on enzymes which can cause juice clarification or browning and thus a combination of treatments to inactivate the enzymes may be necessary (Guerrero-Beltrán & Barbosa-Cánovas, 2004).

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CHAPTER 3

ISOLATION AND IDENTIFICATION OF SPECIES OF *ALICYCLOBACILLUS* FROM ORCHARD SOIL IN THE WESTERN CAPE, SOUTH AFRICA

Abstract

Alicyclobacilli were isolated from orchard soil collected from an apple and pear farm in Elgin, Western Cape, South Africa. Morphological, biochemical and physiological characteristics of the isolates were used to presumptively classify them as belonging to the genus *Alicyclobacillus*. Strains were identified to species level by polymerase chain reaction (PCR) with genus-specific primers, and 16S ribosomal RNA (rRNA) gene sequencing. To our knowledge this is the first report on the isolation of *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius* from orchard soil. The presences of these organisms in the soil suggest a possible source of contamination for the final fruit juice, concentrate or pulp.

Introduction

The alicyclobacilli are thermo-acidophilic, Gram-positive, rod-shaped, spore-forming, aerobic micro-organisms that possess ω-alicyclic fatty acids (ω-cyclohexane or ω-cycloheptane) as the major components of the cellular membrane (Wisotskey *et al*., 1992; Walls & Chuyate, 1998). Species of *Alicyclobacillus* have been isolated from a range of habitats and substrates, such as organic compost, manure, fruit, and heat-processed foods (Deinhard *et al*. 1987; Yamazaki *et al*. 1996; Pettipher *et al*. 1997; Albuquerque *et al*. 2000; Jensen 2000; Walls & Chuyate, 2000; Goto *et al*., 2002; Matsubara *et al*., 2002). At present there are 19 recognized species of *Alicyclobacillus* of which three species have been isolated from spoilt juice products, namely *Alicyclobacillus acidoterrestris*, *Alicyclobacillus acidocaldarius* and *Alicyclobacillus pomorum* (Goto *et al*., 2003; Jensen & Whitfield, 2003; Gouws *et al*., 2005). *Alicyclobacillus* spores are heat resistant and pasteurization does not inactivate the spores (Splittstoesser *et al*., 1994; Eiroa *et al*., 1999; Orr & Beuchat, 2000). This heat

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resistance was observed by Splittstoesser *et al*. (1994) who reported D values for *A. acidoterrestris* of 23 min at 90 ℃ and 2.4 to 2.8 min at 95 ℃, suggesting that spores survive the typical juice pasteurization process that consists of holding at 86° to 96 ℃ for 2 min. In fact pasteurization serves as a heat treatment that stimulates germination of the spores, leading to growth. The ability of *A. acidoterrestris* to grow at a pH range of 2.5 to 6 (Yamazaki *et al*., 1996) and to survive the typical juice pasteurization process has caused concern in the fruit juice industry (Splittstoesser *et al*., 1998; Eiora *et al*., 1999; Gouws *et al*., 2005).

Spoilage caused by this micro-organism is visually difficult to detect. The spoiled juice appears normal or might have light sediment. No gas is produced. Often, the only evidence of spoilage is a medicinal/phenolic off-flavour (Walls & Chuyate, 1998; Jensen, 1999; Jensen, 2000). Fruit juice contamination results from unwashed or poorly washed raw fruit that is processed, as well as contaminated water used during the production of fruit juices (Pontius *et al*., 1998; Orr & Beuchat, 2000; McIntyre *et al*., 1995). Our interest in the presence of species of *Alicyclobacillus* in orchard soil is to investigate a potential source of contamination of the final fruit juice concentrate. In this study, strains of *Alicyclobacillus* were isolated from orchard soil of an apple and pear farm and identified to species level based on genus-specific PCR and rRNA gene sequence analyses.

Materials and methods

Collection of samples

Top soil was collected from 12 random sites in apple and pear orchards on a farm in the Elgin region of the Western Cape, South Africa. The soil samples were sieved through a 2.0 mm width mesh to remove stones and plant debris. A Soil: water ratio of 1:1 was used for the determination of soil pH. Approximately 10 g of soil was placed in sterile 50 mL centrifuge tubes and re-suspended in 30 mL sterile peptone water. After soil particles were allowed to sediment, the supernatants were heat treated at 80˚C for 10 min (Walls & Chuyate, 2000) and diluted in sterile distilled water. Dilution series of 10^{-1} to 10^{-6} were prepared in triplicate on potato dextrose agar (PDA) (Biolab, Biolab Diagnostics, Midrand, SA), orange serum agar (OSA) (Oxoid, Basingstoke, Hampshire, England), YSG (yeast extract starch glucose) agar (Uchino & Doi, 1967) and YSG agar, supplemented with 20% (v/v) sterile apple juice. All media contained 100 µg mL^{-1}

Delvocid (GistBrocades, Delf, Netherlands) to inhibit the growth of yeast and fungi. Tartaric acid (1N) was used after autoclaving to adjust all media to a final pH of 4. One of three plates was incubated at 55°C while the remaining two sets of plates were incubated at 45°C. Plates were incubated aerobically and examined for growth after 72, 96 and 120 h.

Preliminary identification of alicyclobacilli

Colonies were randomLy selected from plates containing between 20 and 300 colonies and re-streaked on corresponding media to obtain pure cultures. All cultures were stored at -80°C in YSG broth adjusted to a pH of 4 with 1 N tartaric acid, supplemented with sterile glycerol (30%, v/v, final concentration). Gram reaction, oxidase and catalase activity were determined according to the methods described by Harrigan and McCance (1976). Gram-positive, oxidase positive, and catalase positive rods were selected for further examination.

Carbohydrate fermentations

Carbohydrate fermentation reactions were recorded by using the API 50 CHB system (BioMerieux, Marcy L'Etoile, France) according to the manufacturer's instructions. Incubation of all API strips was at 45 °C, and results were recorded after 4 and 5 days.

Genus-specific PCR amplification

Isolates were grown in YSG broth adjusted to pH 4 for 4 days. Their genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin) according to the manufacturer's instructions. The DNA was amplified with primers CC16S-F (CGTAGTTCGGATTGCAGGG) and CC16S-R (GTGTTGCCGACTCTCGTG) (Conner *et al*., 2004). PCR reactions were performed in a total volume of 25 µL containing 0.6 µM of each of the primers, 1.25 U *Taq* DNA polymerase (Promega, Madison, Wisconsin), 1 x PCR reaction buffer containing $MgCl_2$, 1 µL of 99% (v/v) dimethyl sulphoxide (DMSO), 0.4 mM deoxyribonucleoside triphosphate (dNTPs) and 2 µL of the extracted DNA. PCR reactions were performed in the Eppendorf Mastercycler Personal. An initial 3 min denaturation at 95ºC was followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s and elongation at 72ºC for

30 s, and a final 2 min chain elongation at 72ºC. *A. acidocaldarius* PMO1 and *A. acidoterrestris* SAO1 (Gouws *et al*., 2005) were used as reference strains.

DNA Sequencing

The genomic DNA of isolates were amplified with primers F8 (5'-CAG GCA TCC AGA CTT TGA TYM TGG CTC AG-3') and R1512 (5'-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3'), as used by Felske *et al*. (1997). PCR was according to the method described by Garbers *et al*. (2004). The amplified fragments of approximately 1.5kb in size were purified using the High Pure PCR Purification Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The fragments were sequenced using the ABI PRISM 377 DNA Sequencer (Perkin Elmer) at the DNA Sequencing Facility, Stellenbosch University and compared to sequences in GenBank using the BLAST search option.

Results and discussion

The soil pH ranged from 5.91 to 6.71 (Table 1), which is typical of orchard soil. YSG plates incubated at 45^{o}C contained the highest amount of microbial cells. From a total of 28 isolates, 5 were selected based on Gram reaction, morphology and catalase activity. All isolates were Gram-positive, rod shaped, oxidase and catalase positive. Carbohydrate fermentation reactions recorded for 4 out of the 5 isolates corresponded most closely to that of the type strain of *A. acidoterrestris* (Table 2). Variations in the fermentation of D-raffinose, starch, trehalose, D-turanose and xylitol were recorded (Table 2). Similar results have been reported for other strains of *A. acidoterrestris* (Chang & Kang, 2001; Silva & Gibbs, 2001; Goto *et al*., 2001). None of the strains fermented ribose and aditinol, which is characteristic for the type strain of *A. acidoterrestris* ATCC 49025^{T} (Goto *et al*., 2001).

DNA amplification with genus-specific primers on all four isolates produced a 134-bp fragment (data not shown), which was identical in size to that reported for *A. acidoterrestris* ATCC 49025^{T}, *A. cycloheptanicus* ATCC 49029 and *A. acidocaldarius* ATCC 43030 (Conner *et al*., 2004). Sequencing of the 16S rRNA gene amplicons for the 5 isolates of *Alicyclobacillus* and comparison with nucleotide sequences in GenBank, revealed DNA homology between 96.8% and 98.7.0% to *A. acidoterrestris*

Table 1 Origin of isolates

Isolate	Location	Soil Type	Soil pH
ESO6	Granny Smith orchard	Sandy Clay Loam	6.69
ESO7	Golden Delicious orchard	Sandy Clay Loam	6.50
ESO14	Forelle orchard	Sandy Clay Loam	6.66
ESO12	Fuji orchard	Sandy Clay Loam	6.71
ESO3	Packham's Triump orchard	Clay Loam	5.91

Table 2 Differential carbohydrate fermentation reactions of *Alicyclobacillus* isolates collected from orchard soil in the Western Cape, South Africa

Isolate	**Glycerol**	**Erythritol**	**Ribose**	**Adonitol**	**L-sorbose**	**Rhamnose**	**Trehalose**	**Inositol**	**Sorbitol**	**α-methyl-D-mannoside**	**Amygdalin**	**Salicin**	**melibiose**	**Melezitose**	**D-raffinose**	**Starch**	**Glycogen**	**Xylitol**	**β-gentiobiose**	**D-turanose**	**D-arabitol**
A.acidoterrestris ATCC 49025[Ta]	+	+	+	+	-	+	-	+	+	+	+	+	-	+	-	-	-	+	+	+	+
ESO6	+	+	-	-	-	+	+	d	+	+	+	+	-	+	+	+	-	+	+	d	+
ESO7	+	+	-	-	-	+	+	d	+	+	+	+	-	+	+	d	-	d	+	d	+
ESO12	+	+	-	-	-	+	-	d	+	+	+	+	-	+	-	-	-	-	+	+	+
ESO14	+	+	-	-	-	+	-	d	+	+	+	+	-	+	-	-	-	-	+	-	+
A.acidocaldarius ATCC 27009[Ta]	+	-	+	-	+	+	-	-	-	-	-	-	+	-	+	-	+	-	-	+	+
ESO3	+	-	-	-	+	-	-	-	-	-	d	+	+	+	d	-	-	-	+	+	-

+, positive reaction; -, negative reaction; d, variable reaction. All strains fermented: aesculin, L-arabinose, arbutin, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose mannitol, D-mannose, α-methyl-D-glucoside, sucrose, trehalose and D-xylose. None of the strains fermented: *N*-acetyl-glucosamine, D-arabinose, L-arabitol, dulcitol, D-fucose, L-fucose, Gluconate, inulin, 2-keto-gluconate, 5-keto-gluconate, β-methyl-xyloside, D-tagatose, D-xylose and L-xylose

[a] Data from Goto *et al.* (2001).

ATCC 49025^T (Table 3). Differences in DNA sequence data suggested that the strains of *A. acidoterrestris*, isolated from orchard soil do not represent a homogeneous collection.

The carbohydrate fermentation pattern of isolate ESO3 corresponded most closely to *A. acidocaldarius* ATCC 2700^T (Table 2). Erythritol was unable to be assimilated, which is a characteristic of *A. acidocaldarius* (Goto *et al*., 2001). PCR with genus-specific primers yielded a DNA fragment of 134 bp (data not shown); indicating isolate ESO 3 belonged to the genus *Alicyclobacillus* (Conner *et al*., 2004). Sequence analysis of isolate ESO3 revealed 98.6% homology to the 16S rDNA of *A. acidocaldarius* ATCC 2700^T (Table 3). Isolate ESO 3 is thus regarded as a member of *A. acidocaldarius*.

To our knowledge, this is the first report on the isolation of *A. acidoterrestris* and *A. acidocaldarius* from orchard soil. Soil adhering to fruit bins, machinery and on fruit that was picked off the ground during harvest can greatly complicate subsequent cleaning operations in the processing plant. The presence of known spoilage causing species of *Alicyclobacillus* in orchard soil suggests a potential source of contamination for the final fruit juice, concentrate or pulp. Further research is needed to establish the role played by these specific strains in the spoilage of fruit juice.

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Table 3 Percentage similarity of isolates from orchard soil to species in the NCBI nucleotide sequence database, based on partial 16S rDNA sequence analysis

Isolate	No of nucleotides	% similarity	Nearest phylogenetic neighbour (GenBank accession number)
ESO6	833	98.7%	*Alicyclobacillus acidoterrestris* (AY686617.1)
ESO7	840	98.1%	*Alicyclobacillus acidoterrestris* (AY686617.1)
ESO14	857	97.0%	*Alicyclobacillus acidoterrestris* (AJ133631.1)
ESO12	845	96.8%	*Alicyclobacillus acidoterrestris* (ABO42058.1)
ESO3	823	98.6%	*Alicyclobacillus acidocaldarius* (ABO59665.1)

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CHAPTER 4

ISOLATION, IDENTIFICATION AND TYPIFICATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* AND *ALICYCLOBACILLUS ACIDOCALDARIUS* STRAINS FROM ORCHARD SOIL AND THE FRUIT PROCESSING ENVIRONMENT IN SOUTH AFRICA

Abstract

Alicyclobacillus acidoterrestris and *A. acidocaldarius* are thermo-acidophilic, non-pathogenic, spore-forming bacteria that can survive the typical heat processing of fruit juices and concentrates. Bacterial endospores then germinate, grow and cause spoilage of acid food products. Species of *Alicyclobacillus* were isolated from orchard soil and a fruit concentrate production factory in South Africa. Preliminary identification of the isolates was based on morphological, biochemical and physiological properties. Identification at species level was done by PCR amplification using genus-specific primers and 16S ribosomal RNA (rRNA) gene sequencing. The majority of isolates belonged to the species *A. acidoterrestris,* but *A. acidocaldarius* was also isolated and identified. As far as we could determine, this is the first report of the isolation of *A. acidoterrestris* from wash water and soil outside a fruit processing plant, as well as the isolation of *A. acidocaldarius* from vinegar flies. The genotypic relatedness between strains of *A. acidoterrestris* and between strains of *A. acidocaldarius* was determined by RAPD-PCR. Sixteen isolates identified as *A. acidoterrestris* grouped into four clusters based on RAPD-PCR banding patterns, suggesting that they belong to at least four genotypic groups. Three isolates identified as *A. acidocaldarius* gave three unique banding patterns.

Introduction

The genus *Alicyclobacillus* consists of a group of thermo-acidophilic, strictly aerobic, heterotrophic, endospore-forming bacteria (Wisotskey *et al.*, 1992; Walls & Chuyate, 1998). Initially, these bacteria were members of the genus *Bacillus*, however, in 1992

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they were allocated to a new genus, *Alicyclobacillus* based on 16S ribosomal RNA (rRNA) gene comparative sequence analysis and the presence of unusual ω-alicyclic fatty acids in their cell membrane (Wizotskey *et al.*, 1992). They were first isolated by Darland and Brock (1971) from various acidic thermal environments in the United States. Subsequently similar acidophilic spore-formers were isolated from other environmental sources, including garden soil, organic compost, and fruit and heat-processed foods (Deinhard *et al.*, 1987; Yamazaki *et al.*, 1996; Pettipher *et al.*, 1997; Albuquerque *et al.*, 2000; Jensen, 2000; Walls & Chuyate, 2000; Goto *et al.*, 2002; Matsubara *et al.*, 2002). Of the currently nineteen recognised species of *Alicyclobacillus*, three have been isolated from spoilt fruit and vegetable juice products, namely *Alicyclobacillus acidoterrestris*, *Alicyclobacillus acidocaldarius* and *Alicyclobacillus pomorum* (Goto *et al.*, 2003; Jensen & Whitfield, 2003; Gouws *et al.*, 2005).

Alicyclobacillus spp. survive thermal acidic conditions in the form of endospores and it is these highly heat resistant spores that can survive the typical pasteurisation regimes applied during juice manufacturing. In single strength juice these micro-organisms find a favorable environment for germination and growth that, under certain conditions, can lead to product deterioration (Chang & Kang, 2004). The first instance of spoilage reported to be caused by members of the genus *Alicyclobacillus* occurred in 1984 in aseptically packaged apple juice in Germany. The impact of these bacteria in fruit juices was not acknowledged until more than a decade later when numerous reports of *A. acidoterrestris* spoilage were reported (Walls, 1994; Yamazaki *et al.*, 1996a; Pettipher *et al.*, 1997; Walls & Chuyate, 1998; Pettipher & Osmundson, 2000). Spoilage caused by *Alicyclobacillus* has to date been reported in apple, pear, orange, peach, mango and white grape juice, as well as in fruit juice blends, fruit juice containing drinks and tomato products, such as tomato juice and canned tomatoes (Borlinghaus & Engel, 1997; Chang & Kang, 2004; Gouws *et al.*, 2005).

Spoilage of fruit juice products by *A. acidoterrestris* is difficult to detect. It causes some clarified fruit juices to have a light sediment, cloudiness or haze. However, the main spoilage characteristic is a medicinal or phenolic off-flavour or odour, caused by guaiacol (Yamazaki *et al.*, 1996), 2,6-dibromophenol (Borlinghaus & Engel, 1997) or 2,6-dichlorophenol (Jensen & Whitfield, 2003).

Since members of the genus *Alicyclobacillus* are soil-borne organisms, it is thought that the source of contamination of the fruit juice is from the harvested, contaminated fresh fruit, which is introduced during processing without proper cleaning

(McIntyre *et al*., 1995; Pontius *et al*., 1998; Orr & Beuchat, 2000). Our interest in the presence of species of *Alicyclobacillus* in the fruit concentrate production environment is to investigate a potential source of contamination of the final product. In this study species of *Alicyclobacillus* were isolated from orchard soil, the factory environment and the fruit concentrate. The orchard soil was from orchards supplying the fruit concentrate manufacturing facility with fruit. The isolates were identified to species level based on 16S rRNA gene sequence analyses and typified using RAPD-PCR.

Materials and methods

Sampling sites

Samples were collected from a Hazard Analysis Critical Control Point (HACCP) accredited fruit processing facility in the Western Cape region of South Africa. This facility processes pear, peach, apricot and apples into fruit concentrate. A simplified and generalised representation of fruit puree manufacturing at this plant is shown in Fig. 1. Samples were taken from: 1) orchard soil and soil on the fruit processing premises; 2) fruit crates; 3) fruit prior to processing; 4) vinegar flies; 5) wash water; 6) water from the flume; 7) pulp/puree; 8) evaporator; 9) factory dust/debris; and 10) final product.

Topsoil was collected from under and around the trees of 4 different pear orchards which supply the factory with fruit. Orchards were located in Montaque and Barrydale in the Western Cape, South Africa. Topsoil was also collected from 4 areas in the immediate vicinity of the fruit processing factory. Composite samples of soil and plant debris were collected from the bottom of 6 different plastic crates used to transport fruit to the factory. The surfaces of pears stored in the processing plant prior to processing were sampled. Vinegar flies, identified as *Drosophila simulans* Stuvervant, were collected from inside the fruit storage area of the manufacturing plant. Vinegar flies were collected by passing a sterile bag through swarming flies. Water samples were collected from the bottom of the wash water reservoir, which stores recycled water after the fruit are washed prior to mashing. Pear skin from the press was sampled and fresh pear puree before concentration was sampled. Water was also taken from the evaporator inlet, which consists of condensed water produced during the process of concentration of the juice. Debris consisting of fruit material and dust were collected from 4 different sites inside the factory. The final product, pasteurised pear concentrate at 32 °Brix, was sampled. All samples, except for the orchard soil, were collected on the

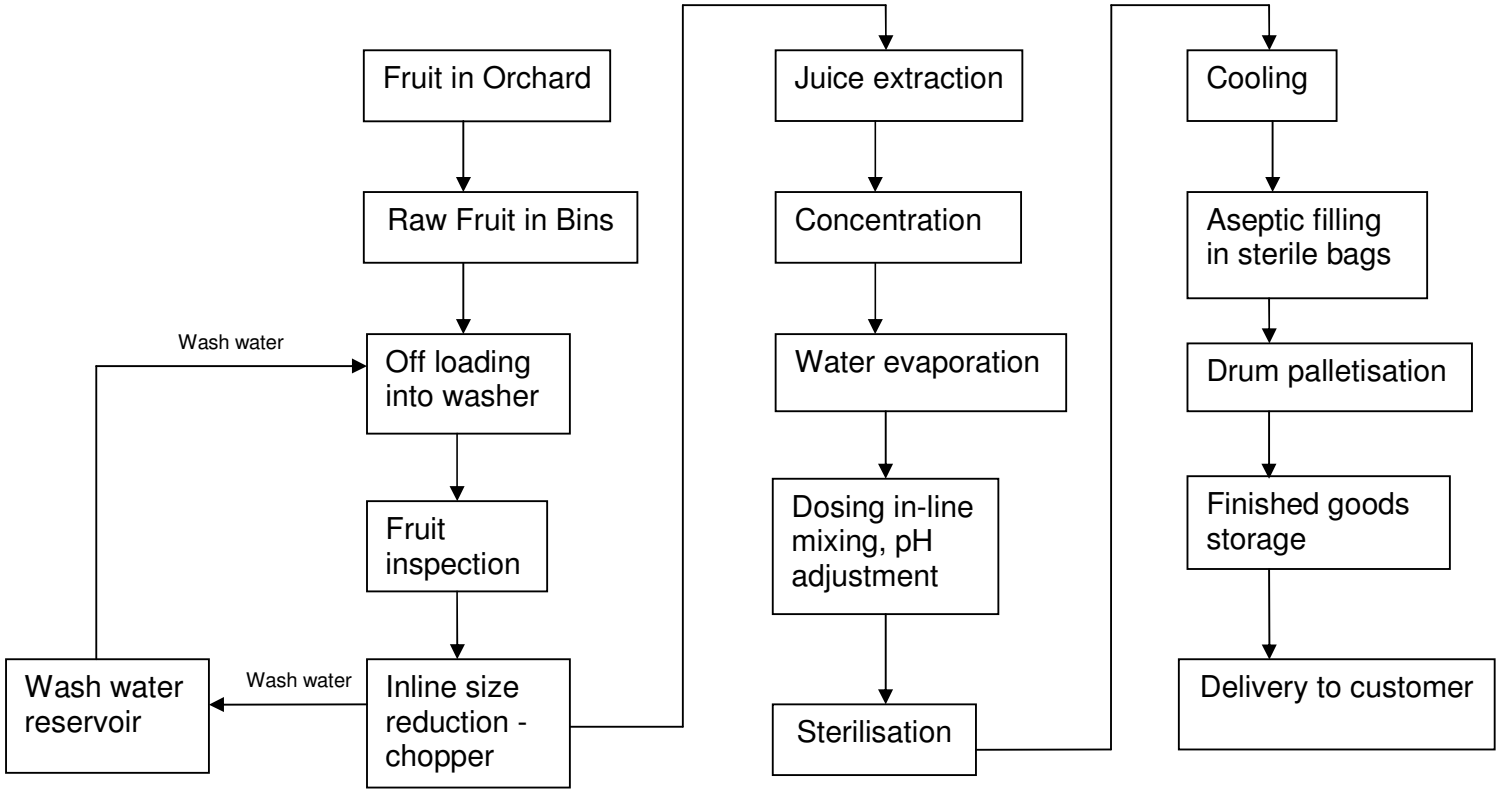

Figure 1 Fruit puree production

same day, stored in sterile sampling bags and processed within 12 to 24 h from the time of sampling. Orchard soil samples were collected and processed one week earlier.

Isolation

Approximately 20 g of top soil, debris or pear pulp were placed in sterile 50 mL centrifuge tubes. Samples were re-suspended in 30 mL sterile 1.5% (m/v) NaCl solution on a vortex for 3 min at 25 ℃. Large particles were then allowed to settle. Around 1 kg of fresh fruit was washed with 100 mL sterile 1.5% (m/v) NaCl solution by thumbling the sample material for 5 min. Around 150 vinegar flies were placed in a sterile 50 mL centrifuge tube with 2 mL sterile 1.5% (m/v) NaCl solution and glass beads (approximately 2 mm in diameter). The flies were homogenised for 3 min at 25 ℃ on a vortex.

Samples were given a heat treatment at 80˚C for 10 min to promote the germination of any alicyclobacilli spores and the elimination of vegetative cells (Walls & Chuyate, 2000). After the heat treatment aliquots of 1 mL of all samples were added to 10 mL Yeast Starch Glucose (YSG) broth prepared according to the according to the formula described by Matsubara *et al.*, (2002). The YSG broth was adjusted to a final pH of 4 using 1N tartaric acid and contained 100 µg mL^{-1} Delvocid (GistBrocades, Delf, Netherlands) to inhibit the growth of yeast and fungi. These were incubated at 45^{o}C for 24 h as an enrichment step. Dilution series of 10^{-1} to 10^{-6} were prepared in sterile distilled water and incorporated in triplicate by pour plating, into potato dextrose agar (PDA) (Chang & Kang, 2004) (Biolab Diagnostics, Midrand, South Africa), orange serum agar (OSA) (Chang & Kang, 2004) (Oxoid, Basingstoke, Hampshire, England), YSG agar (Matsubara *et al.*, 2002) and YSG agar supplemented with 20% (v/v) sterile filtered apple juice. All media contained 100 µg mL^{-1} Delvocid (GistBrocades). Tartaric acid (1N) was used to adjust all media after autoclaving to a final pH of 4. Plates were aerobically incubated at 55^{o}, 45^{o} and 40^{o}C and examined for growth after 72, 96 and 120 h.

Preliminary identification

Colonies were randomLy selected from plates containing between 20 and 300 colonies and re-streaked on corresponding media to obtain pure isolates. All isolates were stored at -80^{o}C in YSG broth (pH 4), supplemented with 30% (v/v) sterile glycerol.

Spore-formation was determined using phase-contrast microscopy, while Gram-reaction and oxidase and catalase activity were determined according to the methods described by Harrigan & McCance (1976). Gram-positive, oxidase-negative, catalase-positive and spore-forming rods were selected for further examination.

Genotypic methods

Isolates were grown for 4 d at 45^{o}C in YSG broth adjusted to pH 4. Their genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin) according to the manufacturer's instructions. For initial screening of the isolates their DNA was amplified using the primers CC16S-F (CGTAGTTCGGATTGCAGGG) and CC16S-R (GTGTTGCCGACTCTCGTG) (Conner *et al.*, 2004). This primer set amplified a 134 bp segment between bases 1254 and 1388 of the 16S rRNA gene.

PCR reactions were performed in a total volume of 25 µL containing 50 pmol of each primer, 1.25 U *Taq* DNA polymerase (Supertherm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 1 x PCR reaction buffer containing 2.5 mM $MgCl_2$, 1 µL of 99% (v/v) dimethyl sulphoxide (DMSO) (Merck), 0.4 mM deoxyribonucleoside triphosphate (dNTPs) (AB gene, supplied by Southern Cross Biotechnologies) and 2 µL of the extracted DNA. Thermal cycling for this PCR and all subsequent PCR reactions were done in an Eppendorf Mastercycler Personal (Eppendorf, Germany). An initial 3 min denaturation at 95ºC was followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s and elongation at 72ºC for 30 s, and a final 2 min chain elongation at 72ºC. *A. acidocaldarius* DSM 446^{T} and *A. acidoterrestris* DSM 3922^{T} obtained from the Deutsche SammLung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures) were included in each reaction as positive controls. Double distilled water instead of extracted DNA was used as a negative control.

Isolates with PCR fragments identical in size to the expected amplification products for the genus *Alicyclobacillus* were selected and their genomic DNA amplified using the primers 8f (5'-CAG GGA TCC AGA CTT TGA TYM TGG CTC AG-3') and 1512r (5'-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3') (Felske *et al.*, 1997). These primers amplify a 1.5 kilobase pair (kb) region of the 16S rRNA gene. The PCR reactions were performed in a total reaction volume of 25 µl containing 0.5 µM of each of the primers, 1 U *Taq* DNA polymerase (Supertherm), 1 × reaction buffer (Southern

Cross), 1 µL 99% (v/v) dimethyl sulphoxide (DMSO) (Merck), 0.5 mM dNTPs (Southern Cross) and 1 µl of the isolated DNA. All the PCR amplifications were initiated at 92˚C for 3 min. The samples were heated to 92˚C for 30 s for denaturation, followed by annealing at 54˚C for 30 s, while primer extension was performed at 72˚C for 1 min. These three steps were repeated for 35 cycles. A final elongation step at 72˚C for 7 min was included and the samples were cooled to 4˚C (Felske *et al.*, 1997).

The amplified fragments were purified using the High Pure PCR Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The fragments were sequenced using the ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA) at the DNA Sequencing Facility, Stellenbosch University and compared to sequences in GenBank using the BLAST search option.

Genomic DNA from all the isolates was amplified with RAPD primers BA-10 (5′-AACGCGCAAC-3′) and F-64 (5′-GCCGCGCCAGTA-3′) from Operon Technologies (Alameda, California, United States) (Yamazaki *et al.*, 1996). PCR reactions were performed in 25 µl volume reactions containing 1.5 ng of template DNA, 1 mM primer, 0.2 mM dNTPs (Southern Cross Biotechnologies), 2 mM $MgCl_2$, 2 U *Taq* DNA polymerase (Super-Therm), and 1 × reaction buffer supplied with the enzyme. Amplification products were separated on a 1.5% (m/v) agarose gel (Whitehead Scientific). A DNA molecular mass marker, O'Generuler™ DNA Ladder Mix (Fermentas, Maryland, United States) was used as a standard. *Alicylcobacillus acidocaldarius* DSM 446^T and *A. acidoterrestris* DSM 3922^T were used as reference strains.

Carbohydrate fermentations

Isolates showing different RAPD-PCR banding patterns were selected for carbohydrate fermentation testing. Carbohydrate fermentation reactions were recorded by using the API 50 CHB system (BioMerieux, Marcy L'Etoile, France) according to the manufacturer's instructions. Incubation of all API strips was at 45^{o}C, and results were recorded after 4 and 5 d.

pH determinations

Five grams of pulverized dry soil samples were mixed with distilled water in a soil: water ratio of 1:1 until the soil and water were in equilibrium. Suspended soil particles were allowed to settle and the pH was read using a pH meter by placing the electrodes in the

slurry, swirling gently and reading the pH immediately. The pH of the flume and wash water was measured by placing the electrodes directly in the water and reading the pH immediately.

Results and discussion

A diverse range of samples from the environment and processing plant were tested in order to provide comprehensive data on the occurrence of species of *Alicyclobacillus* at different stages of fruit processing. YSG plates incubated at 45 °C contained the highest amount of colony forming units compared to plates incubated at 55 °C and 40 °C and contained a higher amount of microbial cells than OSA and PDA plates. Nineteen spore-forming, Gram positive, oxidase-negative and catalase-positive rods were selected for further investigation. After DNA amplification with genus-specific primers all nineteen isolates produced a 134-bp fragment (data not shown), which was identical in size to that reported for *A. acidoterrestris* DSM 3922^T, *A. cycloheptanicus* ATCC 49029 and *A. acidocaldarius* ATCC 43030 (Connor *et al.*, 2005). Species of *Alicyclobacillus* were isolated from orchard soil from Barrydale, soil on the fruit processing premises, vinegar flies, wash water, flume water, pear skin from the press, debris from the factory floor, water from the evaporator inlet and the pear concentrate (Table 1). No *Alicyclobacillus* spp. were isolated from fruit surfaces and debris collected from inside the crates used to transport the fruit to the processing facility.

Between 778 and 1119 bases were sequenced for all isolates (Table 1). Sequencing of the 16S rRNA gene amplicons for the 19 isolates identified them as members of the genus *Alicyclobacillus* and comparison with nucleotide sequences in GenBank, revealed DNA homology between 98.5% and 99.4% to strains of *A. acidoterrestris* for 16 of the isolates (Table 1). The other three isolates yielded amplicons 98.9% to 99.0% homologous to the 16S rRNA gene of a strain of *A. acidocaldarius* (Table 1).

Carbohydrate fermentation reactions recorded for the 8 isolates identified as belonging to the species *A. acidoterrestris* by the use of DNA sequence analysis corresponded most closely to that of the type strain of *A. acidoterrestris* (Table 2). Variations in the fermentation of trehalose, D-turanose and xylitol were recorded (Table 1). Similar results have been reported for other strains of *A. acidoterrestris* (Chang & Kang, 2001; Silva & Gibbs, 2001; Goto *et al.*, 2001). None of the strains fermented

Table 1 Percentage similarity of isolates to species in the NCBI nucleotide sequence database, based on partial 16S rRNA gene sequence analysis.

Isolate	Source	Nearest phylogenetic neighbour (GenBank accession number)	No of nucleotides sequenced	% similarity	RAPD-PCR Clusters
FB-26	Soil outside factory	*A. acidoterrestris* (AB042058.1)	1035	99.2%	I
FB-14	Wash water	*A. acidoterrestris* (AB059676.1)	925	99.3%	II
FB-15	Wash water	*A. acidoterrestris* (AB059676.1)	816	99.2%	II
FB-17	Wash water	*A. acidoterrestris* (AB059676.1)	805	99.2%	II
FB-38	Water from flume	*A. acidoterrestris* (AB059675.1)	788	98.7%	III
FB-39	Water from flume	*A. acidoterrestris* (AB059675.1)	958	98.5%	III
FB-21	Pear skin from press	*A. acidoterrestris* (AJ133631.1)	820	98.5%	IV
FB-22	Pear skin from press	*A. acidoterrestris* (AJ133631.1)	802	98.6%	IV
FB-35	Water from evaporator inlet	*A. acidoterrestris* (AJ133631.1)	963	98.9%	IV
FB-32	Water from evaporator inlet	*A. acidoterrestris* (AJ133631.1)	778	98.8%	IV
FB-41	Debris from factory floor	*A. acidoterrestris* (AB059675.1)	795	98.7%	III
FB-2	Pear concentrate	*A. acidoterrestris* (AB042058.1)	784	99.2%	I
FB-5	Pear concentrate	*A. acidoterrestris* (AB042058.1)	848	99.4%	I
FB-7	Pear concentrate	*A. acidoterrestris* (AB059676.1)	874	99.3%	II
FB-11A	Pear concentrate	*A. acidoterrestris* (AB042058.1)	1119	99.3%	I
FB-13	Pear concentrate	*A. acidoterrestris* (AB059676.1)	1102	99.1%	II
FB-28	Orchard soil from Barrydale	*A. acidocaldarius* (AB042056.1)	1119	99.0%	
FB-19	Vinegar flies	*A. acidocaldarius* (AB042056.1)	1057	98.9%	
FB-1	Pre-pasteurized pear puree	*A. acidocaldarius* (AB042056.1)	821	98.9%	

Table 2 Differential carbohydrate fermentation reactions of *Alicyclobacillus* spp. collected from the fruit processing environment in the Western Cape, South Africa

Isolate (RAPD group)	Glycerol	Erythritol	Ribose	Adonitol	L-sorbose	Rhamnose	Trehalose	Inositol	Sorbitol	α-methyl-D-mannoside	Amygdalin	Salicin	melibiose	Melezitose	D-raffinose	Starch	Glycogen	Xylitol	β-gentiobiose	D-turanose	D-arabitol
A.acidoterrestris DSM 3922[Ta]	+	+	+	+	-	+	-	+	+	+	+	+	-	+	-	-	-	+	+	+	+
FB-2(I)	+	+	-	-	-	+	+	d	+	+	+	+	-	+	+	-	-	-	+	d	+
FB-11A(I)	+	+	-	-	-	+	-	d	+	+	+	+	-	+	+	-	-	-	+	d	+
FB-14(II)	+	+	-	-	-	+	-	d	+	+	+	+	-	+	+	-	-	+	+	d	+
FB-15(II)	+	+	-	-	-	+	-	d	+	+	+	+	-	+	+	-	-	+	+	d	+
FB-38(III)	+	+	-	-	-	+	-	d	+	+	+	+	-	+	+	d	-	d	+	d	+
FB-39(III)	+	+	-	-	-	+	-	d	+	+	+	+	-	+	-	-	-	-	+	+	+
FB-21(IV)	+	+	+	-	-	+	-	d	+	+	+	+	-	+	-	-	-	-	+	+	+
FB-32(IV)	+	+	-	-	-	+	-	d	+	+	+	+	-	+	-	-	-	-	+	+	+
A.acidocaldarius DSM 446[Ta]	+	-	+	-	+	+	-	-	-	-	-	-	+	-	+	+	+	-	-	+	+
FB-1	-	-	-	-	+	-	-	-	-	-	d	+	+	+	d	+	-	-	+	+	-
FB-28	-	-	-	-	-	-	-	-	-	-	d	+	+	+	d	+	-	-	-	+	-
FB-19	-	-	-	-	+	-	-	-	-	-	d	+	+	+	d	+	-	-	-	+	-

+, positive reaction; -, negative reaction; d, variable reaction. All strains fermented: aesculin, L-arabinose, arbutin, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, mannitol, D-mannose, α-methyl-D-glucoside, sucrose, trehalose and D-xylose. None of the strains fermented: *N*-acetyl-glucosamine, D-arabinose, L-arabitol, dulcitol, D-fucose, L-fucose, gluconate, inulin, 2-keto-gluconate, 5-keto-gluconate, β-methyl-xyloside, D-tagatose, D-xylose and L-xylose.

[a] Data from Goto *et al.* (2003).

ribose and adonitol, which is characteristic for the type strain of *A. acidoterrestris* DSM 3922^T (Goto *et al*., 2001).

The carbohydrate fermentation pattern of isolates FB-1, FB-28 and FB-19 corresponded most closely to that of *A. acidocaldarius* DSM 446^T (Table 2). These three isolates were previously classified as strains of *A. acidocaldarius* according to DNA sequence analysis. For all strains erythritol was unable to be assimilated, which is a characteristic of *A. acidocaldarius* (Goto *et al*., 2001).

Confirmation of genotypic diversity was provided by RAPD-PCR, which grouped the 16 isolates identified as *A. acidoterrestris* into 4 genotypically well-separated clusters. The profiles generated by RAPD-PCR with the two primers were composed of three to eight bands for both primers (Fig. 2). All isolates, as well as the type strains for *A. acidoterrestris* and *A. acidocaldarius* showed amplification with the primers used. The RAPD-PCR profiles of all strains differed from that of the type strain of *A. acidoterrestris*, which did not group in any of the four RAPD-PCR clusters (Fig. 2). Isolates in clusters I were isolated from pear concentrate and soil outside of the factory. The identical banding patterns obtained for the isolates indicate that soil in the vicinity of the factory, which could be carried into the processing facility by employees or through windborne action, is a potential source of contamination for the final fruit juice, concentrate or pulp. The factory soil had a pH of 6.67 which was similar to that of the orchard soil from Barrydale which had a pH of 6.52. These results confirm the ability of cells *A. acidoterrestris* and *A. acidocaldarius* or at least spores of these species to survive in soil which are slightly acidic to neutral (Groenewald *et al*., 2008). Isolates in cluster II were isolated from pear concentrate and wash water. The pH of the wash water was 5.84. Wash water could also act as a potential reservoir of *A. acidoterrestris* which results in the contamination of the final product. Wash water is used during the production of fruit to wash the fruit, removing dust, soil and any foreign objects from the fruit immediately prior to the pulping of the fruit. This water is conserved by recycling it during the manufacturing process (Fig. 1). The presence of strains of *A. acidoterrestris* displaying identical banding patterns isolated from the factory environment before pasteurisation and in the pear concentrate confirms the ability of strains of *A. acidoterrestris* to survive the commercial pasteurisation process.

Cluster III contained two strains from flume water and one strain isolated from debris found on the factory floor. Flume water had a pH of 5.91. During the movement of fruit along the flume, water is spilled onto the floor of the processing plant leading to

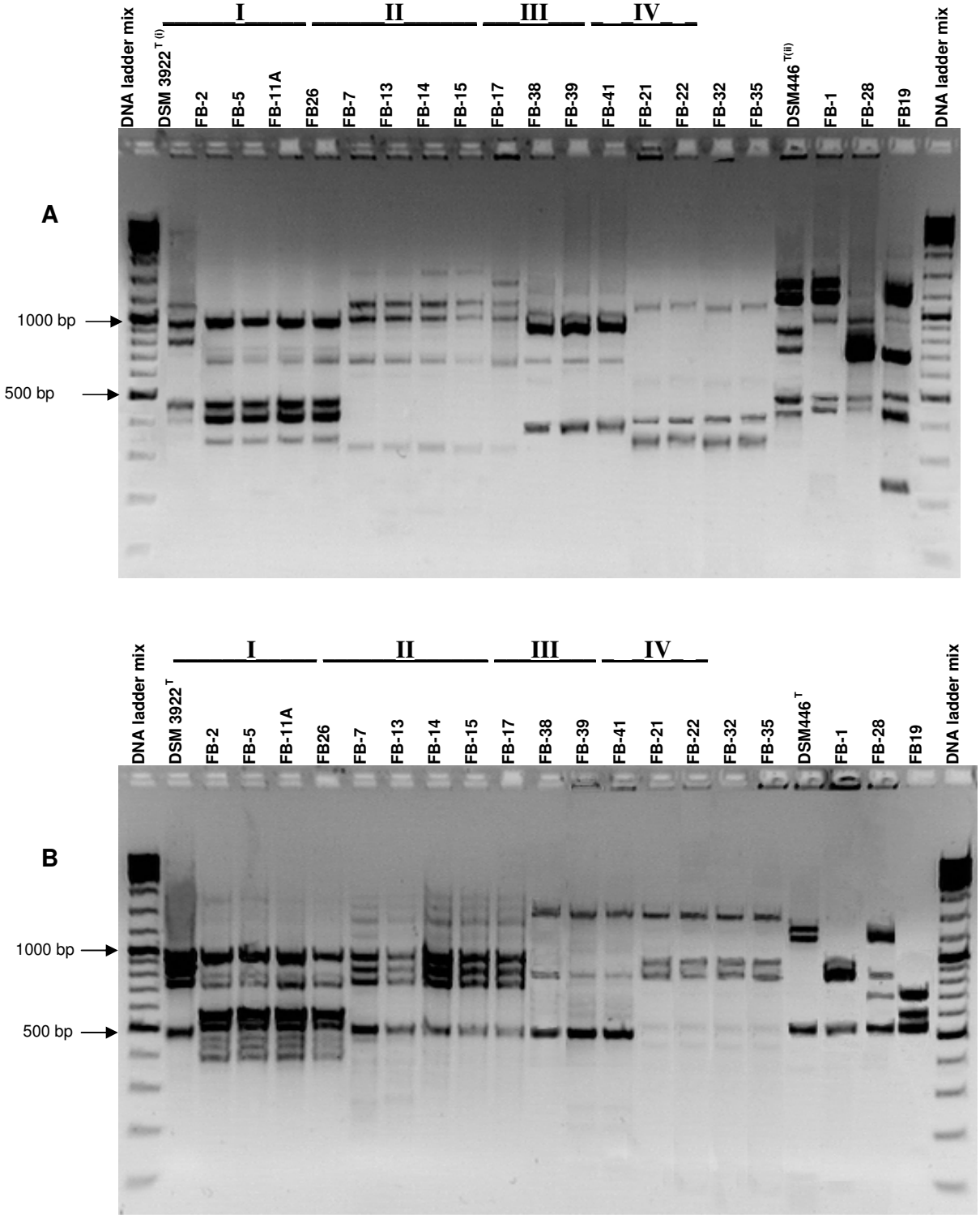

Figure 2 DNA fragments obtained after RAPD-PCR of the genomic DNA of isolates identified as *A. acidoterrestris* and *A. acidocaldarius*. Isolates identified as *A. acidoterrestris* are in groups labelled I, II, III and IV, while isolates FB1, FB28 and FB19 were identified as *A. acidocaldarius* by 16S RNA gene sequence analysis. Lanes 1 and 23 consist of the DNA molecular mass marker O'Generuler™ DNA Ladder Mix. Lane 2: A. *acidoterrestris* DSM 3922^{T}; Lane 19: *Alicylcobacillus acidocaldarius* DSM 446^{T}. A: Primer BA-10 (5′-AACGCGCAAC-3′); B: Primer F-64 (5′-GCCGCGCCAGTA-3′)

possible contamination of the floor surface area. Water from the evaporator inlet and and pear skin from the press provided isolates that grouped into genetically distinct cluster IV (Fig. 2). Inside the evaporator, vacuum and heat are used to remove excess water in order to obtain a base concentrate of 32 °Brix.

Isolates FB1, FB28 and FB19 identified as *A. acidocaldarius* by DNA sequence analysis gave unique RAPD banding patterns, suggesting they do not represent a homogeneous collection, despite their almost identical 16S rRNA sequence homologies (Table 1). Furthermore, the RAPD-PCR profiles of these strains differed from that of the type strain *A. acidocaldarius* (Fig. 2). *Alicyclobacillus acidocaldarius* does not produce the taint chemical guaiacol; however, its isolation from spoilt fruit juice suggests a possible role in the spoilage of fruit juice (Gouws *et al.*, 2005).

Conclusions

As far as we are aware this is the first report on the isolation of *A. acidoterrestris* from wash water, water from the evaporator inlet, soil outside of fruit concentrate factory and flume water. Strains of *A. acidocaldarius* were isolated from pre-pasteurised pear puree, orchard soil and for the first time to our knowledge, from vinegar flies. Further research is needed to establish the role played by these specific strains of *A. acidoterrestris* and *A. acidocaldarius* in the spoilage of fruit juice. The widespread occurrence of strains of *A. acidoterrestris* in the fruit concentrate manufacturing environment suggests that good manufacturing practices play an essential role in controlling instances of spoilage caused by these bacteria.

Acknowledgments

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CHAPTER 5

THE USE OF ULTRAVIOLET RADIATION AS A NON-THERMAL TREATMENT FOR THE INACTIVATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* SPORES IN WATER, WASH WATER FROM A FRUIT PROCESSING PLANT AND GRAPE JUICE CONCENTRATE

Abstract

Alicyclobacillus acidoterrestris is a non-pathogenic, spore-forming bacterium that can survive the commercial pasteurisation processes commonly used during fruit juice production. Surviving bacterial endospores germinate, grow and cause spoilage of high acid food products. Fruit juices can be treated using ultraviolet (UV-C) with a wavelength of 254 nm, which has a germicidal effect against micro-organisms. In this study *A. acidoterrestris* was inoculated into water, used wash water from a fruit processing plant and grape juice concentrate. Ultraviolet dosage levels (J L^{-1}) of 0, 61, 122, 183, 244, 305 and 367 were applied using a novel UV-C turbulent flow system. The UV treatment method was shown to reliably achieve in excess of a 4 $\log_{10}$ reduction (99.99%) per 0.5 kJ L^{-1} of UV-C dosage in all the liquids inoculated with *A. acidoterrestris*. The applied novel UV technology could serve as an alternative to thermal treatments of fruit juices for the inactivation of *Alicyclobacillus* spores or in the treatment of contaminated processing wash water.

Introduction

Alicyclobacillus acidoterrestris is a Gram-positive, thermo-acidophilic, non-pathogenic, spore-forming bacterium that has been isolated and identified in spoiled commercial pasteurised fruit juices (Wisotskey *et al*., 1992; Walls & Chuyate, 1998; Silva & Gibbs, 2001). This bacterium was firstly isolated by Hippchen *et al*. (1981) from a variety of different soils such as garden soil, oak wood soil, woodland soil and in moor lands. *Alicyclobacillus acidoterrestris* has subsequently been found in a range of habitats and substrates, including organic composting, manure, crop fields, orchards, heat-processed foods such as fruit juice concentrate, and the fruit juice concentrate processing environment (Deinhard *et al*., 1987; Yamazaki *et al*. 1996; Pettipher *et al*.,

1997; Albuquerque *et al*., 2000; Walls & Chuyate, 2000; Goto *et al*., 2002; Matsubara *et al*., 2002; Groenewald *et al*., 2008, 2009).

The threat that *A. acidoterrestris* pose to the fruit juice industry is the ability of its spores to survive thermal pasteurisation and hot-fill and hold pasteurisation processes used during fruit processing and fruit juice production (Splittstoesser *et al*., 1994; Eiroa *et al*., 1999; Orr & Beuchat, 2000). This heat resistance was observed by Splittstoesser *et al*. (1994) who reported D-values for *A. acidoterrestris* spores of 23 min at 90 ℃ and 2.4 to 2.8 min at 95 ℃, suggesting that spores survive the juice pasteurisation process of 88° to 96 ℃ for 30 s to 2 min. In fact, pasteurisation serves as a heat treatment that stimulates germination of the spores. The resulting growth of *A. acidoterrestris* at the low pH (3-3.5) typically found in fruit juice may lead to spoilage (Splittstoesser *et al*., 1998; Eiora *et al*., 1999; Gouws *et al*., 2005).

Spoilage caused by *A. acidoterrestris* has to date been reported in apple, pear, orange, peach, mango and white grape juice, as well as in fruit juice blends, fruit juice containing drinks and tomato products, such as tomato juice and canned tomatoes (Borlinghaus & Engel, 1997; Chang & Kang, 2004). Spoilage caused by this bacterium is difficult to detect visually. The spoiled juice appears normal, or might have a light sediment with no gas formation. Often, the only evidence of spoilage is apparent as a medicinal/phenolic off-flavour (Walls & Chuyate, 1998; Jensen, 1999). The chemicals responsible for this off-odour was identified as guaiacol (2-methoxyphenol) and other halophenols such as 2,6-dichlorophenol (2,6-DCP) and 2,6-dibromophenol (2,6-DBP). Guaiacol can be detected by smell in fruit juices at 2 ppb and was detected in orange and apple juices in the presence of around 5 log CFU mL^{-1} of *A. acidoterrestris* cells (Gocmen *et al*., 2005).

Fruit juice contamination results from unwashed or poorly washed raw fruit that is processed, as well as contaminated water used during the production of fruit juices (Pontius *et al*., 1998; Orr & Beuchat, 2000; McIntyre *et al*., 1995; Groenewald *et al*., 2009). Due to their thermo-acidophilic properties and their occurrence in several spoiled pasteurised products, Silva *et al*. (1999) recommended *A. acidoterrestris* spores as the target microbe for the pasteurisation of high acidic food products. Subsequently, the fruit juice industry acknowledges *A. acidoterrestris* as an important target micro-organism that must be managed by an effective quality control program during the production of fruit juices and fruit juice concentrates.

Ultraviolet (UV) light is one of a number of non-thermal technologies currently being used in food processing, together with pulsed electric fields, high-pressure

processing and ultrasound. These alternative technologies can deliver food products that do not contain spoilage or pathogenic micro-organisms and enzymes that may decrease the nutritional and sensory characteristics of foods (Butz & Tauscher, 2002).

UV wavelengths of between 220 and 300 nm are considered germicidal against micro-organisms such as bacteria, viruses, protozoa, fungi and algae (Sizer & Balasubramaniam, 1999; Bintsis *et al*., 2000). The highest germicidal effect is obtained between 250 and 270 nm, decreasing as the wavelength is increased. Above 300 nm the germicidal effect of UV light is annulled. Therefore, a wavelength of 254 nm is used for disinfection of surfaces, water and some food products (Guerrero-Beltrán & Barbosa-Cánovas, 2004).

Liquids such as water and fruit juices have been successfully treated with UV light to reduce bacterial counts (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Keyser *et al*., 2008). The efficacy of the microbial reduction in fruit juices by UV-C light at 254 nm depends on a number of factors. These include the organisms (including different strains) present in the liquid, the contamination level, the % UV transmittance of the liquid (opaqueness) and suspended particles in the liquid. It is known that the penetration depth of UV-C light through the surface of liquids is very short, with the exception of clear water (Shama, 1999). The penetration of UV light into juices is about 1 mm for absorption of 90% of the light (Sizer & Balasubramaniam, 1999). Greater amounts of soluble and insoluble solids lower the intensity of penetration of the UV-C light (Shama, 1999; Bintsis *et al*., 2000). For these reasons a turbulent flow during liquid food processing is recommended and is a legal requirement by the USA Food and Drug Administration (US FDA, 2001; Keyser *et al*., 2008). The objective of this study was to determine the reduction of *A. acidoterrestris* spores inoculated in water, fruit concentrate factory wash water and 80 °Brix grape juice concentrate using a novel UV treatment system.

Materials and methods

Novel pilot-scale UV system

The UV reactor system was designed and manufactured by SurePure, Milnerton, South Africa. The UV reactor (Fig. 1) consists of NW100 stainless steel inlet and outlet chambers with a stainless steel corrugated spiral tube between the chambers. Inside

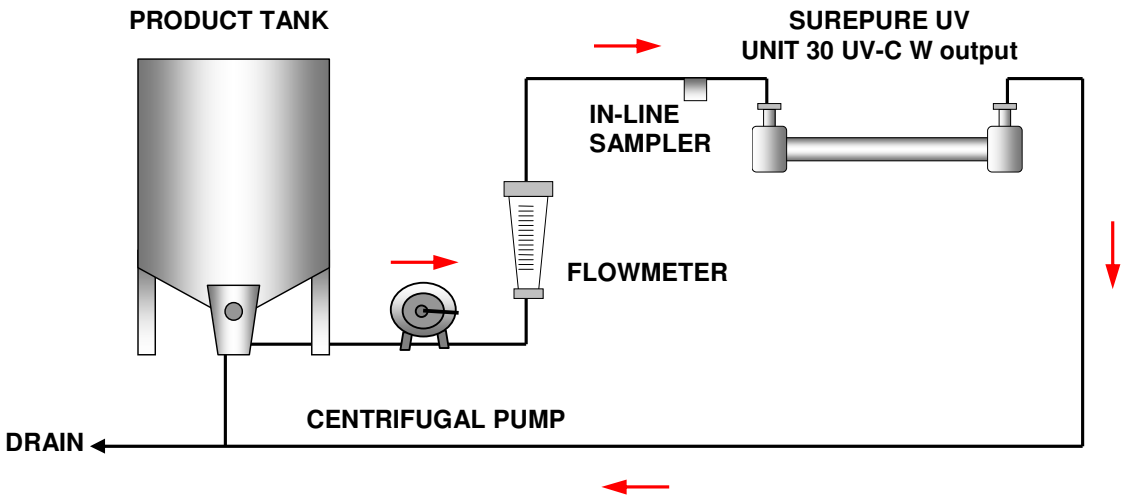

Figure 1 Schematic representation of the novel pilot-scale UV treatment system containing one UV-C lamp.

the spiral tube is a low pressure mercury UV lamp (30 UV-C Watt, 90% 254 nm and 90% emittance) which is protected by a quartz sleeve. The liquid flows between the corrugated spiral tube and the quartz sleeve. The tangential inlet of the reactor creates a high velocity and turbulence in the inlet chamber which helps to prevent clumping of micro-organisms and assists in the efficiency of UV radiation by increasing the exposure of the liquid to the light. From the inlet chamber the liquid is forced into the actual reactor, the space between the quartz sleeve and the corrugated spiral tubing. The corrugation of the tubing creates very high turbulence which is then carried with the spiral over the length of the reactor chamber. At flow rates (Fr) above 2800 L h^{-1} the Reynolds value is calculated to be more than 4000, indicating turbulent flow patterns. The UV reactor operates at a flow rate capacity of between 3800 and 4200 L h^{-1}.

The time needed for a UV treatment depends on the quantity of product to be treated and the flow rate of the product feed. The design of the pilot-scale batch system (Fig. 1) used consists of only one UV lamp. This unit was used for the treatment of 20 L batches of liquid which was circulated at a flow rate of 4000 L h^{-1}. The time for the liquid to pass through the system once was 18 s, therefore, delivering a UV-C dose of 22.97 J L^{-1} to the liquid being treated after one passage. The contact time or retention time was determined theoretically, assuming that the system was in steady state operation with uniform product and product flow, and that the liquid was non-expandable and non-volatile.

Cleaning of the units

The pilot-scale unit was cleaned after every treatment using standard 'Cleaning In Place' (CIP) processes. The equipment was rinsed with warm water (50 °C) for 10 min, where after a 1.0% alkaline solution was circulated for 30 min at 75 °C, followed by a warm water rinse at 50 °C for 5 min. Finally, a 0.5% Perasan solution (Divosan System, Johnson Diversey, South Africa) was circulated for 10 min before a final rinse with cold water.

Dosage measurement

UV light was used initially to disinfect surfaces, therefore the irradiance is generally expressed as watts per square centimetre (W cm^{-2}), whilst the radiant exposure (dosage) is expressed as watts per second per square centimeter (W s cm^{-2}) or joules

per square centimeter ($J.cm^{-2}$) and characterises the energy delivered per surface area of the treatment device (Matak *et al.,* 2005). UV dosage (D) is, therefore, determined as time (T) multiplied by irradiance (I). As the UV-C energy penetrates into the medium, therefore working with volume rather than area, Keyser *et al*. (2007) proposed an alternative method to characterize UV as dosage per volume of liquid. For liquids, the UV dosage was expressed as $J\ L^{-1}$. A comparison between UV-C dosage as $J\ L^{-1}$ and $W.cm^{-2}$ was then determined (Table 1) by calculating the dose per area as well as the dose per volume, together with time of UV-C exposure.

UV dosage per area

The length of the quartz sleeve used was 0.860 m, with an outer surface area (As) of 661.93 cm^2. The area between the quartz sleeve and corrugated spiral tubing is termed the annulus and the volume thereof was determined as being 0.675 L or 0.00068 m^3. The effective area (As) of UV-C is at a distance of 5 mm for the as the lamp is 5 mm away from the outer surface of the sleeve. According to the manufacturers, the energy transmission rate (total UV-C output) to the constant surface of the quartz sleeve (As = 661.93 cm^2) from the UV lamp is 25.5W (watts) UV-C. Ignoring the volume of the annulus and disregarding the type of product in the annulus the following calculations is based on the effective A_s of the quartz sleeve alone. The following calculations are based on the A_s of the quartz sleeve alone, not taking into account the volume of the annulus and the type of liquid treated. The intensity (I) per reactor can be calculated as follow:

The retention time (T) of the product per reactor can be calculated as follow:

Retention time (T) = Volume of the reactor (L) / Flow rate $L\ h^{-1}$
= 0:675 l/4 000 $L\ h^{-1}$
= 0:675 l/1:111 $L\ s^{-1}$
= 0:608 s

Thus, at a flow rate (Fr) of 4000 $L\ h^{-1}$ the product retention time (T) is 0.608 s per reactor, therefore the UV dosage (D) per surface area for one reactor with continuous flow is calculated as follows:

Table 1 The log_{10} microbial reduction of *A. acidoterrestris* (CFU mL^{-1}) (average value calculated from 4 repetitions) at a starting concentration of around 5 x 10^5 CFU mL^{-1} in inoculated tap water, used wash water from a fruit processing plant and 80 °Brix grape juice concentrate after UV (J L^{-1}) treatment

Treated medium	Log_{10} *A. acidoterrestris* reduction						
	Applied UV dosages						
	0	**61**	**122**	**183**	**244**	**305**	**367**
Inoculated tap water	0.00	1.04 (0.89-1.28)	2.15 (1.96-2.31)	3.15 (2.96-3.29)	4.32 (4.06-4.29)	5.13 (4.99-5.23)	5.13 (4.99-5.23)
Inoculated used wash water	0.00	0.925 (0.43-1.13)	1.84 (1.43-2.21)	2.83 (2.59-3.03)	3.65 (3.49-3.95)	4.49 (4.29-4.91)	5.19 (5.11-5.27)
Inoculated 80 °Brix grape juice concentrate	0.00	0.76 (0.60-0.85)	1.85 (1.51-2.14)	2.59 (2.55-2.66)	3.59 (3.43-3.71)	3.97 (3.73-4.24)	4.61 (4.56-4.64)

The values given are averages (n = 4); values in parentheses are the minimum and maximum values of four samples.

Dosage = Intensity (I) × Time (T)
= 38:50 Mw cm^{-2} × 0:608 s
= 23:408 mW s cm^{-2}
= 23:408 mJ cm^{-2}

UV dosage per volume

At a flow rate (Fr) of 4000 L h^{-1} the product retention time (T) is 0.608 s per reactor (as calculated in paragraph 2.3.1), therefore the UV dosage per L of liquid treated for one reactor with continuous flow is calculated as follows:

Dosage = Total UV-C output per unit (W) / Flow rate (L s^{-1})
= 25.50 W / 1.11 L s^{-1}
= 25.50 J.s^{-1} / 1.11 L s^{-1}
= 22:972 J L^{-1}

Growth of *Alicyclobacillus acidoterrestris*

Alicyclobacillus acidoterrestris K47 (Witthuhn *et al*., 2007), a strain isolated from spoilt grape juice, was grown in 2 L yeast starch glucose (YSG) (Matsubara *et al*., 2002) broth adjusted with tartaric acid (1N) (Saarchem, Krugersdorp, South Africa) to a final pH of 4 and incubated at 45 °C for 5 d. This culture was then heat treated at 80˚C for 10 min to promote the germination of any alicyclobacilli spores and eliminate vegetative cells (Walls & Chuyate, 2000) before being used as an inoculum. *Alicyclobacillus acidoterrestris* were inoculated by the addition of the whole pellet into either water, used wash water from a fruit processing plant or 80 °Brix grape juice concentrate. A final concentration of approximately 5 x 10^5 CFU mL^{-1}, as determined by sampling at time 0, was obtained.

UV-C processing of wash water and water

Used wash water was obtained from a Hazard Analysis Critical Control Point (HACCP) accredited fruit processing facility in the Western Cape region of South Africa and kept at 22 °C. The wash water, containing foliage and dust, was used to wash off fruit debris and dust before processing and was recycled several times. Wash water and the tap

water were inoculated with around 5 log CFU mL^{-1} *A. acidoterrestris* K47 and processed in a similar way to grape concentrate except that these liquids were processed at 22 °C. All the UV-C treatments were done in quadruplicate.

UV-C processing of 80 °Brix grape juice concentrate

Grape juice 80 °Brix concentration was received from a concentrate manufacturer in the Western Cape, South Africa and kept at 4 – 8 °C. A sample volume of 20 L 80 °Brix grape juice concentrate was inoculated with *A. acidoterrestris* K47 as previously described and placed into the holding tank of the pilot UV treatment unit. To achieve a flow rate of 4000 L h^{-1} in the unit a speed controlled sanitary Prolac centrifugal pump (Inoxpa, Brackenfell, South Africa) was used. The concentrate was treated at 4 – 8 °C, and due to the short contact time, no heat transfer from the lamps to the concentrate was recorded after processing. Samples were subjected to UV dosages of 0, 61, 122, 183, 244, 305 and 367 J L^{-1}. After each dosage, a 50 mL sample was taken aseptically using an in-line sampler. The concentrate was extracted from the flow stream without halting the process in order to avoid excessive UV-C exposure of the grape juice concentrate. Microbiological analyses were performed on each 50 mL sample within 24 h. All the UV-C treatments were done in quadruplicate.

pH determinations

Suspended particles in the wash water were allowed to settle and the pH was measured using a HI 221 pH meter (Hanna Instruments, Bedfordshire, United Kingdom).

Microbiological analysis

A 100 µL sample of concentrate, tap water or wash water was aseptically transferred to 900 µL sterile distilled water and mixed thoroughly. Serial dilutions of the samples were then prepared (10^{-1}–10^{-6}) and 100 µL of each of the different sample dilutions were plated in triplicate onto YSG agar. Tartaric acid (1N) (Saarchem) was used to adjust the YSG agar after autoclaving to a final pH of 4. Plates were incubated aerobically at 45 °C and examined for growth after 96 h. The results obtained were expressed as colony forming units per milliliter (CFU mL^{-1}).

Statistical analysis

All statistical analyses were performed using Statistica™ 7.1 (StatSoft, Inc., 2006). A two way cross classification of the log CFU mL^{-1} on UV-C dosage and treated medium was carried out. Since these interaction was highly significant ($F_{12,63}$ = 5.31) with a P-value of 0.000004, the interactions between the treated media (water, wash water and grape juice concentrate) and UV-C dosage were investigated. As the residuals were not normally distributed, a Bootstrap multiple comparison was performed on the interactions.

Results and discussion

UV radiation was successfully applied to reduce *A. acidoterrestris* spores inoculated into tap water, used wash water from a fruit concentrate manufacturing facility and 80 ˚Brix grape juice concentrate (Table 1). In water inoculated with *A. acidoterrestris* spores a 5.3 log_{10} reduction of the alicyclobacilli was achieved after a UV dosage of only 305 J L^{-1}, resulting in no viable spores (Fig. 2). The UV treatment method was shown to be capable of reliably achieving in excess of a 4 log_{10} reduction (99.99%) after 500 J $L^{-1}$of applied UV-C dosage in *A. acidoterrestris* inoculated in used fruit juice concentrate factory wash water (Fig. 2).

Alicyclobacillus acidoterrestris has been previously isolated from wash water in a factory processing fruit (Groenewald *et al*., 2008). This wash water can act as a potential reservoir of *A. acidoterrestris*, resulting in the contamination of the fruit concentrate or juice product. Wash water is used during the production to wash the fruit, removing dust, soil and any foreign objects from the fruit immediately prior to pulping. This water is conserved by recycling it during the manufacturing process, thus fruit can be potentially re-inoculated *with A. acidoterrestris* spores. The wash water, which had a pH of 3.98, can be subjected to UV treatment to decrease the contamination of the fruit by *A. acidoterrestris* during processing.

Figure 2 represents the log reduction of *A. acidoterrestris* spores in 80 ºBrix grape juice concentrate after UV treatment. It can be observed that a total inactivation of spores was obtained after 367.2 J L^{-1} was applied. This equates to around a 4.61 log_{10} reduction in spores. The grape juice concentrate used had a pH of 2.8 and was a clear liquid without any suspended solids, making it easier for the UV light to penetrate than for an opaque liquid. Koutchma *et al*. (2004) identified the factor that consistently affect

the efficacy of UV light inactivation in juice was absorbance, while factors unique to juice, such as °Brix and pH did not exhibit a profound effect on the efficacy of the treatment. Although grape juice concentrate itself is not susceptible to spoilage due to its high sugar content (Chang & Kang, 2004), its contamination with *A. acidoterrestris* spores can lead to spoilage when the concentrate is diluted to single strength fruit juice and *A. acidoterrestris* spores find a favourable environment for growth. It has, therefore, been suggested that *A. acidoterrestris* becomes the target spoilage organism for effective pasteurisation and that the processes must be designed to eliminate the spores (Silva & Gibbs, 2001). However, the high thermal resistance of these spores would necessitate pasteurisation at elevated temperatures resulting in unacceptable changes in the organoleptic and nutritional characteristics of the treated fruit concentrate or juice.

The UV inactivation curves for *A. acidoterrestris* showed mainly linear regression with only a slight tailing effect in all three treated liquids (Fig. 2). The sigmoidal shape of the curve with a shoulder and tailing is described as typical for UV light inactivation of micro-organisms (Koutchma *et al*., 2004; Hoyer, 1998). The shoulder is attributed to the requirement for more than one UV light hit to kill a micro-organism. Tailing has been attributed to either variability in the UV light sensitivity of the targeted population, inculding variabilty of UV resistance genes turned on, non-uniform processing conditions due to laminar type of flow and shading effects owing to insufficient exposure to UV light in solutions of lower transmittance (Hoyer, 1998). In this study the linear regression observed might be as a result of a single strain of *A. acidoterrestris* being used and also suggests that the novel UV treatment system reached sufficient turbulent flow inside the reactor to ensure an even UV exposure. It is important to note that the current USA FDA regulations on the use of UV light for fresh juice stipulate the use of a turbulent flow system (US FDA, 2001).

A bootstrap multiple comparison between UV-C dosage and media (tap water, wash water and grape juice concentrate) found significant differences at applied UV-C dosages of 244 and 305 J L^{-1}. At an applied UV-C dosage of 244 J L^{-1} on wash water, surviving spores (1.54 log CFU mL^{-1}) was significantly higher than a similar treatment on tap water (0.81 log CFU mL^{-1}) and 80 °Brix grape juice concentrate (1.02 log CFU mL^{-1}) with P = 0.0035. At an applied UV-C dosage of 305 J L^{-1}, surviving spores (log CFU mL^{-1}) in tap water was significantly lower (0.01) than for a similar treatment on wash water (0.70) and 80 °Brix grape juice concentrate (0.64) (Table 1). The greater

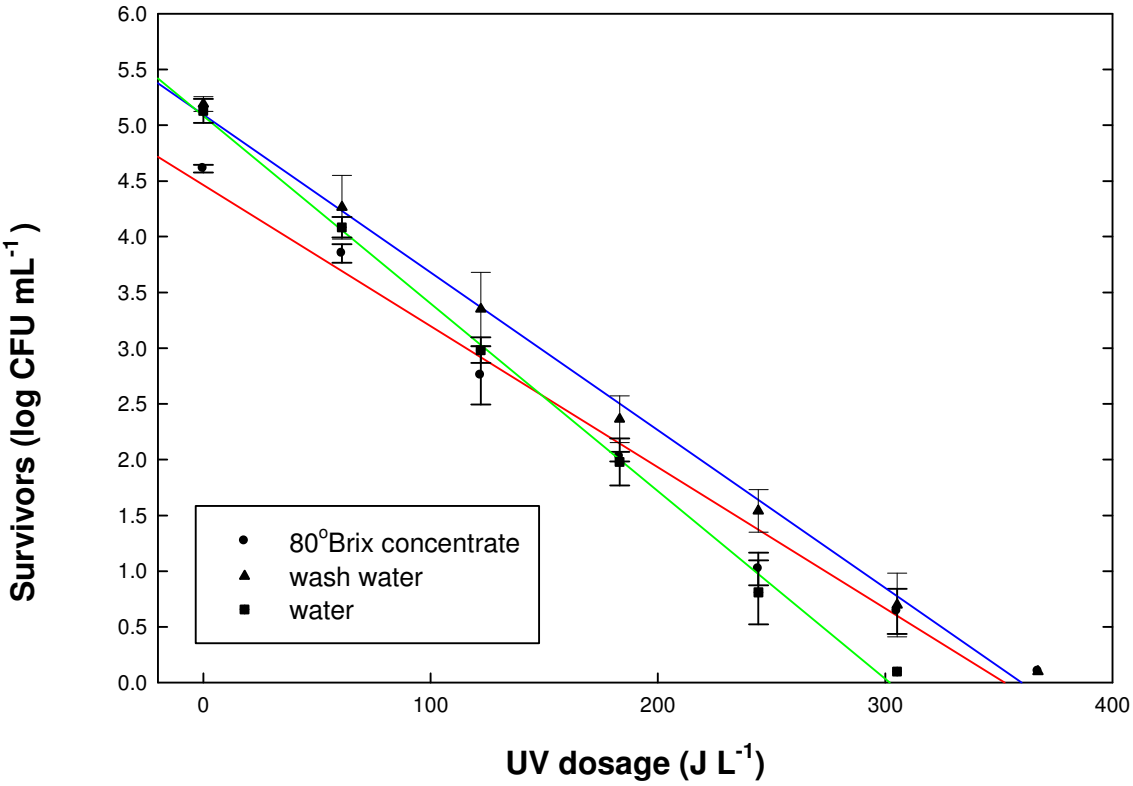

Figure 2 The $\log_{10}$ reduction of *A. acidoterrestris* K47 spores) at a starting concentration of approximately 5 x 10^5 CFU mL^{-1} in 80 °Brix grape juice concentrate, used fruit concentrate factory wash water and tap water. (Each data point represents quadruplicate values. The standard deviation was used as the error-bar.

UV-C absorptivity of the used wash water and grape juice concentrate is due to the presence of suspended matter and soluble solids respectively in the liquids.

Optimisation of the parameters is essential to ensure the maximum reduction of the microbial load of different fruit juices and concentrates without affecting the taste of the product. These parameters include the magnitude of turbidity, UV light transmittance through the media in the reactor, flow pattern and flow rate. Based on the results from this study, it can be recommended that the use of the novel UV treatment system is a promising way to control contamination of juice concentrate by species of *Alicyclobacillus*. However additional research needs to be done to further evaluate the effect of UV on the organoleptic and nutritional characteristics of the concentrate and subsequent single strength juice produced from the treated concentrate.

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CHAPTER 6

THERMAL INACTIVATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* SPORES ISOLATED FROM A FRUIT PROCESSING PLANT AND GRAPE JUICE CONCENTRATE IN SOUTH AFRICA

Abstract

Alicyclobacillus acidoterrestris is a non-pathogenic, spore-forming bacterium that can survive the commercial pasteurisation processes commonly applied to fruit juices and concentrates. Surviving bacterial endospores then germinate, grow and can under certain conditions cause spoilage of high acid food products. In this study, thermal inactivation at 95 °C for two strains of *A. acidoterrestris* isolated from contaminated fruit juice concentrates were investigated in a 0.1% (m/v) peptone buffer solution (pH 7.04) and grape juice (pH 4.02, 15.5 °Brix). The thermal inactivation of *A. acidoterrestris* spores followed first-order kinetics, suggesting that as the microbial population is exposed to a specific high temperature, the spores inactivated at a constant rate. D-values determined in the buffer solution were calculated to be 1.92 min and 2.29 min, while in grape juice D-values were found to be 2.25 min and 2.58 min for the two strains tested. From this study it is clear that the D-value is dependant on the strain tested, but also on the soluble solids of the solution the cells are suspended in. The soluble solids, therefore play a role in protecting the bacterial spores. The results indicated that the spores of *A. acidoterrestris* isolated from South African fruit juice concentrate may survive after the pasteurisation treatment commonly applied during manufacturing. Since the implementation of a more severe heat process required to inactivate spores of *A. acidoterrestris* will produce unacceptable organoleptic changes in the product, it is recommended the risk of spoilage should be minimised through the use of good manufacturing practices during fruit processing and the implementation of HACCP procedures, substituting Food Safety Hazards, normally associated with HACCP studies, with the risk of spoilage by *A. acidoterrestris* in the final product.

Introduction

Fruit concentrate has traditionally been regarded as resistant to spoilage by deteriogenic micro-organisms due its physical and chemical characteristics. These characteristics include a low pH of between 3.5 to 4.0, low water activity, high sugar concentration (typically around 66 °Brix), and reduced aeration capacity and dissolved oxygen (Palop *et al*., 2000). The addition of a hot-fill and hold pasteurisation process as used in the fruit beverage industry, where the product is held at 86° to 95 °C for approximately 2 min, is also sufficient to destroy most non-spore forming micro-organisms (Palop *et al*., 2000; Chang & Kang, 2004).

Spoilage of commercially available pasteurised fruit juice was first reported by Cerny *et al.* (1984) who found shelf-stable, aseptically packaged apple juice to have an off-flavour. Following this report, an increasing number of spoilage incidents arose and almost all of these were caused by the spore-forming, thermo-acidophilic bacteria *Alicyclobacillus acidoterrestris*. Spoilage has been to date reported in apple, pear, orange, peach, mango and white grape juice, with shelf-stable apple juice most frequently being spoiled (Borlinghaus & Engel, 1997; Chang & Kang, 2004; Walker & Phillips, 2008). More diverse products such as shelf-stable iced tea containing berry juice, the ingredients of rose hip and hibiscus teas (Duong & Jensen, 2000), a carbonated fruit drink (Pettipher, 2000) and diced canned tomatoes (Chang & Kang, 2004) have also seen incidences of spoilage caused by *A. acidoterrestris*. The fruit juice industry now acknowledges *A. acidoterrestris* as a major quality control target for pasteurisation (Yamazaki *et al*., 1996; Pettipher *et al*., 1997; Silva & Gibbs, 2004; Walker & Phillips, 2008; Bevilacque *et al*., 2008).

Spoilage caused by this bacterium is difficult to detect visually. The spoiled juice appears normal, or might have light sediment with no gas formation. Often, the only evidence of spoilage is apparent as a medicinal or phenolic off-flavour (Walls & Chuyate, 1998; Jensen, 1999). The chemicals responsible for this off-odour were identified as guaiacol (2-methoxyphenol) and other halophenols such as 2,6-dichlorophenol (2,6-DCP) and 2,6-dibromophenol (2,6-DBP). Guaiacol can be detected by smell in fruit juices at 2 ppb and was detected in orange and apple juices in the presence of around 5 log CFU mL^{-1} of *A. acidoterrestris* cells (Gocmen *et al*., 2005).

A wide range of D-values have been reported by researchers for the heat resistance of *A. acidoterrestris* spores, as the experimental conditions and protocols vary and the taxonomy of this group is still unclear. A review of D-values determined in

different fruit products and McIlvaine buffer are presented in Table 1. The $D_{95\ ^{\circ}C}$ ranged from 0.1 to 9.98 min, suggesting that spores survive the typical juice pasteurisation process applied during fruit juice and concentrate production and in fact provide a heat-shock treatment that may stimulate spore germination and outgrowth (Splittstoesser *et al*., 1994; Eiroa *et al*., 1999; Orr & Beuchat, 2000). Differences between the D-values reported in literature may be attributed to differences in strains, sporulation temperature, nutrient composition and pH of the heating medium, water activity, presence or absence of divalent cations and antimicrobial compounds (Bahçeci & Acar, 2007). Fruit juice contamination results from unwashed or poorly washed raw fruit that is processed, as well as contaminated water used during the production of fruit juices (Pontius *et al*., 1998; Orr & Beuchat, 2000; McIntyre *et al*., 1995; Groenewald *et al*., 2009).

The objective of this research was to determine the D-values in buffered water and single strength grape juice of spores *A. acidoterrestris* strains isolated from a South African fruit concentrate processing environment. Strains were tested at 95 °C, which is in the region of the highest temperature used during flash pasteurisation, to determine their ability to survive commercial pasteurisation regimes.

Materials and methods

Bacterial strains

Alicyclobacillus acidoterrestris K47 (Witthuhn *et al*., 2007), a strain isolated from grape juice concentrate and *Alicyclobacillus acidoterrestris* FB2 (Groenewald *et al*., 2009) isolated from pear juice concentrate were used in this study. Potato dextrose agar (PDA) (Biolab, Biolab Diagnostics, Midrand, SA) adjusted with tartaric acid (1N) (Saarchem, Krugersdorp, South Africa) after autoclaving to a final pH of 4 was used as a culture medium (Witthuhn *et al*., 2007).

Alicyclobacillus acidoterrestris spore suspension

Spores were produced on PDA (Biolab) incubated at 45 °C for 5 – 7 days until approximately 70% of cells sporulated, as determined by microscopic examination. Spores were removed by gently agitating each plate using a glass spreader after adding 5 mL of sterile distilled water. The spore suspension was centrifuged at 5 000 x *g*

Table 1 Previously reported values of thermal inactivation parameters of *A. acidoterrestris* spores

Heating medium	Strain	pH	Soluble solids (˚Brix)	Temperature (˚C)	D-value	Reference
Concord grape juice	WAC	3.5	16	85 90 95	53 11 1.9	Splittstoesser *et al.*, 1998
Grape juice	WAC	3.3	15.8	85 90 95	57 16 2.4	Splittstoesser *et al.*, 1994
Non-clarified lemon juice	nr	2.45	9.8	82 86 92 95	16.72 11.32 10.58 9.98	Maldonado *et al.*, 2008
Berry juice	nr	nr	nr	81.8 91.1 95	11.0 3.8 1.0	McIntyre *et al.*, 1995
McIlvaine buffer	AB-1	7.0	nr	88 90 92 95	24.7 15.7 6.7 2.2	Murakami *et al.*, 1998

[b]nr = not reported

(Beckman Coulter TJ-25 Centrifuge, Beckman Coulter Inc., USA) for 15 min after which the supernatant was discarded and the pellet was resuspended. Spores were cleaned by washing of the pellets with sterile distilled water, followed by centrifugation and this was repeated five times. Pellets were then resuspended in sterile saline solution (SSS) (0.85% (m/v) NaCl (Merck, Halfway House, Gauteng, SA). The spore suspension was heated at 80 °C for 10 min to eliminate vegetative cells and stored at 4 °C.

Thermal inactivation and enumeration

A Colworth House submerged-coil heating apparatus (Protrol Limited, Surrey, United Kingdom) was used for the investigation of the thermal inactivation of *A. acidoterrestris* spores. The apparatus has a narrow bore stainless steel coil (9.5 mL total volume, 3.175 mm outer diameter, 0.5 mm thickness), fully submerged in a thermostatically controlled water bath and a automatic sampler with the sampling frequency controlled by a DOS based computer program (Fig. 1). The time for the sample to reach the water bath temperature was 1 s. Cleaning was performed by first injecting industrial alcohol followed by sterile water. The water bath was set at a temperature of 95 °C and ten sampling times at 1 min intervals were programmed, for up to 9 min heating time in total. A temperature of 95 °C was chosen as being representative of the highest temperature used in commercial pasteurisation regimes. Ten mL of either inoculated 0.1% (m/v) peptone (Biolab) buffer solution (pH 7.04) or 15.5 °Brix single strength grape juice (pH 4.02) (made by the dilution of 80 °Brix grape concentrate with distilled water) were injected into the submerged coil, followed by immediate initiation of the timing sequence. The remaining peptone buffer solution (Biolab) or grape juice were used for the time zero determination of the viable spore count. Times were selected in order to cover spore inactivation until approximately 10^2 CFU mL^{-1} of *A. acidoterrestris* spores remained. For each sampling time, 500 µL of the heated spores were collected and promptly cooled by dilution with 5 mL SSS at room temperature. The samples were left at ambient temperature for approximately 4 h to allow further cooling and recovery of the heat-shocked spores.

Serial dilutions of the samples were then prepared (10^{-1} – 10^{-6}) and 100 µL of each of the different sample dilutions were plated in triplicate onto PDA (Biolab) (pH 4). Plates were incubated aerobically at 45 °C and examined for growth after 96 h. The results obtained were expressed as colony forming units per milliliter (CFU mL^{-1}).

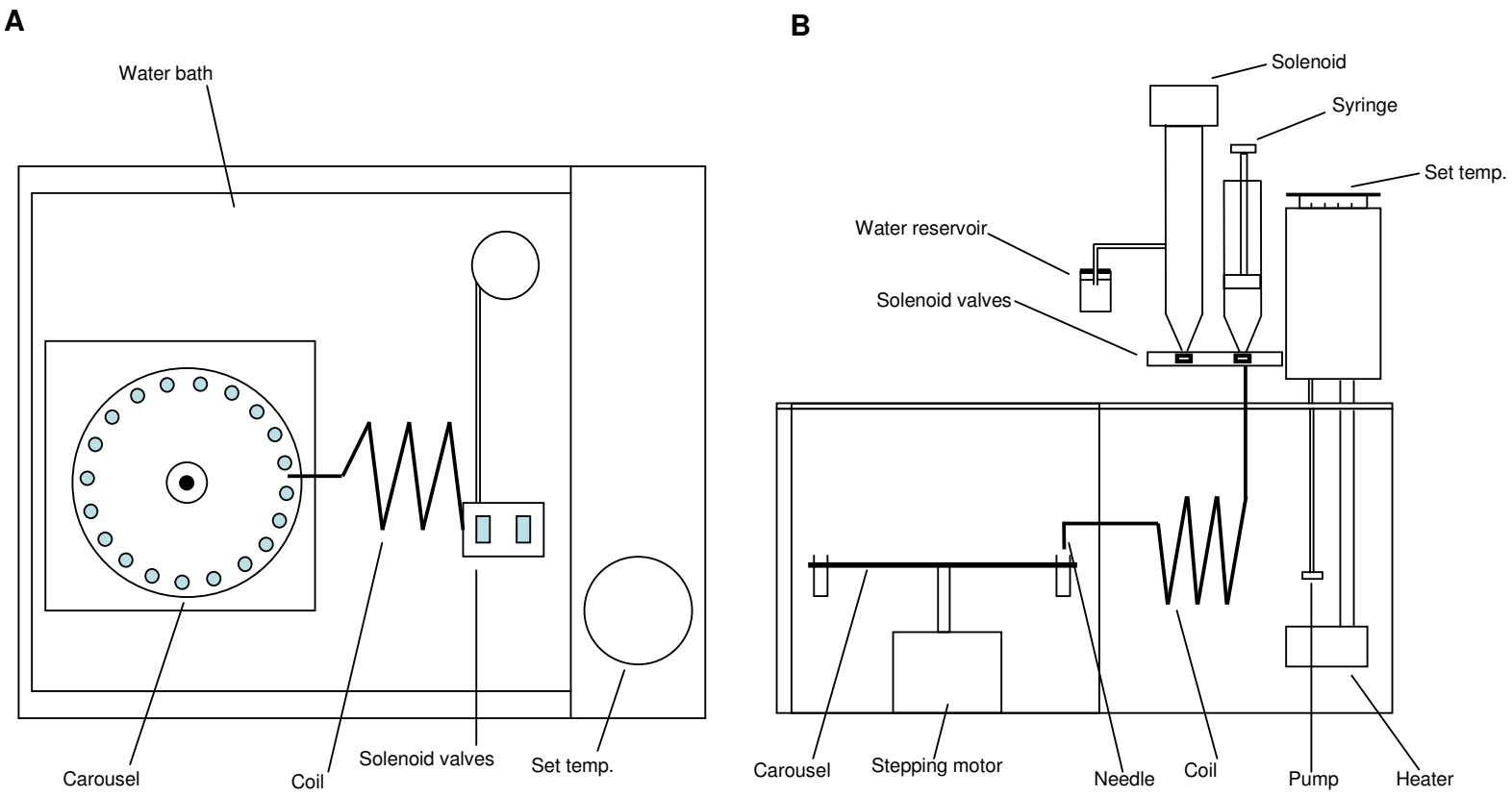

Figure 1 Submerged coil heating apparatus (Protrol Limited, Surrey, United Kingdom). Plan (A) and side view (B) of water bath, coil and sampling device. The electronic system for sampling frequency control and solenoid valve switching is not shown.

The efficacy of thermal treatments in terms of eliminating *A. acidoterrestris* spore was measured by their decimal reduction time (D) which, for this study, was defined as the time (min) of a given treatment for the number of survivors to be reduced by one log cycle. In this study, the D-value at 95 °C was abbreviated as D_{95}. D-values were calculated from the slope of the regression line when time (x-axis) was plotted against cell counts (CFU mL^{-1}) (y-axis). Experiments were performed in triplicate.

Results and discussion

A linear-log relation was observed between the endospore concentration and time (Figs. 2 and 3) suggesting that as the microbial population was heated at a specific temperature, the spores inactivated at a constant rate. A similar first-order kinetic relationship was observed by other authors (Pontius *et al*., 1998; Silva *et al*., 1999). In this study D_{95}-values were calculated to be 1.92 min and 2.29 min for *A. acidoterrestris* strains K47 and FB2, respectively in a 0.1% (m/v) peptone buffer solution (Biolab) (pH 7) (Fig. 2), and 2.25 min and 2.58 min in grape juice (pH 4.05, 15.5 °Brix) (Fig. 3)

Splittstoeser *et al*. (1999) reported similar D_{95}-values in Concord grape juice (pH 3.5, 16 °Brix) for *A. acidoterrestris* strain WAC spores of 1.9 min and a value of 2.4 min for the same strain in grape juice (pH 3.3, 15.8 °Brix). However, D-values amongs strains of *A. acidoterrestris* varied greatly when tested in different fruit juices with similar levels of acidity and concentration of dissolved sugars. Maldonado *et al*., (2008), reported D_{95}-values in non clarified lemon juice (pH 2.45, 9.8 °Brix) of 9.98 min, while McIntyre *et al.* (1995) found *A. acidoterrestris* spores to have a D_{95}-value of only 1.0 min in berry juice. These differences could be explained due to differing compositions of fruit products, including soluble solids, which might increase the heat resistance of spores (Maldonado *et al*., 2008).

Heat resistance between strains of *A. acidoterrestris* also varies greatly (Murakami *et al*., 1998; Pontius *et al*., 1998; Eiora *et al*., 1999; Bahçeci & Acar, 2007). Confirmation of strain differences is provided in this study with *A. acidoterrestris* FB2 showing more thermal resistance than *A. acidoterrestris* K47 in a peptone buffer solution (Biolab), as well as grape juice (D_{95}-values of 1.92 min and 2.29 min and 2.25 min and 2.58 min, respectively). The steeper slope of the regression line for the 6×10^5 CFU mL^{-1} *A. acidoterrestris* inoculum in peptone buffer solution (Biolab) (Fig. 2) as opposed to the grape juice, and the subsequent lower D_{95}-values of 1.92 and 2.29 min

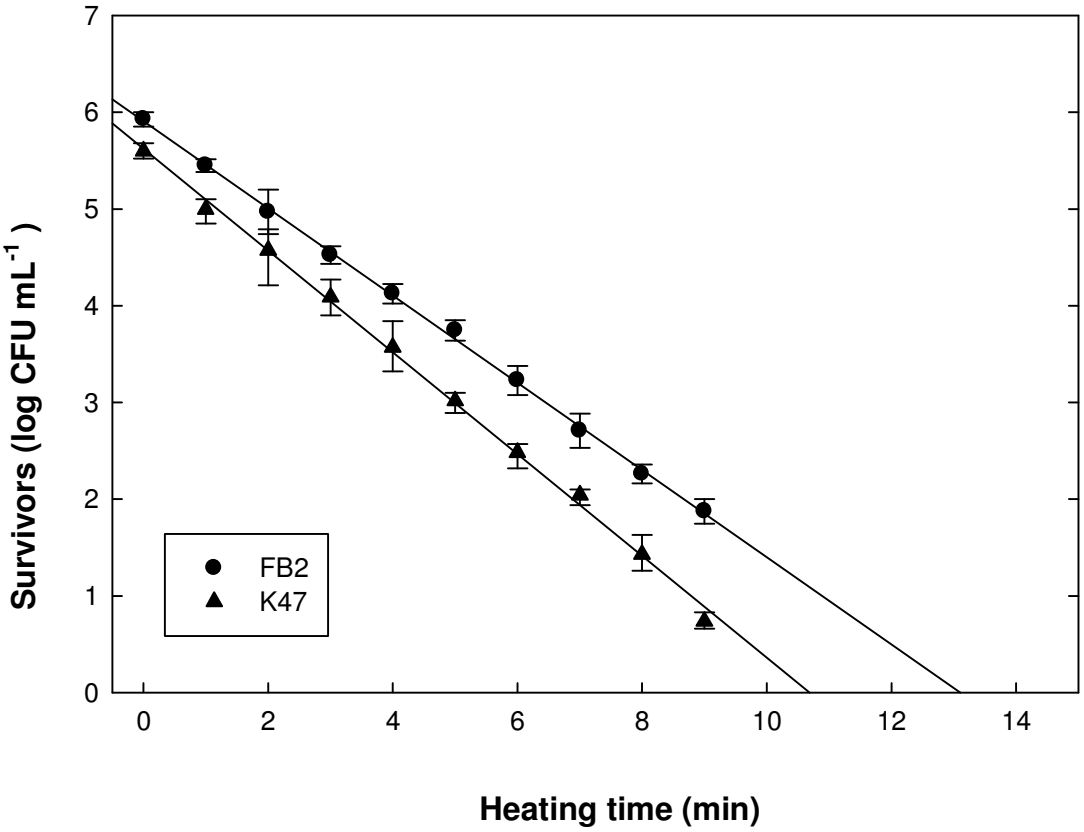

Figure 2 Impact of temperature at 95 ℃ on *A. acidoterrestris* strains K47 and FB2 at a starting concentration of 6 x 10^5 CFU mL^{-1} in a peptone buffer solution (Each data point represents triplicate values. The standard deviation was used as the error-bar).

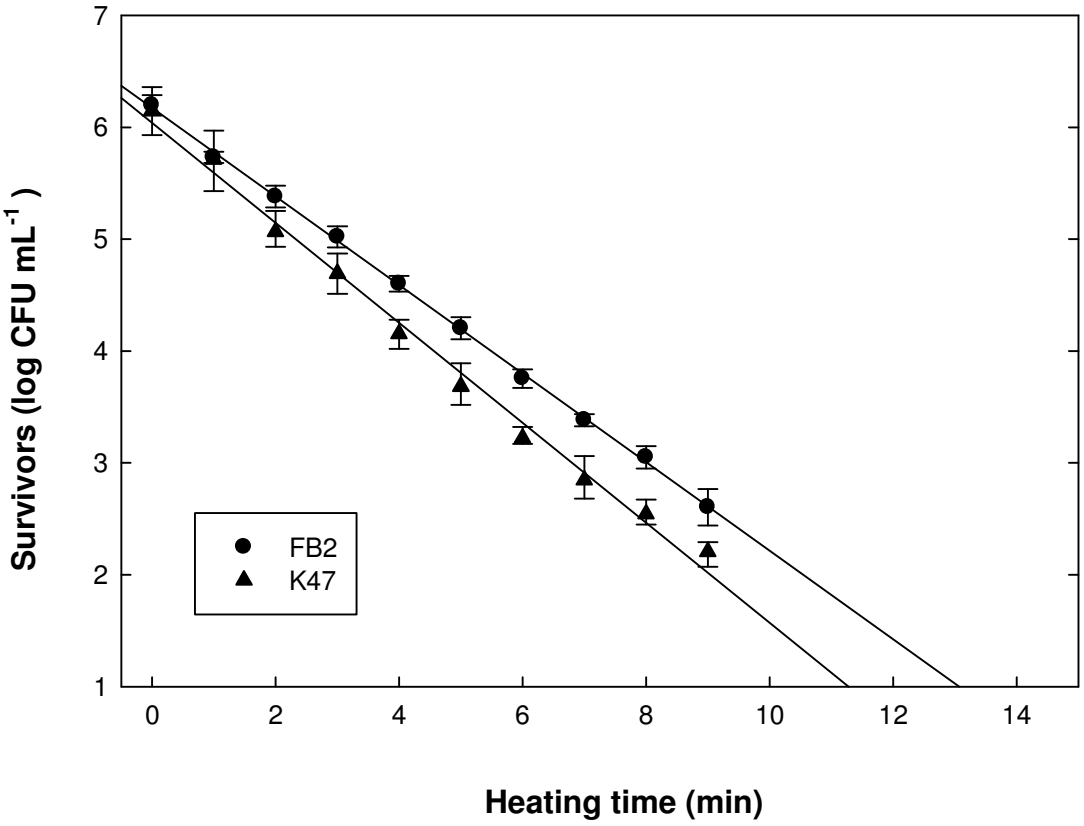

Figure 3 Impact of temperature at 95 °C on *A. acidoterrestris* strains K47 and FB2 at a starting concentration of 6 x 10^5 CFU mL^{-1} in a 15.5 °Brix single strength grape juice (pH 4.02) (Each data point represents triplicate values. The standard deviation was used as the error-bar).

for *A. acidoterrestris* K47 and FB2, respectively can be ascribed to the lower pH and higher percentage of soluble solids (°Brix) in the grape juice.

Conclusion

Results from this study indicated that the spores of *A. acidoterrestris* may survive in fruit juices after pasteurisation treatment commonly applied in the food industry, at least under the conditions described in the current study. Since the implementation of a more severe heat process required to inactivate spores of *A. acidoterrestris* will also produce unacceptable organoleptical changes in the product, and the fact that no species of *Alicyclobacillus* have shown any pathogenic potential, it would serve little purpose to set pasteurisation temperatures to target *A. acidoterrestris.* It is important to note that incidence of *A. acidoterrestris* in fruit juice is not directly associated with deterioration. Detection of *A. acidoterrestris* in non-deteriorated fruit juices (Previdi *et al*., 1997; Cerny *et al*., 1999; Bahçeci *et al*., 2005; Walker & Phillips, 2008) suggests deterioration to be incidental, requiring adequate conditions for its development. The susceptibility of fruit juice to spoilage is dependant on initial levels of contamination and the conditions of storage of the fruit juice. Manufacturers of fruit juice concentrate should minimised the risk of spoilage by *A. acidoterrestris* through the use of good manufacturing practices during fruit processing and the implementation of HACCP procedures, substituting Food Safety Hazards, normally associated with HACCP studies, with the risk of spoilage by *A. acidoterrestris* in the final product. Storage of pasteurised fruit products below 20 °C would also prevent spoilage since the growth of this bacterium is suppressed at these temperatures.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Since 1994 the numbers of incidences of spoilage by *A. acidoterrestris* of fruit juices and fruit juice product have been on the increase (Komitopoulou *et al*., 1999; Walker & Phillips, 2008). Spoilage by alicylobacili has become regarded as an industry-wide problem that requires solutions to be found and effective control measures developed and implemented (Duong & Jensen, 2000). As members of the genus *Alicyclobacillus* are soil-borne organisms, it is thought that the source of contamination of the fruit juice is from the harvested, contaminated fresh fruit, which is introduced during processing without proper cleaning (Pontius *et al*., 1998; Orr & Beuchat, 2000). However, the exact route of contamination of the final product remains unclear and, therefore, strategies to combat contamination cannot be fully developed.

In this study alicylcobacilli were isolated from a diverse range of samples from the environment and fruit processing plant in order to provide comprehensive data on the occurrence of species of *Alicyclobacillus* at different stages of fruit processing. Species of *Alicyclobacillus* were isolated from orchard soil, soil on the fruit processing premises, vinegar flies, wash water, flume water, pear skin from the press, debris from the factory floor, water from the evaporator inlet and the pear concentrate. As far as we are aware this is the first report on the isolation of *A. acidoterrestris* from orchard soil, wash water, water from the evaporator inlet, soil outside a fruit concentrate factory and flume water. Strains of *A. acidocaldarius* were isolated from pre-pasteurised pear puree, orchard soil and for the first time to our knowledge, from vinegar flies. Results from this study indicate that species of *A. acidoterrestris* and *A. acidocaldarius* are found in orchard soil and throughout the processing environment.

The isolates identified as *A. acidoterrestris* grouped into four clusters based on RAPD-PCR band pattern, suggesting that they belong to at least four genotypic groups. The identical banding patterns obtained for the isolates in each respective cluster suggests that they are descendants from the same strain and, therefore, could indicate potential cross contamination between the sources of isolation within isolates represented by the same cluster. Wash water and soil outside the factory can act as a reservoir of *A. acidoterrestris* leading to the contamination of the final fruit concentrate product. It is, therefore, recommended to treat fruit during processing and wash water to reduce the microbial load of these important spoilage bacteria. The widespread

occurrence of strains of *A. acidoterrestris* in the fruit concentrate manufacturing environment suggests that good manufacturing practices play an essential role in controlling instances of spoilage caused by these bacteria.

Consumers are increasingly demanding minimally processed foods that are fresher, more natural (Kouchma, 2009) and with a better nutritional content and higher overall quality. The use of ultraviolet (UV) light in food processing is one of a number of non-thermal technologies being studied as a possible substitute for thermal processing. UV radiation was successfully applied using a novel pilot-scale UV system to reduce *A. acidoterrestris* spores inoculated into tap water, used wash water from a fruit concentrate manufacturing facility and 80 ˚Brix grape juice concentrate. In water inoculated with *A. acidoterrestris* spores a 5.3 $\log_{10}$ reduction of alicyclobacilli spores was achieved after a UV dosage of only 305 J L^{-1}, resulting in no viable spores. The UV treatment method was shown to be capable of reliably achieving in excess of a 4 $\log_{10}$ reduction (99.99%) per 0.5 kJ L^{-1} of UV-C dosage in *A. acidoterrestris* spores inoculated in used fruit juice concentrate factory wash water. For total deactivation of *A. acidoterrestris* spores in 80 °Brix grape juice concentrate, 367.2 J L^{-1} was needed, which equates to around a 4.61 $\log_{10}$ reduction in spores.

The UV inactivation curves for *A. acidoterrestris* showed mainly linear regression with only a slight tailing effect in all three treated liquids. This suggests that the spores and cells inactivated at a fairly constant rate in relation to the UV dosage received. The sigmoidal shape of the curve with an initial lag and tailing is described as typical for UV light inactivation of micro-organisms (Hoyer, 1998; Koutchma *et al*., 2004). In this study the linear regression observed might be as a result of a single strain of *A. acidoterrestris* being used and also suggests that the novel UV treatment system reached sufficient turbulent flow inside the reactor to ensure an even UV exposure. It is important to note that the current USA FDA regulations on the use of UV light for fresh juice stipulate the use of a turbulent flow system (US FDA, 2001). The greater UV-C absorptivity of the used wash water and grape juice concentrate is due to the presence of suspended matter and soluble solids in the liquids. Optimisation of the parameters is essential to ensure the maximum reduction of the microbial load of different fruit juices and concentrates without affecting the taste of the product. These parameters include the magnitude of turbidity, UV light transmittance through the media in the reactor, flow pattern and flow rate. Based on the results from this study, it can be recommended that the use of the novel UV treatment system is a promising way to control contamination of juice concentrate by species of *Alicyclobacillus*. In addition, UV radiation has been

shown to be less energy-intensive, more cost-effective and environmentally friendly than conventional pasteurisation (Kouchma, 2009). However, additional research needs to be done to further evaluate the effect of UV on the organoleptic and nutritional characteristics of the concentrate and subsequent single strength juice produced from the treated concentrate. The effects of the different treatments on the organoleptic and nutritional characteristics of the fruit juice and concentrate, the real shelf life of the product, and the exact mode of action of some compounds or treatments against *A. acidoterrestris* needs to be established. An alternate use is to treat wash water with UV radiation prior to the washing of the fruit, as wash water can act as a reservoir of *A. acidoterrestris* leading to contamination of the final product.

A wide range of D-values have been reported by researchers for the heat resistance of *A. acidoterrestris* spores, as the experimental conditions and protocols vary and the taxonomy of this group is still unclear. In this study, thermal inactivation at 95 °C for two strains of *A. acidoterrestris* isolated from contaminated fruit juice concentrates were investigated in a 0.1% (m/v) peptone buffer solution (pH 7.04) and grape juice (pH 4.02, 15.5 °Brix). The thermal inactivation of *A. acidoterrestris* spores followed first-order kinetics, suggesting that as the microbial population is exposed to a specific high temperature, the spores inactivated at a constant rate. D-values determined in the buffer solution were calculated to be 1.92 min and 2.29 min, while in grape juice D-values were found to be 2.25 min and 2.58 min for the two strains tested. From this study it is clear that the D-value is dependant on the strain, but also on the soluble solids of the solution the cells are suspended in. The soluble solids play a role in protecting the bacterial spores. The results indicated that the spores of *A. acidoterrestris* isolated from South African fruit juice concentrate may survive after the pasteurisation treatment commonly applied during manufacturing.

Concluding remarks

Alicyclobacillus acidoterrestris, or at least the spores of this species, appears to be endemic to the fruit processing environment. The susceptibility of fruit juice to spoilage is dependant on initial levels of contamination and the conditions of storage of the fruit juice, rather than the presence or absence of *A. acidoterrestris* (Bahçeci *et al*., 2005; Walker & Phillips, 2008). The creation of a sterile environment in a fruit processing plant to prevent contamination of the final product is not practical. Moreover, *A. acidoterrestris* spores can survive the commercial thermal treatments applied during manufacturing

and the implementation of a more severe heat process required to inactivate spores of *A. acidoterrestris* will produce unacceptable organoleptic changes in the product. Manufacturers of fruit juice concentrate should minimised the quantity of *A. acidoterrestris* spores in the final product and thus the risk of spoilage through the use of good manufacturing practices during fruit processing. Particular attention should be paid to water used during the processing of fruit in the plant. A standardised quantitative method of detection of *A. acidoterrestris* spores needs to be developed coupled with research on fruit juice to establish the levels of contamination by *A. acidoterrestris* needed in fruit juice for the probability of spoilage to occur.

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