

**GROWTH AND GUAIACOL PRODUCTION OF SPECIES OF
ALICYCLOBACILLUS ISOLATED FROM THE SOUTH AFRICAN FRUIT
PROCESSING ENVIRONMENT**

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The crest of the University of Stellenbosch is centered behind the text. It features a shield with a blue and red design, topped with a crown and surrounded by red and blue decorative elements. A banner at the bottom of the crest contains the Latin motto "Pacta sunt quibus recti".

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DECLARATION

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ABSTRACT

Bacteria belonging to the genus *Alicyclobacillus* are thermo-acidophilic spore-formers that are able to spoil acidic food and beverage products through the production of guaiacol and other taint compounds, which causes a medicinal off-flavour and/or odour in the products. This thesis reports on the comparison of methods used for the isolation of species of *Alicyclobacillus*, as well as the growth behaviour and guaiacol production of different strains isolated from the South African fruit processing environment. Two methods for guaiacol detection were also evaluated and compared.

Three isolation methods frequently used by South African fruit processors were compared with regards to their ability to isolate a strain of *A. acidoterrestris* from diluted peach juice concentrate. Method 1, the International Federation of Fruit Juice Producers (IFU) Method No. 12, makes use of spread plating onto *Bacillus acidoterrestris* (BAT) agar plates; Method 2 involves pour plating using acidified potato dextrose agar (PDA); and Method 3 makes use of membrane filtration and incubation of the membrane on K agar. The IFU Method No. 12 was the most effective method for the isolation of *A. acidoterrestris*, with a recovery of 75.97%. These results support the use of the IFU Method No. 12 as a standard international method for the isolation and detection of species of *Alicyclobacillus*.

Seven strains of *Alicyclobacillus*, including the type strains *A. acidoterrestris* DSM 3922^T and *A. acidocaldarius* DSM 446^T and five strains isolated from a South African fruit processing plant, *A. acidoterrestris* FB2, FB14, FB32, FB38 and *A. acidocaldarius* FB19, were analysed based on their growth characteristics and guaiacol production under optimum conditions. Strains were inoculated into BAT medium at pH 4.00, supplemented with 100 mg.L⁻¹ vanillin, and incubated at 45°C for 7 d. All the strains had similar growth patterns, with cell concentrations increasing rapidly from 0-24 h, followed by a stabilisation around maximum cell concentrations of 10⁵-10⁷ cfu.mL⁻¹. Cell concentrations after heat shock, measured as an indication of spore formation, increased to maximum values of 10⁵-10⁷ cfu.mL⁻¹, indicating an increase in spores as the cell density and competition for resources increased. All the strains were able to produce guaiacol in detectable concentrations [as measured by the peroxidase enzyme colourimetric assay (PECA)], and, therefore, possess the potential to cause product spoilage.

The influence of temperature on the growth and guaiacol production of the *Alicyclobacillus* strains was also investigated and two guaiacol detection methods, the PECA and headspace gas-chromatography mass-spectrometry (HS GC-MS), were compared with regards to their ability to detect guaiacol. The strains were incubated at 25°C and 45°C for 6 d and samples analysed every 24 h. Growth of the *A. acidoterrestris* strains was slower at 25°C, and maximum cell concentrations were lower than at 45°C. A decrease in cell concentrations was observed in the *A. acidocaldarius* strains at 25°C, as this temperature is below their growth temperature range. All the strains were able to produce guaiacol at 45°C, with guaiacol only being detected once a cell concentration of 10^4 - 10^5 cfu.mL⁻¹ had been reached. The maximum guaiacol concentrations detected at 45°C in the samples containing *A. acidoterrestris* were significantly higher than those detected in the *A. acidocaldarius* samples. At 25°C there was a longer lag phase before guaiacol was detected in the *A. acidoterrestris* samples, while no guaiacol was detected in the samples containing *A. acidocaldarius*. Because guaiacol is produced at ambient temperatures, cooling of products is recommended to control spoilage by *A. acidoterrestris*. The sensitivity of the two guaiacol detection methods also differed significantly and, therefore, the PECA is recommended for presence/absence detection of guaiacol, while HS GC-MS is recommended where accurate quantification of guaiacol is required.

Alicyclobacillus acidoterrestris FB2 was investigated for its ability to grow and produce guaiacol in white grape juice supplemented with vanillin at different concentrations. *Alicyclobacillus acidoterrestris* FB2 was inoculated into white grape juice concentrate diluted 1:10 with distilled water containing 0-500 mg.L⁻¹ vanillin and incubated at 45°C for 6 d. Similar growth patterns were observed in all the samples, except in the sample containing 500 mg.L⁻¹ vanillin, which had a longer lag phase of growth. Guaiacol concentrations, detected using the PECA, increased as the vanillin concentration increased, with the exception of the sample containing 500 mg.L⁻¹ vanillin, where less guaiacol was detected than in the sample containing 250 mg.L⁻¹ vanillin, due to growth inhibition caused by the higher vanillin concentration. A number of conditions need to be favourable for detectable guaiacol production to occur and it could, therefore, be possible to minimise or prevent guaiacol production by controlling or eliminating some of these factors. Good manufacturing practices should be employed in order to minimise contamination and, therefore, spoilage, by *Alicyclobacillus* species.

UITTREKSEL

Bakterieë wat aan die genus *Alicyclobacillus* behoort, is termo-asidofiliese spoorvormers wat suur voedsel en drank produkte kan bederf deur die produksie van guaiakol en ander bederf verbindings, wat 'n medisinale geur en/of reuk in die produkte veroorsaak. Hierdie tesis doen verslag oor die vergelyking van metodes wat vir die isolasie van spesies van *Alicyclobacillus* gebruik word, sowel as die groei kenmerke en guaiakol produksie van verskillende stamme wat uit die Suid-Afrikaanse vrugte prosesseringsomgewing geïsoleer is. Twee metodes vir die deteksie van guaiakol is ook geëvalueer en vergelyk.

Drie isolasie metodes wat algemeen deur Suid-Afrikaanse vrugteprosesseerders gebruik word, is vergelyk ten opsigte van hul vermoë om 'n *A. acidoterrestris* stam uit verdunde perskesap konsentraat te isoleer. Metode 1, die Internasionale Federasie van Vrugtesap Produseerders (IFU) Metode No. 12, maak gebruik van spreiplating op *Bacillus acidoterrestris* (BAT) agar plate; Metode 2 behels gietplating met aartappel dekstrose agar (PDA) and Metode 3 maak gebruik van membraan filtrasie en inkubasie van die membraan op K agar. Die IFU Metode No. 12 was die mees effektiewe metode vir die isolasie van *A. acidoterrestris*, met 'n sel herwinning van 75.97%. Hierdie resultate ondersteun die gebruik van die IFU Metode No. 12 as 'n standaard internasionale metode vir die isolasie en deteksie van spesies van *Alicyclobacillus*.

Sewe *Alicyclobacillus* stamme, insluitende die tipe stamme *A. acidoterrestris* DSM 3922^T en *A. acidocaldarius* DSM 446^T en vyf stamme geïsoleer uit 'n Suid-Afrikaanse vrugte prosesseringsaanleg, *A. acidoterrestris* FB2, FB14, FB32, FB38 en *A. acidocaldarius* FB19, is geanaliseer met betrekking tot hul groei kenmerke en guaiakol produksie onder optimum toestande. Stamme is in BAT medium by pH 4.00, aangevul met 100 mg.L⁻¹ vanillin, geïnkuleer en geïnkubeer teen 45°C vir 7 d. Al die stamme het soortgelyke groeipatrone getoon, met selgetalle wat vinnig toegeneem het van 0-24 h, gevolg deur 'n stabilisering rondom maksimum selgetalle van 10⁵-10⁷ kve.mL⁻¹. Selgetalle na hitte behandeling, gemeet as 'n aanduiding van spoorvorming, het toegeneem tot maksimum waardes van 10⁵-10⁷ kve.mL⁻¹, wat aandui dat spore toegeneem het soos die seldigheid en kompetisie vir voedingsbronne toegeneem het. Al die stamme kon guaiakol in bespeurbare

konsentrasies produseer [soos gemeet deur die peroksidase ensiem kolorimetriese bepaling (PEKB)] en besit dus die potensiaal om produkte te bederf.

Die invloed van temperatuur op groei en guaiakol produksie van die *Alicyclobacillus* stamme is ook ondersoek en twee guaiakol deteksie metodes, die PEKB en topspasie gas-kromatografie massa-spektrometrie (TS GK-MS) is vergelyk ten opsigte van hul vermoë om guaiakol op te spoor. Die stamme is geïnkubeer teen 25°C en 45°C vir 6 d en monsters is elke 24 h geanaliseer. Groei van die *A. acidoterrestris* stamme was stadiger by 25°C en maksimum selgetalle was laer as by 45°C. 'n Vermindering in selgetalle is waargeneem in die *A. acidocaldarius* stamme by 25°C, aangesien hierdie temperatuur buite hul groei temperatuur grense val. Al die stamme kon guaiakol produseer by 45°C, met guaiakol deteksie wat eers 'n aanvang geneem het nadat 'n sel konsentrasie van 10^4 - 10^5 kve.mL⁻¹ bereik is. Die maksimum guaiakol konsentrasies wat by 45°C in die monsters met *A. acidoterrestris* opgespoor is, was beduidend hoër as die konsentrasies wat in die *A. acidocaldarius* monsters opgespoor is. By 25°C was daar 'n langer sloefase voor guaiakol opgespoor is in die *A. acidoterrestris* monsters, terwyl geen guaiakol opgespoor is in die monsters wat *A. acidocaldarius* bevat het nie. Aangesien guaiakol by kamertemperatuur geproduseer word, word verkoeling van produkte aanbeveel ten einde bederf deur *A. acidoterrestris* te beheer. Die sensitiwiteit van die twee guaiakol deteksie metodes het ook beduidend verskil en dus word die gebruik van die PEKB aanbeveel vir teenwoordigheid/afwesigheid deteksie van guaiakol, terwyl TS GK-MS aanbeveel word waar akkurate kwantifisering van guaiakol vereis word.

Ondersoek is ingestel na die vermoë van *A. acidoterrestris* FB2 om te groei en guaiakol te produseer in witdruiwesap aangevul met verskillende vanillin konsentrasies. *Alicyclobacillus acidoterrestris* FB2 is geïnkuleer in witdruiwesap konsentraat 1:10 verdun met gedistilleerde water wat 0-500 mg.L⁻¹ vanillin bevat het en is geïnkubeer teen 45°C vir 6 d. Soortgelyke groeipatrone is waargeneem in al die monsters, behalwe die monster wat 500 mg.L⁻¹ vanillin bevat het, wat 'n langer sloefase van groei gehad het. Guaiakol konsentrasies, soos gemeet deur die PEKB, het toegeneem soos die vanillin konsentrasie toegeneem het, met die uitsondering van die monster wat 500 mg.L⁻¹ vanillin bevat het, waar minder guaiakol opgespoor is as in die monster wat 250 mg.L⁻¹ bevat het as gevolg van groei inhibisie veroorsaak deur die hoër vanillin konsentrasie. 'n Aantal toestande moet gunstig wees vir guaiakol produksie om plaas te vind en dit kan dus moontlik wees om

guaiakol produksie te minimaliseer of te voorkom deur die beheer of uitskakeling van sommige van hierdie faktore. Goeie vervaardigingspraktyke moet in plek gestel word ten einde kontaminasie en bederf deur *Alicyclobacillus* spesies tot 'n minimum te beperk.

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- Philippians 4: 13

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

CHAPTER 1

INTRODUCTION

The production of fruit juice forms an important part of the global beverage industry (Roethenbaugh, 2005). The products are consumed by a large percentage of the population, especially since consumers have become more health conscious and greater emphasis has been placed on the consumption of healthier, natural products. Fruit juice and fruit based products also forms an important part of the rapidly expanding functional foods market (Gordon & Kubomura, 2003). However, because these products are considered to be healthy and nutritious (Gordon & Kubomura, 2003), consumers have developed greater expectations with regards to their quality and safety. Spoilage incidents resulting in the loss of consumer confidence is severely damaging to the manufacturer as well as the product image.

Until recently acidic products such as fruit juice and fruit based products were only thought to be susceptible to spoilage by yeasts, fungi and lactic acid bacteria, as the low pH ($\text{pH} \leq 4.00$) of these products acts as a natural control measure against spoilage by most bacteria, especially spore-formers (Vieira *et al.*, 2002; Jay *et al.*, 2005a; Jay *et al.*, 2005b). A pasteurisation treatment is sufficient to destroy the conventional spoilage organisms that may occur in fruit products as they are not heat resistant (Blocher & Busta, 1983; Silva *et al.*, 2000; Vieira *et al.*, 2002) and the low pH makes storage at ambient temperatures after pasteurisation possible.

A large scale spoilage incident reported in Germany in 1984 involving pasteurised shelf-stable apple juice (Cerny *et al.*, 1984) cast doubt on the efficiency of pasteurisation treatments applied to acidic products for the control of spoilage organisms. A species from the genus *Alicyclobacillus*, *A. acidoterrestris*, was identified as the causative organism in this spoilage incident (Deinhard *et al.*, 1987a; Wisotzkey *et al.*, 1992). This was the first report to implicate these bacteria in a food spoilage incident. Initial isolations of *Alicyclobacillus* spp. had been almost exclusively from soil (Hippchen *et al.*, 1981; Deinhard *et al.*, 1987b; Nicolaus *et al.*, 1998) and thermal acid environments such as hot springs (Uchino & Doi, 1967; Darland & Brock, 1971), but they have subsequently also been isolated from a variety of acidic food and beverage products, including fruit juice and fruit products

(Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996; Walls & Chuyate, 1998; Jensen, 2000; Pettipher & Osmundson, 2000; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Jensen & Whitfield, 2003; Gouws *et al.*, 2005), iced tea (Duong & Jensen, 2000) and canned diced tomatoes (Walls & Chuyate, 1998).

Members of the genus *Alicyclobacillus* are thermo-acidophilic, spore-forming bacteria and are able to grow at temperatures of 4°-70°C and pH values ranging from 0.50-6.50 (Wisotzkey *et al.*, 1992; Goto *et al.*, 2002; Karavaiko *et al.*, 2005). The thermo-acidophilic nature and spore-forming abilities of *Alicyclobacillus* spp. presents a problem to the fruit processing industry, as this allows them to survive the pasteurisation treatment normally applied to these products (Splittstoesser *et al.*, 1998; Eiroa *et al.*, 1999; Vieira *et al.*, 2002). In fact, the pasteurisation treatment may act as a heat shock treatment that activates spores (Jensen, 1999; Gouws *et al.*, 2005) and because they favour the acidic environment they can germinate and grow to cell populations high enough to produce spoilage taints.

Spoilage takes the form of an off-flavour and/or odour in the products, most often attributed to the production of the chemical compound guaiacol (Yamazaki *et al.*, 1996; Splittstoesser *et al.*, 1998; Jensen, 2000; Walls & Chuyate, 2000; Gocmen *et al.*, 2005; Siegmund & Pöllinger-Zierler, 2006; Goto *et al.*, 2008), although the halophenols 2,6-dichlorophenol (2,6-DCP) (Jensen, 2000; Jensen & Whitfield, 2003; Gocmen *et al.*, 2005) and 2,6-dibromophenol (2,6-DBP) (Borlinghaus & Engel, 1997; Jensen, 1999; Jensen, 2000; Jensen & Whitfield, 2003; Gocmen *et al.*, 2005; Siegmund & Pöllinger-Zierler, 2006) have also been identified as the source of taint in some products. Spoilage caused by members of *Alicyclobacillus* is most often ascribed to the presence of the species *A. acidoterrestris* (Yamazaki *et al.*, 1996; Walls & Chuyate, 1998; Jensen, 2000; Pettipher & Osmundson, 2000; Jensen & Whitfield, 2003), although other species have also been implicated due to their ability to produce taint compounds or their isolation from spoiled products (Matsubara *et al.*, 2002; Goto *et al.*, 2003; Niwa & Kawamoto, 2003; Gocmen *et al.*, 2005; Gouws *et al.*, 2005; Goto *et al.*, 2008).

Even though *Alicyclobacillus* spp. seem to be quite prevalent in products (Borlinghaus & Engel, 1997; Pettipher *et al.*, 1997; Pinhatti *et al.*, 1997; Eiroa *et al.*, 1999; Jensen, 2005), their presence does not always lead to product spoilage (Pettipher *et al.*, 1997; Pinhatti *et al.*, 1997). A number of factors contribute to create an environment favourable for product spoilage to occur, including the

Alicyclobacillus strain and cell concentration, temperature, the medium pH and the specific type of product and its constituents.

There is currently no standard accepted method for the isolation of *Alicyclobacillus* species, as a number of methods have been shown to be effective to varying degrees. The growth characteristics and spoilage potential of species of *Alicyclobacillus* occurring in the South African fruit processing environment are also not well characterised. As new *Alicyclobacillus* strains are isolated, it is important to investigate these characteristics in order to determine whether they pose a threat to processors and manufacturers and if steps need to be taken to eliminate them from the processing environment.

In this research three methods for *Alicyclobacillus* spp. isolation were compared to establish which method was most effective. Strains of *Alicyclobacillus* isolated from the South African fruit processing environment were also incubated at different temperatures and their growth, spore formation and guaiacol production was analysed and two guaiacol detection methods were compared. Furthermore, a strain of *A. acidoterrestris* was incubated in white grape juice containing different concentrations of vanillin, a known guaiacol precursor, to establish the ability of the strain to grow in juice and also the minimum concentration of vanillin that needs to be present for detectable guaiacol production to occur.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

Food and beverage products are classified as acidic if they have a pH of between 3.70-4.00 and 4.60 and highly acidic if they have a pH lower than 4.00-3.70. Most fruits fall in the latter category, with a few, such as tomatoes, pears and figs falling in the former (Jay *et al.*, 2005a). The low pH of acidic foods and beverages such as fruit products and fruit juice serves as a natural control measure against spoilage, as there are very few micro-organisms that can survive in the acidic environment (Jay *et al.*, 2005b). Spoilage of fruit juices had previously been attributed primarily to the growth of yeasts, fungi and lactic acid bacteria (Jay *et al.*, 2005a; Jay *et al.*, 2005b). Spore-forming bacteria were traditionally not of concern in the spoilage of fruit juices as the majority of spore-formers cannot survive in the acidic environment after spore germination (Jay *et al.*, 2005b; Jay *et al.*, 2005c). Because of this, fruit juices are traditionally only subjected to a pasteurisation treatment as this is sufficient to inactivate the spoilage micro-organisms of concern (Blocher & Busta, 1983). Products are then stored at ambient temperatures (Solberg *et al.*, 1990).

A new spoilage threat for acidic products emerged in 1984, with the report of a spoilage incident in Germany involving shelf-stable apple juice (Cerny *et al.*, 1984). The organism responsible for the incident was identified as the thermo-acidophilic bacterium *Alicyclobacillus acidoterrestris* (Cerny *et al.*, 1984; Deinhard *et al.*, 1987a; Wisotzkey *et al.*, 1992). Heat resistance studies revealed the ability of this bacterium to survive pasteurisation procedures normally applied to fruit juice and acidic products (Splittstoesser *et al.*, 1998; Eiroa *et al.*, 1999; Vieira *et al.*, 2002) and because of their acidophilic nature (Wisotzkey *et al.*, 1992) the spores can germinate and increase in products to cell concentrations high enough to produce taint compounds, leading to product spoilage (Pettipher *et al.*, 1997; Orr *et al.*, 2000; Gocmen *et al.*, 2005). Since their implication in the latter and other subsequent spoilage incidents (Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996a; Walls & Chuyate, 1998; Duong & Jensen, 2000; Jensen, 2000; Matsubara *et al.*, 2002;

Gouws *et al.*, 2005), species in the genus *Alicyclobacillus*, especially *A. acidoterrestris*, have become the focus of much research investigating their involvement in the spoilage of acidic food products, their production of taint compounds, and the development of isolation, detection and control procedures for these micro-organisms.

Surveys have shown that there is great potential for substantial product and consumer confidence losses, should a spoilage incident occur (Walls & Chuyate, 1998; Howard, 2006). *Alicyclobacillus* species have become a great concern to manufacturers and processors in the fruit industry and it has been suggested as a possible target organism in the design of pasteurisation processes for acidic products (Silva *et al.*, 2000; Vieira *et al.*, 2002; Silva & Gibbs, 2004).

B. HISTORY AND SPECIES CLASSIFICATION

Uchino and Doi reported the first case of the isolation of thermo-acidophilic bacteria in 1967. Three bacterial strains were isolated from hot-springs in the Tohoku district in Japan and were identified as part of the genus *Bacillus*. Even though they were dissimilar to *Bacillus coagulans* and *Bacillus stearothermophilus*, the two most well known thermophilic species at that time, they were tentatively classified as new strains of *B. coagulans* based on morphological and cultural characteristics.

Darland and Brock (1971) and De Rosa *et al.* (1971) isolated similar organisms from aqueous and terrestrial acid thermal environments in Yellowstone National Park in the United States of America (USA), Volcano National Park in Hawaii and Piciarelli in Italy. These isolates differed considerably more from *B. coagulans* than the isolates of Uchino and Doi (1967), especially in their pH optimum and DNA base composition. They also contained ω -cyclohexane fatty acids as the major components (up to 65%) in the saponifiable lipid fraction of their membranes (De Rosa *et al.*, 1971). It was proposed that they be classified into a new species, *Bacillus acidocaldarius* (Darland & Brock, 1971).

Hippchen *et al.* (1981) set out to identify relatives of *B. acidocaldarius* and isolated several thermo-acidophiles from a variety of neutral soils. These organisms possessed similar membrane properties to *B. acidocaldarius*, but their precise relationship to this bacterium could not be determined. Even though the potential of these organisms to be involved in food spoilage had already been recognised

(Uchino & Doi, 1967), it was only confirmed in 1984 when Cerny *et al.* (1984) reported the isolation of a bacterial strain closely related to those of Hippchen *et al.* (1981) from spoiled apple juice. Subsequently, this organism was classified into a new species, *Bacillus acidoterrestris* (Deinhard *et al.*, 1987a). A third thermo-acidophilic bacillus distinct from *B. acidocaldarius* and *B. acidoterrestris* was described by Poralla and König (1983). It differed from *B. acidocaldarius* and *B. acidoterrestris* in that it contained primarily ω -cycloheptane fatty acids in its membrane and it was subsequently classified into a new species, *Bacillus cycloheptanicus* (Poralla & König, 1983; Deinhard *et al.*, 1987b). Comparative sequence analyses carried out on the 16S ribosomal RNA (rRNA) genes of the three existing thermo-acidophilic *Bacillus* strains showed that they were distinct from any other *Bacillus* species. These findings led to the proposal of a new genus, *Alicyclobacillus*, to accommodate these unique bacteria (Wisotzkey *et al.*, 1992).

During the following years several new species belonging to the genus *Alicyclobacillus* were isolated from a variety of environments (Table 1). Species first classified in the genus *Sulfobacillus* were also reclassified into the genus *Alicyclobacillus* (Karavaiko *et al.*, 2005). The isolation of *A. pomorum* led to an amendment of the description of the genus *Alicyclobacillus*, since this species did not contain ω -alicyclic fatty acids in its membrane (Goto *et al.*, 2003). An amendment of the description of the species *A. acidocaldarius* was suggested by Goto *et al.* (2006) to include *A. acidocaldarius* subsp. *rittmannii* in the *A. acidocaldarius* species instead of classifying it as a separate subspecies. *Alicyclobacillus acidocaldarius* subsp. *rittmannii* is, however, still recognised as a subspecies (Anon., 2009). To date, 19 species, two subspecies and two genomic species belonging to the genus *Alicyclobacillus* have been identified, although the two genomic species are not formally recognised (Anon., 2009).

C. CHARACTERISTICS

General characteristics

The characteristics of all the *Alicyclobacillus* species identified to date are summarised in Table 1. *Alicyclobacillus* species are thermo-acidophilic, rod-shaped spore-formers. All species known to date are gram-positive, with the exception of

A. sendaiensis (Tsuruoka *et al.*, 2003). In many of the species old cultures have a tendency to be gram variable (Darland & Brock, 1971; Walls & Chuyate, 1998; Goto *et al.*, 2003; Karavaiko *et al.*, 2005; Goto *et al.*, 2007). All species are aerobic, with *A. pohliae* sometimes being facultatively anaerobic (Imperio *et al.*, 2008). Most are motile, with the exception of *A. acidocaldarius* subsp. *rittmannii* (Nicolaus *et al.*, 1998), *A. hesperidum* (Albuquerque *et al.*, 2000), *Alicyclobacillus* genomic species 1 (Albuquerque *et al.*, 2000), *A. sendaiensis* (Tsuruoka *et al.*, 2003), *A. tolerans* (Karavaiko *et al.*, 2005), *A. disulfidooxidans* (Karavaiko *et al.*, 2005), *A. fastidiosus* (Goto *et al.*, 2007) and *A. ferrooxydans* (Jiang *et al.*, 2008).

Alicyclobacillus spores are described as oval (Wisotzkey *et al.*, 1992; Walls & Chuyate, 1998; Goto *et al.*, 2002a; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Karavaiko *et al.*, 2005; Goto *et al.*, 2007), ellipsoidal (Wisotzkey *et al.*, 1992; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Tsuruoka *et al.*, 2003; Goto *et al.*, 2007) or round (Tsuruoka *et al.*, 2003; Imperio *et al.*, 2008) and located terminally (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Karavaiko *et al.*, 2005; Goto *et al.*, 2007; Imperio *et al.*, 2008), subterminally (Wisotzkey *et al.*, 1992; Walls & Chuyate, 1998; Goto *et al.*, 2002a; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Karavaiko *et al.*, 2005; Goto *et al.*, 2007) or centrally (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998), depending on the species and/or strain. Sporangia are mostly swollen (Goto *et al.*, 2002a; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Tsuruoka *et al.*, 2003; Karavaiko *et al.*, 2005; Goto *et al.*, 2007; Imperio *et al.*, 2008), although only slight swelling (Wisotzkey *et al.*, 1992; Walls & Chuyate, 1998) and sometimes no swelling (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002c) is observed for some species. Formation of endospores by bacteria is induced as a survival mechanism during adverse conditions (Brown, 2000). Bacterial spores are more resistant to heat, chemicals, irradiation and dehydration than vegetative cells and allow the micro-organism to survive hostile environments for long periods of time (Brown, 2000). Nutrient depletion/starvation conditions is the primary trigger for spore formation in bacteria (Errington, 1993; Bogdanova *et al.*, 2002; Setlow & Johnson, 2007) and increased spore formation is observed in bacterial cultures with a high cell density (Grossman & Losick, 1988).

Alicyclobacillus colonies on a variety of different growth media are round or circular (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Goto *et al.*, 2002a; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Tsuruoka *et al.*, 2003; Goto *et al.*, 2007; Jiang *et al.*, 2008), non-pigmented (Wisotzkey *et al.*, 1992; Albuquerque *et al.*, 2000; Goto *et al.*, 2002a; Goto *et al.*, 2003; Jiang *et al.*, 2008) or creamy white (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Goto *et al.*, 2007; Imperio *et al.*, 2008), translucent (Wisotzkey *et al.*, 1992; Walls & Chuyate, 1998; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004) to opaque (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Matsubara *et al.*, 2002; Goto *et al.*, 2007) and 0.30-5.00 mm in diameter (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002a; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Karavaiko *et al.*, 2005; Goto *et al.*, 2007; Imperio *et al.*, 2008; Jiang *et al.*, 2008). The temperature range of growth for all species except *A. disulfidooxidans* (Karavaiko *et al.*, 2005), *A. tolerans* (Karavaiko *et al.*, 2005) and *A. ferrooxydans* (Jiang *et al.*, 2008) is 20°-70°C (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002a; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Goto *et al.*, 2007; Imperio *et al.*, 2008; Jiang *et al.*, 2008), with the latter three species also able to grow at temperatures below 20°C. The optimum growth temperatures for these bacteria range from 35°-65°C (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002a; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Karavaiko *et al.*, 2005; Goto *et al.*, 2007; Imperio *et al.*, 2008; Jiang *et al.*, 2008). The pH range for growth is between 2.00-6.50 (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002a; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Goto *et al.*, 2007; Imperio *et al.*, 2008; Jiang *et al.*, 2008), again with the exception of *A. disulfidooxidans* and *A. tolerans* (Karavaiko *et al.*, 2005). These two species are able to grow at a pH of below 1.50. The pH optima range is 3.00-5.50 (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002a; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Goto *et al.*,

Table 1 Cultural, morphological and colony characteristics of species belonging to the genus *Alicyclobacillus*

<i>Alicyclobacillus</i> species	Source	Cultural characteristics			Morphological characteristics						Colony morphology			Reference
		pH range (optimum)	T-range (°C) (optimum)	Oxygen requirement	Gram stain	Shape	Cell Size (length x width μm)	Motility	Endospore characteristics	Sporangia swollen	Colour	Shape	Size (diameter mm)	
<i>A. acidocaldarius</i>	Thermal acid waters	2.00-6.00 (3.50-4.00)	45-71 (53-65)	Aerobic	+ to variable	Rod	1.5-3.0 x 0.5-0.8	Yes	Oval or ellipsoidal, 1.0-1.1 x 0.7-0.8 μm , terminal to subterminal	No to slightly	Unpigmented, cream yellow	Circular, flat or convex, smooth, irregular margins	1.0-2.0	Uchino & Doi, 1967; Darland & Brock, 1971; Wisotzkey <i>et al.</i> , 1992
<i>A. acidocaldarius</i> subsp. <i>acidocaldarius</i>	Subspecies automatically created according to Rule 40d (previously Rule 46) of the International Code of Nomenclature of Bacteria (1990 Revision). Characteristics the same as for <i>A. acidocaldarius</i> .											Goto <i>et al.</i> , 2006; Anon., 2009		
<i>A. acidocaldarius</i> subsp. <i>rittmannii</i>	Geothermal soil of Mount Rittmann, Antarctica	2.50-5.00 (4.00)	45-70 (63)	Aerobic	+	Rod	2.0-4.0 x 0.5-2.0	No	Central to terminal	No	Cream, opaque	Convex, circular, entire margins	0.8-1.0	Nicolaus <i>et al.</i> , 1998
<i>A. acidoterrestris</i>	Soil / apple juice	2.50-5.80 (4.50-5.00)	20-70 (36-53)	Aerobic	+ to variable	Rod	2.9-4.3 x 0.6-0.8	Yes	Oval, 1.5-1.8 x 0.9-1.0 μm , terminal, subterminal and central	No to slightly	Creamy white to yellowish, translucent to opaque	Round	3.0-5.0	Hippchen <i>et al.</i> , 1981; Deinhard <i>et al.</i> , 1987a; Wisotzkey <i>et al.</i> , 1992; Walls & Chuyate, 1998
<i>A. cycloheptanicus</i>	Soil	3.00-5.50 (3.50-4.50)	40-53 (48)	Aerobic	+	Rod	2.5-4.5 x 0.35-0.55	Yes	Oval, 1.0 x 0.75 μm , subterminal	Slightly	Creamy white, opaque	Round, small, smooth	nr	Poralla & König, 1983; Deinhard <i>et al.</i> , 1987b; Wisotzkey <i>et al.</i> , 1992
<i>A. hesperidum</i>	Solfataric soils of São Miguel, Azores	3.50-4.00	35-60 (50-53)	Aerobic	+	Rod	2.1-3.9 x 0.5-0.7	No	Terminal	No	Not pigmented	nr	1.0-2.0	Albuquerque <i>et al.</i> , 2000
<i>Alicyclobacillus</i> genomic species 1 (<i>A. mali</i>)	Solfataric soils of São Miguel, Azores	3.50-4.00	40-70 (60-63)	Aerobic	+	Rod	2.1-4.2 x 0.5-0.8	No	Terminal	No	Not pigmented	nr	1.0-2.0	Albuquerque <i>et al.</i> , 2000

Table 1 Continued

<i>Alicyclobacillus</i> species	Source	Cultural characteristics			Morphological characteristics						Colony morphology			Reference
		pH range (optimum)	T (°C) range (optimum)	Oxygen requirement	Gram stain	Shape	Cell Size (length x width µm)	Motility	Endospore characteristics	Sporangia swollen	Colour	Shape	Size (diameter mm)	
<i>Alicyclobacillus</i> genomic species 2	Soil near a geyser in Kirishima, Japan	2.00-6.50 (4.00-4.50)	35-70 (55-60)	Aerobic	+	Rod	2.0-4.5 x 0.5-1.0	Yes	Ellipsoidal, terminal or subterminal	No	Creamy white, slightly mucous	Round	1.0-4.0	Goto <i>et al.</i> , 2002c
<i>A. herbarius</i>	Herbal tea	3.50-6.00 (4.50-5.00)	35-65 (55-60)	Aerobic	+	Rod	nr	Yes	Oval, subterminal	Yes	Not pigmented	Circular	2.0-3.0	Goto <i>et al.</i> , 2002a
<i>A. acidiphilus</i>	Acidic beverage	2.50-5.50 (3.00)	20-55 (50)	Aerobic	+	Rod	0.9-1.1 x 4.8-6.3	Yes	Ellipsoidal to oval, terminal to subterminal	Yes	Creamy white, opaque	Round, smooth	1.1-3.8	Matsubara <i>et al.</i> , 2002
<i>A. pomorum</i>	Mixed fruit juice	3.00-6.00 (4.00-4.50)	30-60 (45-50)	Aerobic	+ to variable	Rod	2.0-4.0 x 0.8-1.0	Yes	Oval, subterminal	Yes	Not pigmented	Circular	3.0-4.0	Goto <i>et al.</i> , 2003
<i>A. sendaiensis</i>	Soil, Japan	2.50-6.50 (5.50)	40-65 (55)	Aerobic	-	Rod	2.0-3.0 x 0.8	No	Round or ellipsoidal, terminal	Yes	White and semi-transparent	Circular, convex	1.0	Tsuruoka <i>et al.</i> , 2003
<i>A. vulcanalis</i>	Geothermal pool, Coso hot springs, California	2.00-6.00 (4.00)	35-65 (55)	Aerobic	+	Rod	1.5-2.5 x 0.4-0.7	nr	Terminal	nr	Semi-transparent to white	Convex	1.0	Simbahan <i>et al.</i> , 2004
<i>A. tolerans</i>	Oxidizable lead-zinc ores	1.50-5.00 (2.50-2.70)	<20-55 (37-42)	Aerobic	+	Rod	3.0-6.0 x 0.9-1.0	No	Oval, terminal or subterminal	Yes	nr	nr	0.3-0.5	Karavaiko <i>et al.</i> , 2005
<i>A. disulfidooxidans</i>	Waste water sludge	0.50-6.00 (1.50-2.50)	4-40 (35)	Aerobic	+ to variable	Rod	0.9-3.6 x 0.3-0.5	No	Oval, 0.9-1.8 x 0.7-0.9, subterminal or terminal	Yes	nr	nr	nr	Dufresne <i>et al.</i> , 1996; Karavaiko <i>et al.</i> , 2005
<i>A. contaminans</i>	Soil from crop fields in Fuji city	3.50-5.50 (4.00-4.50)	35-60 (50-55)	Aerobic	+ to variable	Rod	4.0-5.0 x 0.8-0.9	Yes	Ellipsoidal, subterminal	Yes	Non-pigmented (creamy white), opaque	Circular, entire, umbonate	3.0-5.0	Goto <i>et al.</i> , 2007

Table 1 Continued

<i>Alicyclobacillus</i> species	Source	Cultural characteristics			Morphological characteristics						Colony morphology			Reference
		pH range (optimum)	T (°C) range (optimum)	Oxygen requirement	Gram stain	Shape	Cell Size (length x width µm)	Motility	Endospore characteristics	Sporangia swollen	Colour	Shape	Size (diameter mm)	
<i>A. fastidiosus</i>	Apple juice	2.50-5.00 (4.00-4.50)	20-55 (40-45)	Aerobic	+ to variable	Rod	4.0-5.0 x 0.9-1.0	No	Ellipsoidal, subterminal	Yes	Non-pigmented (creamy white), opaque	Circular, entire, flat	3.0-4.0	Goto <i>et al.</i> , 2007
<i>A. kakegawensis</i>	Soil from crop fields in Kakegawa city	3.50-6.00 (4.00-4.50)	40-60 (50-55)	Aerobic	+ to variable	Rod	4.0-5.0 x 0.6-0.7	Yes	Oval, subterminal	Yes	Non-pigmented (creamy white), opaque	Circular, entire, flat	2.0-3.0	Goto <i>et al.</i> , 2007
<i>A. macrosporangioidus</i>	Soil from crop fields in Fujieda city	3.50-6.00 (4.00-4.50)	35-60 (50-55)	Aerobic	+ to variable	Rod	5.0-6.0 x 0.7-0.8	Yes	Oval, terminal	Yes	Non-pigmented, (creamy white), opaque	Circular, entire, convex	2.0-4.0	Goto <i>et al.</i> , 2007
<i>A. sacchari</i>	Liquid sugar	2.50-5.50 (4.00-4.50)	30-55 (45-50)	Aerobic	+ to variable	Rod	4.0-5.0 x 0.6-0.7	Yes	Ellipsoidal, subterminal	Yes	Non-pigmented (creamy white), opaque	Circular, entire, umbonate	3.0-5.0	Goto <i>et al.</i> , 2007
<i>A. shizuokensis</i>	Soil from crop fields in Shizuoka city	3.50-6.00 (4.00-4.50)	35-60 (45-50)	Aerobic	+ to variable	Rod	4.0-5.0 x 0.7-0.8	Yes	Oval, subterminal	Yes	Non-pigmented (creamy white), opaque	Circular, entire, convex	1.0-2.0	Goto <i>et al.</i> , 2007
<i>A. pohliae</i>	Geothermal soil of Mount Melbourne, Antarctica	4.50-7.50 (5.50)	42-60 (55)	Aerobic, facultatively anaerobic	+	Rod	1.5-2.5 x 0.4-0.6	nr	Round, terminal	Yes	Cream-coloured	Entire, convex	1.5-2.0	Imperio <i>et al.</i> , 2008
<i>A. ferrooxydans</i>	Solfataric soil	2.00-6.00 (3.00)	17-40 (28)	Aerobic	+	Rod / coccus	1.0-1.5 x 0.4-0.6	No	nr	nr	Non-pigmented	Pinpoint, circular, entire	0.3-0.5	Jiang <i>et al.</i> , 2008

nr - not reported

2003; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Goto *et al.*, 2007; Imperio *et al.*, 2008; Jiang *et al.*, 2008), except for *A. disulfidooxidans* and *A. tolerans*, having much lower pH optima ranging from 1.50-2.00 (Karavaiko *et al.*, 2005).

The soluble solids (SS) content of juices affects the growth of *Alicyclobacillus* spp. Splittstoesser *et al.* (1994) observed that *A. acidoterrestris* VF was able to grow in Riesling grape juice with a soluble solids content ranging from 5.40°-16.20°Brix, while a SS content of 21.60°Brix inhibited growth. Thus, growth of *Alicyclobacillus* spp. in juice concentrates would be inhibited, but upon dilution to form single strength juice, spores present in the concentrate could multiply to numbers high enough to cause spoilage (Pettipher & Osmundson, 2000).

Since all species of *Alicyclobacillus* are aerobic (Table 1) the amount of oxygen present in the growth medium influences the growth of the organisms. Walker and Phillips (2005) found that containers with 0% headspace showed a significantly lower level of growth when compared to containers containing a headspace. In contrast, Cerny *et al.* (2000) found that the presence or absence of a headspace in the packaging system did not significantly influence the growth of *A. acidoterrestris* and no spoilage of juices was observed under either condition (Cerny *et al.*, 2000). In apple juice, low residual oxygen concentrations (7-3%) resulted in more rapid growth than atmospheric concentrations (21%), although final cell counts were higher at atmospheric concentrations. In orange juice only anaerobic conditions could prevent growth of *A. acidoterrestris* (Walker & Phillips, 2005). Siegmund and Pöllinger-Zierler (2007) also found that a limited oxygen supply slowed the growth rate of *A. acidoterrestris*, but did not prevent it from reaching high cell concentrations. In contrast to the observations made by Cerny *et al.* (2000), Siegmund & Pöllinger-Zierler (2007) found that a limited oxygen supply did not prevent guaiacol production and spoilage occurred under both free and limited oxygen supply.

Pathogenicity

When *Alicyclobacillus* species became apparent as spoilage organisms, concerns arose about pathogenicity. Walls and Chuyate (2000a) undertook a study to determine the pathogenicity of several strains of *A. acidoterrestris*, as well as a strain of *A. acidocaldarius*. Mice were injected intraperitoneally with a mixture of cells

grown in orange serum broth and observed for one week for signs of illness. Guinea pigs were fed with spoiled apple juice containing 5×10^6 cfu.mL⁻¹ *A. acidoterrestris* and also observed for one week. No adverse symptoms, illnesses or deaths were observed in either the mice or the guinea pigs and it was concluded that species of *Alicyclobacillus* were not pathogenic at the levels tested. Although *Alicyclobacillus* bacteria pose an economic threat to the fruit processing industry, consumption of products containing *Alicyclobacillus* spp. does not pose a health or safety risk (Borlinghaus & Engel, 1997; Walls & Chuyate, 2000b).

Membrane structure

One of the characteristics that distinguish species of *Alicyclobacillus* from other *Bacillus* species is the predominance of ω -alicyclic fatty acids in their cellular membranes. In a strain of *A. acidocaldarius* isolated in Italy, up to 70% of the saponifiable membrane lipid extract consisted of ω -cyclohexane fatty acids (De Rosa *et al.*, 1971). In agreement with this Oshima and Ariga (1975) found that the total fatty acid content of strains of *A. acidocaldarius* isolated from Japanese thermal acid environments consisted of 74% to 93% ω -cyclohexane fatty acids. Investigations into the lipid content of the membranes of *A. acidoterrestris* showed that, depending on the strain, ω -cyclohexane fatty acids comprised 15-91% of the total fatty acid content (Hippchen *et al.*, 1981).

The types of ω -alicyclic fatty acids found in the membranes of *Alicyclobacillus* spp. are not limited to ω -cyclohexane fatty acids. These thermo-acidophilic bacteria were also found to contain ω -cycloheptane fatty acids (Poralla & König, 1983; Deinhard *et al.*, 1987b). Of the 23 species, subspecies and genomic species known to date, 14 possess predominantly ω -cyclohexane fatty acids in their membranes. These are *A. acidocaldarius* (Uchino & Doi, 1967; Darland & Brock, 1971; Wisotzkey *et al.*, 1992), *A. acidocaldarius* subsp. *acidocaldarius* (Goto *et al.*, 2006; Anon., 2009;), *A. acidoterrestris* (Hippchen *et al.*, 1981; Deinhard *et al.*, 1987a; Wisotzkey *et al.*, 1992; Walls & Chuyate, 1998), *A. hesperidum* (Albuquerque *et al.*, 2000), *Alicyclobacillus* genomic species 1 (Albuquerque *et al.*, 2000), *Alicyclobacillus* genomic species 2 (Goto *et al.*, 2002c), *A. acidocaldarius* subsp. *rittmannii* (Nicolaus *et al.*, 1998), *A. acidiphilus* (Matsubara *et al.*, 2002), *A. sendaiensis* (Tsuruoka *et al.*, 2003), *A. vulcanalis* (Simbahan *et al.*, 2004), *A. tolerans* (Karavaiko *et al.*, 2005),

A. disulfdooxidans (Dufresne *et al.*, 1996; Karavaiko *et al.*, 2005), *A. fastidiosus* (Goto *et al.*, 2007) and *A. sacchari* (Goto *et al.*, 2007). Four species of *Alicyclobacillus*, namely *A. cycloheptanicus* (Poralla & König, 1983; Deinhard *et al.*, 1987b; Wisotzkey *et al.*, 1992), *A. herbarius* (Goto *et al.*, 2002a), *A. kakegawensis* (Goto *et al.*, 2007) and *A. shizuokensis* (Goto *et al.*, 2007) possess predominantly ω -cycloheptane fatty acids.

Alicyclobacillus pomorum was found not to contain ω -alicyclic fatty acids in its membrane, but rather straight- and/or branched-chain saturated fatty acids also found in the membranes of *Bacillus* species. Nevertheless, *A. pomorum* was classified into the genus *Alicyclobacillus* based on phylogenetic analyses of the 16S rRNA and DNA gyrase B subunit (*gyrB*) gene sequences. This led to an amendment of the description of the genus *Alicyclobacillus* to include organisms not containing ω -alicyclic fatty acids in their membranes (Goto *et al.*, 2003). Four other *Alicyclobacillus* species, namely *A. contaminans* (Goto *et al.*, 2007), *A. macrosporangioides* (Goto *et al.*, 2007), *A. pohliae* (Imperio *et al.*, 2008) and *A. ferrooxydans* (Jiang *et al.*, 2008) have this fatty acid profile.

A number of species contain hopanoids in their membranes (Poralla *et al.*, 1980; Hippchen *et al.*, 1981; Cerny *et al.*, 1984). The hopane ring is structurally similar to cholesterol, which is known to affect membrane lipid organisation (Poralla *et al.*, 1980). It has been shown that the hopane glycolipids have a condensing effect on the membrane, which decreases the mobility of the acyl chains of the lipids and stabilises the membrane. This condensing action is also advantageous at low pH, since it hinders the passive diffusion of protons through the membrane, thereby facilitating the establishment of an approximately neutral cytoplasmic pH (Poralla *et al.*, 1980). The membrane stabilisation effect of hopanoids is further confirmed by the observation that mutant cells containing only branched-chain fatty acids have significantly higher hopanoid contents when compared to cells containing ω -cyclohexane fatty acids. The presence of a higher concentration of hopanoids compensates for the low membrane viscosity induced by the branched-chain fatty acids, leading to a more stable membrane (Krischke & Poralla, 1990).

Function of ω -alicyclic fatty acids in the membrane

There has been speculation on the function of ω -alicyclic fatty acids found in the

membranes of most *Alicyclobacillus* species. Some researchers have suggested that they contribute to the heat resistance and thermo-acidophilic nature of the organisms. Kannenberg *et al.* (1984) studied the properties of ω -cyclohexane fatty acids in model membranes and found that the presence of the cyclohexane ring increased the acyl chain density, leading to a denser packing of the lipids in the membrane core, structural stabilisation of the membrane, lower membrane fluidity and reduced permeability. This may contribute to the maintenance of the barrier function of the membrane, protecting the organism against acidic conditions and high temperatures (Oshima & Ariga, 1975; Kannenberg *et al.*, 1984; Krischke & Poralla, 1990; Chang & Kang, 2004). Mutants of *A. acidocaldarius* that were unable to synthesise ω -cyclohexane fatty acids had a lower growth yield at low pH and high temperature conditions compared to wild-type organisms. Their sensitivity to heat shock and ethanol was also increased, as growth was inhibited after a heat shock treatment at 72°C for 20-80 min or at an ethanol concentration of 3% (v/v) (Krischke & Poralla, 1990).

Heat resistance

Several studies have been conducted to investigate the heat resistance of *Alicyclobacillus* spores under different conditions and in a variety of media. A summary of heat resistance parameters for strains of *A. acidoterrestris* and *A. acidocaldarius* in fruit products and buffers is given in Tables 2, 3 and 4. D_{95} -values determined for different strains of *A. acidoterrestris* in apple juice (pH 3.50-3.51, 11.40°Brix) (Splittstoesser *et al.*, 1994; Komitopoulou *et al.*, 1999), grape juice (pH 3.30, 15.80°Brix) (Splittstoesser *et al.*, 1994), berry juice (McIntyre *et al.*, 1995), orange juice (pH 3.15-4.10, 5.30°-9.00°Brix) (Splittstoesser *et al.*, 1994; Baumgart *et al.*, 1997; Eiroa *et al.*, 1999; Komitopoulou *et al.*, 1999), a fruit drink (pH 3.50, 4.80°Brix) (Baumgart *et al.*, 1997), a fruit nectar (pH 3.50, 6.10°Brix) (Baumgart *et al.*, 1997), concord grape juice (pH 3.50, 16.00°-30.00°Brix) (Splittstoesser *et al.*, 1998), cupuaçu extract (pH 3.60, 11.30°Brix) (Silva *et al.*, 1999), grapefruit juice (pH 3.42) (Komitopoulou *et al.*, 1999), mango pulp (pH 4.00) (De Carvalho *et al.*, 2008), clarified lemon juice (pH 3.50, 6.20-9.80°Brix) (Maldonado *et al.*, 2008) and non-clarified lemon juice (pH 2.45, 6.20-9.80°Brix) (Maldonado *et al.*, 2008) range from 1.00 to 9.98 min. The D_{90} -values in apple juice (pH 3.20-3.68, 11.40°-12.20°Brix)

(Cerny *et al.*, 1984; Splittstoesser *et al.*, 1994; Komitopoulou *et al.*, 1999; Bahçeci & Acar, 2007b), grape juice (pH 3.30, 15.80°Brix) (Splittstoesser *et al.*, 1994), concord grape juice (pH 3.50, 16.00°-30.00°Brix) (Splittstoesser *et al.*, 1998), orange juice (pH 3.15-3.90 9.00°Brix) (Eiroa *et al.*, 1999; Komitopoulou *et al.*, 1999), grapefruit juice (pH 3.42) (Komitopoulou *et al.*, 1999), a clear apple drink (Yamazaki *et al.*, 2000), an orange drink (Yamazaki *et al.*, 2000), apple nectar without ascorbic acid (pH 2.97, 14.00°Brix) (Bahçeci & Acar, 2007b), apple nectar with ascorbic acid (pH 2.95, 14.00°Brix) (Bahçeci & Acar, 2007b) and mango pulp (pH 4.00) (De Carvalho *et al.*, 2008) range from 5.95 to 23.10 min. The z-values range from 6.90 to 21.27 in different fruit products (Splittstoesser *et al.*, 1994; McIntyre *et al.*, 1995; Baumgart *et al.*, 1997; Splittstoesser *et al.*, 1998; Eiroa *et al.*, 1999; Komitopoulou *et al.*, 1999; Silva *et al.*, 1999; Bahçeci & Acar, 2007b; De Carvalho *et al.*, 2008) and from 5.90 to 10.00 in buffers (Pontius *et al.*, 1998; Alpas *et al.*, 2003; Bahçeci & Acar, 2007b).

Heat resistance values obtained in fruit products are higher when compared to those obtained in buffers at the same heating temperature and pH. This could be due to constituents of the fruit products that increase the heat resistance of spores (Bahçeci & Acar, 2007b). The range of D-values observed between different studies may be attributed to differences in strains, sporulation temperature, nutrient composition and pH of the heating medium, water activity and presence or absence of divalent cations and antimicrobial compounds (Bahçeci & Acar, 2007b).

Since *A. acidoterrestris* is the *Alicyclobacillus* species mostly associated with spoilage, most studies have focused on the investigation of the heat resistance of this species. However, Palop *et al.* (2000) investigated the heat resistance of *A. acidocaldarius* in McIlvaine buffers of different pH, as well as in distilled water and orange juice. No significant differences were observed in the heat resistance of *A. acidocaldarius* between the different heating mediums, with recorded D₁₂₀-values of 0.087 to 0.11 min. Z-values also did not differ significantly and ranged between 6.50°C and 7.50°C. Thus, neither the pH, nor the composition of the heating medium, affected the heat resistance at any of the evaluated temperatures. This strain of *A. acidocaldarius* was significantly more heat resistant than *A. acidoterrestris* strains investigated by other researchers (Splittstoesser *et al.*, 1994; McIntyre *et al.*, 1995; Murakami *et al.*, 1998; Pontius *et al.*, 1998; Splittstoesser *et al.*, 1998; Eiora *et al.*, 1999), but had z-values comparable to those obtained by these authors, indicating a similar thermodependence.

Table 2 Heat resistance of *A. acidoterrestris* in various fruit juices and concentrates

Heating medium	pH	SS (°Brix)	Strain	T (°C)	D-value ± SD / SE (min)	z-value (°C)	Reference
Apple juice	3.20	nr	nr	90	15.00±nr	nr	Cerny <i>et al.</i> , 1984
Apple juice	3.50	11.40	VF	85 90 95	56.00±14.00 23.00±7.50 2.80±0.70	7.70	Splittstoesser <i>et al.</i> , 1994
Grape juice	3.30	15.80	WAC	85 90 95	57.00±13.00 16.00±4.10 2.40±0.90	7.20	Splittstoesser <i>et al.</i> , 1994
Berry juice	nr	nr	nr	81.8 91.1 95	11.00±nr 3.80±nr 1.00±nr	7.20	McIntyre <i>et al.</i> , 1995
Orange juice	4.10	5.30	nr	95	5.30±nr	9.50	Baumgart <i>et al.</i> , 1997
Fruit drink	3.50	4.80	nr	95	5.20±nr	10.80	Baumgart <i>et al.</i> , 1997
Fruit nectar	3.50	6.10	nr	95	5.10±nr	9.60	Baumgart <i>et al.</i> , 1997
Concord grape juice	3.50	16.00	WAC	85 90 95	53.00±nr 11.00±nr 1.90±nr	6.90	Splittstoesser <i>et al.</i> , 1998
		30.00	WAC	85 90 95	76.00±nr 18.00±nr 2.30±nr	6.60	Splittstoesser <i>et al.</i> , 1998
		65.00	WAC	85 90 95	276.00±nr 127.00±nr 12.00±nr	7.40	Splittstoesser <i>et al.</i> , 1998
Orange juice	3.15	9.00	46	85 90 95	60.8±nr 10±nr 2.5±nr	7.20	Eiroa <i>et al.</i> , 1999
			70	85 90 95	67.30±nr 15.60±nr 8.70±nr	11.30	Eiroa <i>et al.</i> , 1999
			145	85 90 95	94.50±nr 20.60±nr 3.80±nr	7.20	Eiroa <i>et al.</i> , 1999
			DSM 2498	85 90 95	50.00±nr 16.90±nr 2.70±nr	7.90	Eiroa <i>et al.</i> , 1999
Cupuaçu extract	3.60	11.30	NCIMB 13137	85 91 95 97	17.50±1.10 5.35±0.57 2.82±0.27 0.57±0.034	9.00	Silva <i>et al.</i> , 1999
Orange juice	3.50	11.70	NCIMB 13137	85 91	65.60±5.50 11.90±0.60	7.80	Silva <i>et al.</i> , 1999
Light blackcurrant concentrate	2.50	26.10	NCIMB 13137	91	3.84±0.49	nr	Silva <i>et al.</i> , 1999
Blackcurrant concentrate	2.50	58.50	NCIMB 13137	91	24.10±2.70	nr	Silva <i>et al.</i> , 1999

Table 2 Continued

Heating medium	pH	SS (°Brix)	Strain	T (°C)	D-value ± SD / SE (min)	z-value (°C)	Reference
Apple juice	3.51	nr	Z CRA 7182	80 90 95	41.15±0.24 7.38±0.85 2.30±0.03	12.20	Komitopoulou <i>et al.</i> , 1999
Grapefruit juice	3.42	nr	Z CRA 7182	80 90 95	37.87±0.20 5.95±0.32 1.85±0.05	11.60	Komitopoulou <i>et al.</i> , 1999
Orange juice	3.90	nr	Z CRA 7182	80 90 95	54.30±0.42 10.30±0.30 3.59±0.04	12.90	Komitopoulou <i>et al.</i> , 1999
Clear apple drink	nr	nr	AB-5	90	20.80±nr	nr	Yamazaki <i>et al.</i> , 2000
Orange drink	nr	nr	AB-5	90	23.10±nr	nr	Yamazaki <i>et al.</i> , 2000
Apple juice	3.68	12.20	DSM 2498	90 93 96 100	11.10±1.60 4.20±0.70 2.10±0.20 0.70±0.00	8.50	Bahçeci & Acar, 2007b
Apple nectar without ascorbic acid	2.97	14.00	DSM 2498	90 93 96 100	14.40±0.80 6.70±0.60 3.30±0.30 1.20±0.00	9.20	Bahçeci & Acar, 2007b
Apple nectar with ascorbic acid (250 mg/L)	2.95	14.00	DSM 2498	90 93 96 100	14.10±0.50 6.40±0.50 3.10±0.30 1.00±0.00	8.80	Bahçeci & Acar, 2007b
Mango pulp	4.00	nr	DSM 2498	80 85 90 95	40.00±1.5 25.00±0.10 11.66±1.8 8.33±2.00	21.27	De Carvalho <i>et al.</i> , 2008
Clarified lemon juice / concentrate	2.28	50.00	nr	82 86 92 95	17.36±nr 18.06±nr 7.60±nr 6.20±nr	nr	Maldonado <i>et al.</i> , 2008
	2.80	50.00	nr	82 86 92 95	25.81±nr 22.01±nr 15.35±nr 11.32±nr	nr	Maldonado <i>et al.</i> , 2008
	3.50	50.00	nr	82 86 92 95	33.66±nr 68.95±nr 16.87±nr 12.63±nr	nr	Maldonado <i>et al.</i> , 2008
		9.80	nr	82 86 92 95	11.23±nr 10.54±nr 9.47±nr 8.55±nr	nr	Maldonado <i>et al.</i> , 2008
		6.20	nr	82 95	13.21±nr 9.38±nr	nr	Maldonado <i>et al.</i> , 2008
	4.00	50.00	nr	82 86 92 95	21.95±nr 35.16±nr 23.19±nr 9.72±nr	nr	Maldonado <i>et al.</i> , 2008

Table 2 Continued

Heating medium	pH	SS (°Brix)	Strain	T (°C)	D-value ± SD / SE (min)	z-value (°C)	Reference	
Non-clarified lemon juice / concentrate	2.28	68.00	nr	82	15.50±nr	nr	Maldonado <i>et al.</i> , 2008	
				86	14.54±nr			
				92	8.81±nr			
				95	8.55±nr			
	2.45	50.00	nr	nr	82	15.50±nr	nr	Maldonado <i>et al.</i> , 2008
					86	14.54±nr		
					92	8.81±nr		
					95	8.56±nr		
	9.80	nr	nr	nr	82	16.72±nr	nr	Maldonado <i>et al.</i> , 2008
					86	11.32±nr		
					92	10.58±nr		
					95	9.98±nr		
	6.20	nr	nr	nr	82	17.82±nr	nr	Maldonado <i>et al.</i> , 2008
					95	9.44±nr		
	2.80	68.00	nr	nr	82	50.50±nr	nr	Maldonado <i>et al.</i> , 2008
					86	31.67±nr		
					92	39.30±nr		
					95	22.02±nr		
	3.50	68.00	nr	nr	82	38.00±nr	nr	Maldonado <i>et al.</i> , 2008
					86	95.15±nr		
					92	59.50±nr		
					95	17.22±nr		
	4.00	68.00	nr	nr	82	27.48±nr	nr	Maldonado <i>et al.</i> , 2008
					86	58.15±nr		
92					85.29±nr			
95					23.33±nr			

nr, not reported; SD, standard deviation; SE, standard error

Table 3 Heat resistance of *A. acidoterrestris* spores in various buffers

Heating medium	pH	SS (°Brix)	Strain	T (°C)	D-value ± SD / SE (min)	z-value	Reference	
Buffers representing a model fruit juice system acidified with: Malic acid	2.80	nr	VF	94	12.30±nr	nr	Pontius <i>et al.</i> , 1998	
	3.10	nr	VF	91	31.30±nr	10.00		
				97	7.90±nr			
	3.40	nr	VF	VF	88	81.20±nr		5.90
					94	16.60±nr		
					100	0.80±nr		
3.70	nr	VF	VF	91	54.30±nr	7.70		
97	8.80±nr							
4.00	nr	VF	VF	94	20.70±nr	nr		
Citric acid	3.10	nr	VF	91	46.10±nr	8.50	Pontius <i>et al.</i> , 1998	
				97	8.20±nr			
	3.70	nr	VF	VF	91	57.90±nr		8.20
				97	10.80±nr			

Table 3 Continued

Heating medium	pH	SS (°Brix)	Strain	T (°C)	D-value ± SD / SE (min)	z-value	Reference
Tartaric acid	3.10	nr	VF	91 97	49.10±nr 8.40±nr	7.80	Pontius <i>et al.</i> , 1998
	3.70	nr	VF	91 97	69.50±nr 10.00±nr	7.10	
Buffers representing a model fruit juice system acidified with:							
Malic acid	3.10	nr	WAC	91 97	40.50±nr 8.00±nr	8.50	Pontius <i>et al.</i> , 1998
	3.70	nr	WAC	91 97	53.20±nr 9.00±nr	7.70	
Buffers representing a model fruit juice system acidified with:							
Malic acid	3.10	nr	IP	91 97	20.30±nr 3.60±nr	8.00	Pontius <i>et al.</i> , 1998
	3.70	nr	IP	91 97	32.60±nr 3.80±nr	6.50	
Citrate buffer:							
20 mM	6.00	nr	AB-1	90	13.60±0.16	nr	Murakami <i>et al.</i> , 1998
100 mM	6.00	nr	AB-1	90	14.40±0.31	nr	
Phosphate buffer:							
20 mM	6.00	nr	AB-1	90	12.90±0.20	nr	Murakami <i>et al.</i> , 1998
100 mM	6.00	nr	AB-1	90	12.30±0.21	nr	
Mcllvaine buffer							
	3.00	nr	AB-1	88	24.10±1.63	nr	Murakami <i>et al.</i> , 1998
				90	14.80±1.28		
				92	6.20±0.37		
				95	2.70±0.50		
	4.00	nr	AB-1	88	25.9±1.45	nr	
				90	16.1±0.59		
				92	6.1±0.30		
				95	2.8±0.21		
	5.00	nr	AB-1	88	29.10±1.87	nr	
				90	16.60±1.68		
				92	7.10±0.18		
				95	2.70±0.11		
	6.00	nr	AB-1	88	25.90±0.35	nr	
				90	16.80±0.28		
				92	6.80±0.40		
				95	2.30±0.41		
	7.00	nr	AB-1	88	24.70±0.21	nr	
				90	15.70±0.71		
				92	6.70±1.20		
				95	2.20±0.56		
	8.00	nr	AB-1	88	25.70±1.01	nr	
				90	16.10±1.58		
				92	5.70±0.13		
				95	2.30±0.42		

Table 3 Continued

Heating medium	pH	SS (°Brix)	Strain	T (°C)	D-value ± SD / SE (min)	z-value	Reference
Bam broth	3.00	nr	DSM 2492	50	18.86±nr	8.50	Alpas <i>et al.</i> , 2003
Mcllvaine buffer	3.00	nr	DSM 2498	90	6.00±0.7	8.20	Bahçeci & Acar, 2007b
				93	2.80±0.3		
				96	1.10±0.2		
				100	0.40±0.2		
	3.50	nr	DSM 2498	90	6.50±1.4	8.40	
				93	3.20±0.8		
				96	1.30±0.0		
				100	0.40±0.0		
	4.00	nr	DSM 2498	90	7.30±2.1	8.50	
				93	3.80±1.3		
				96	1.70±0.3		
				100	0.50±0.1		

nr, not reported; SD, standard deviation; SE, standard error

Table 4 Heat resistance of *A. acidocaldarius* spores in distilled water, buffer and orange juice

Heating medium	pH	SS (°Brix)	Strain	T (°C)	D-value ± SD / SE (min)	z-value	Reference
Mcllvaine buffer	7.00	nr	STCC 5137	110	2.60±0.30	6.70±1.00	Palop <i>et al.</i> , 2000
				115	0.54±0.07		
				120	0.097±0.010		
				125	0.014±0.002		
	4.00	nr	STCC 5137	110	2.60±0.60	7.50±2.40	Palop <i>et al.</i> , 2000
				115	0.99±0.43		
				120	0.11±0.07		
				125	0.035±0.002		
Distilled water	nr	nr	STCC 5137	110	3.70±1.60	6.70±0.30	Palop <i>et al.</i> , 2000
				115	0.48±0.17		
				120	0.11±0.01		
				125	0.024±0.007		
Orange juice	nr	nr	STCC 5137	110	3.90±0.10	6.80±1.50	Palop <i>et al.</i> , 2000
				115	0.61±0.34		
				120	0.087±0.009		
				125	0.027±0.007		

nr, not reported; SD, standard deviation; SE, standard error

Spores of a variety of *A. acidoterrestris* strains (Splittstoesser *et al.*, 1994; Baumgart *et al.*, 1997; Splittstoesser *et al.*, 1998; Eiora *et al.*, 1999; Komitopoulou *et al.*, 1999; Silva *et al.*, 1999; Yamazaki *et al.*, 2000; Bahçeci & Acar, 2007b; De Carvalho *et al.*, 2008; Maldonado *et al.*, 2008) and a strain of *A. acidocaldarius* (Palop *et al.*, 2000) are, therefore, sufficiently heat resistant to enable them to survive the hot-fill-hold pasteurisation process to which fruit juice and similar products are exposed in order to render them commercially sterile. This process involves heating the product at 90°-95°C for 15-20 s, followed by package filling while the product cools to 82°-84°C. The product is then held at this temperature for approximately 2 min before chilling (Solberg *et al.*, 1990). Due to its high heat resistance and involvement in several spoilage incidents, it has been suggested that *A. acidoterrestris* be designated the target organism in the design of pasteurisation processes for acidic foods and beverages (Silva *et al.*, 1999; Silva *et al.*, 2000; Silva & Gibbs, 2001; Silva & Gibbs, 2004).

Factors influencing heat resistance

Temperature

Temperature has the greatest influence on D-values, with a non-linear decrease in D-values (indicating a decreased heat resistance) observed with an increase in temperature (Silva *et al.*, 1999; Bahçeci & Acar, 2007b; Maldonado *et al.*, 2008). The effect of temperature on D-values is three times greater than that of pH and slight changes in temperature have a considerable effect on D-values (Silva *et al.*, 1999; Bahçeci & Acar, 2007b). Temperature also affects the role that other parameters such as pH and SS play in the overall effect on D-values, as their effects are more pronounced at lower temperatures. Manufacturers need to also take these effects into account when processing in a lower temperature range (Pontius *et al.*, 1997; Komitopoulou *et al.*, 1999; Silva *et al.*, 1999).

pH

Most studies found that pH had an effect on heat resistance, with a linear decrease in D-values being observed with a decrease in pH (Silva *et al.*, 1999). This effect seems to be more pronounced at lower temperatures (Pontius *et al.*, 1997; Komitopoulou *et al.*, 1999, Silva *et al.*, 1999). In contrast to this, Murakami *et al.*

(1998) found that pH did not have a significant influence on heat resistance, as there were no significant differences between D-values of *A. acidoterrestris* AB-1 spores in McIlvaine buffer at pH values ranging from 3.00-8.00 at a given temperature (Murakami *et al.*, 1998). Temperature and specific properties of different juices seem to play a bigger role than pH in contributing to heat sensitivity, as *A. acidoterrestris* still had a lower heat resistance in grapefruit juice than in orange juice, even though grapefruit juice had a higher pH of 4.00 compared to 3.90 of the orange juice (Komitopoulou *et al.*, 1999). Furthermore, the type of acid used to acidify the heating medium does not influence the heat resistance, as the D-values obtained in a model fruit juice system acidified with malic, tartaric or citric acids did not differ significantly from one another in the temperature range (91°-100°C) studied (Pontius *et al.*, 1998).

Soluble solids (SS) content

The total SS also influences the heat resistance of species of *Alicyclobacillus*. There is a linear relationship (Silva *et al.*, 1999) between SS and D-values, with an increase in SS content leading to an increase in D-values and a higher heat resistance. Therefore, destruction of spores would be more difficult in fruit juice concentrate than in single strength juice (Splittstoesser *et al.*, 1998). The effect of SS is also less pronounced at higher temperatures (Silva *et al.*, 1999). Silva *et al.* (1999) suggested that water activity, rather than total SS, should be measured, as different sugars at the same concentrations generate different water activities and could have different effects on D-values. The clarity of the juice influences the SS, with non-clarified juice having a higher °Brix. *Alicyclobacillus* spores in non-clarified lemon juice concentrate (68.00°Brix) had a correspondingly higher heat resistance than in clarified lemon juice concentrate (50.00°Brix) (Maldonado *et al.*, 2008).

Alicyclobacillus species/strain

Different strains of *A. acidoterrestris* differ in their heat resistance. A study on three *A. acidoterrestris* strains (VF, WAC and IP) showed that in a model fruit juice system acidified with malic acid to pH 3.70, strains VF and WAC had approximately the same heat resistance, while strain IP was less heat resistant (Pontius *et al.*, 1998). In McIlvaine buffer at pH 4.00, strain AB-1 (Murakami *et al.*, 1998) was approximately twice as heat resistant as strain DSM 2498 (Bahçeci & Acar, 2007b). In orange juice (pH 3.15, 9.00°Brix) the heat resistances of four *A. acidoterrestris* strains (46, 780,

145 and the type strain DSM 2498) were studied (Eiora *et al.*, 1999). Strains DSM 2498 and 46 had similar D-values, while strains 145 and 70 were more heat resistant. It has been suggested that differences in the heat sensitivity of different *Alicyclobacillus* strains can be correlated with differences in optimum growth temperature and the type, pH and temperature of the sporulation medium (Bahçeci & Acar, 2007b).

Divalent cations

Divalent cations can also influence the heat resistance of spores. Mineralisation of spores with divalent cations, such as calcium or manganese, contributes to the stabilisation of spores against heat (Bender & Marquis, 1985). Calcium also chelates dipicolinic acid (DPA) to form Ca-DPA, which further stabilises spores and contributes to heat resistance (Yamazaki *et al.*, 1997a). *Alicyclobacillus acidoterrestris* spores bind Ca^{2+} and Mn^{2+} more strongly at a low pH compared to *Bacillus* species and are also able to keep Ca-DPA levels constant. Thus, stabilisation of Ca-DPA concentrations and their ability to strongly bind divalent cations contribute to the heat resistance of *A. acidoterrestris* spores (Yamazaki *et al.* 1997a).

Sporulation temperature

It has been reported that incubation of cultures at a higher temperature could increase the heat resistance of spores (Jay *et al.*, 2005a). Palop *et al.* (2000) found this to be true for *A. acidocaldarius*, as an approximately linear increase in D_{110} -values (and thus heat resistance) was observed when the sporulation temperature was increased from 45°C to 65°C. Even with the decreased heat resistance observed at the lower sporulation temperature, *A. acidocaldarius* was still approximately 30 times more heat resistant than *A. acidoterrestris* that had been sporulated at the same temperature (Palop *et al.*, 2000), indicating the greater thermophilic properties of this species.

Heat resistance prediction models

Models have been developed for predicting the D-values of *A. acidoterrestris* using response surface methodology. However, the predicted values were consistently

lower than those observed in real fruit systems. The models made use of McIlvaine buffer or malt extract broth (MEB) as heating medium. The difference between the predicted and observed D-values could be attributed to other components present in the fruit products that could increase the heat resistance of *A. acidoterrestris* spores. Further challenge tests and model validation studies were recommended by researchers before the prediction models can be used in industry (Silva *et al.*, 1999; Bahçeci & Acar, 2007b).

C. SPOILAGE

Interest in *Alicyclobacillus* spp. focused on their significance as spoilage organisms after a report by Cerny *et al.* in 1984 was published, implicating *A. acidoterrestris* as the causative organism in a large-scale spoilage incident in Germany involving shelf-stable, aseptically packaged apple juice. Subsequently, spoilage incidents attributed to *Alicyclobacillus* species were reported in various fruit juices (Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996a; Walls & Chuyate, 1998; Jensen, 2000; Matsubara *et al.*, 2002), fruit juice blends (Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996a; Walls & Chuyate, 1998; Jensen & Whitfield, 2003; Goto *et al.*, 2003), carbonated fruit juice drinks (Pettipher & Osmundson, 2000; Gouws *et al.*, 2005), fruit pulps (Gouws *et al.*, 2005), lemonade (Yamazaki *et al.*, 1996a), iced tea (Duong & Jensen, 2000), isotonic water (Yamazaki *et al.*, 1996a) and even canned diced tomatoes (Walls & Chuyate, 1998) worldwide, including in the USA (Splittstoesser *et al.*, 1994; Walls & Chuyate, 1998), Australia (Duong & Jensen, 2000; Jensen, 2000; Jensen & Whitfield, 2003), Japan (Yamazaki *et al.*, 1996a; Matsubara *et al.*, 2002; Goto *et al.*, 2003), the United Kingdom (Pettipher & Osmundson, 2000) and South Africa (Gouws *et al.*, 2005).

Alicyclobacillus spp. related problems are relatively widespread, as indicated by a survey conducted by the National Food Processors Association (NFPA) of the USA in 1998. Out of the 60% of companies that responded to the survey (34 out of 57), 35% had experienced spoilage incidents consistent with the presence of acidophilic spore-formers such as *Alicyclobacillus* spp. (Walls & Chuyate, 1998). Most companies had experienced one or two such spoilage incidents in the five years preceding the survey, with apple juice being the product most often involved. Spoilage incidents occurred in spring or summer and spoilage was mainly apparent as an off-flavour or -odour, with or without sediment (Duong & Jensen, 2000) and in

some products discolouration or cloudiness occurred. Consumer complaints were often the only reason for companies becoming aware of the problem, since the absence of gas production (Splittstoesser *et al.*, 1994; Duong & Jensen, 2000) made spoilage difficult to detect (Walls & Chuyate, 1998). The European Fruit Juice Association (AIJN) conducted a survey in 2005 amongst a total of 68 participants involved in various areas of the fruit processing industry, including packers, producers and canners (Howard, 2006). Forty five percent of the respondents had experienced *Alicyclobacillus* spp. related problems in the three years preceding the survey, with 33% of these experiencing more than three incidents. Of those that had experienced spoilage problems, 35% of the incidents were reported as being intermediately to majorly severe. Problems occurred primarily in apple raw materials and the type of product involved was primarily concentrates (Howard, 2006).

The off-flavour and -odour caused by *Alicyclobacillus* spp. has been described as medicinal, disinfectant-like, antiseptic, phenolic, smoky and hammy (Wasserman, 1966; Walls & Chuyate, 1998; Duong & Jensen, 2000; Orr *et al.*, 2000; Pettipher, 2000; Pettipher & Osmundson, 2000; Gocmen *et al.*, 2005). The compound responsible for the taint has, in most cases, been identified as guaiacol (Yamazaki *et al.*, 1996a; Splittstoesser *et al.*, 1998; Jensen, 2000; Walls & Chuyate, 2000b; Gocmen *et al.*, 2005; Siegmund & Pöllinger-Zierler, 2006), although the halophenols 2,6-dichlorophenol (2,6-DCP) (Jensen, 2000; Jensen & Whitfield, 2003; Gocmen *et al.*, 2005) and 2,6-dibromophenol (2,6-DBP) (Borlinghaus & Engel, 1997; Jensen, 1999; Jensen, 2000; Jensen & Whitfield, 2003; Gocmen *et al.*, 2005; Siegmund & Pöllinger-Zierler, 2006) have also been implicated.

Alicyclobacillus acidoterrestris is the species primarily responsible for spoilage incidents (Yamazaki *et al.*, 1996a; Walls & Chuyate, 1998; Jensen, 2000; Pettipher & Osmundson, 2000; Jensen & Whitfield, 2003) although other species, including *A. acidiphilus* (Matsubara *et al.*, 2002; Goto *et al.*, 2008), *A. pomorum* (Goto *et al.*, 2003), *A. hesperidum* (Gocmen *et al.*, 2005; Goto *et al.*, 2008), *A. cycloheptanicus* (Gocmen *et al.*, 2005), *A. herbarius* (Goto *et al.*, 2008) and *A. acidocaldarius* (Gouws *et al.*, 2005) have also been implicated due to their ability to produce taint compounds or because they were isolated from spoiled products. A recent report has identified *A. acidocaldarius* as the causative organism in the spoilage of non-concentrated tomato products. Although no guaiacol was detected, 2-methyltetrahydrothiophene-3-one was identified using gas-chromatography mass-

spectrometry (GC-MS) as the compound responsible for the off-flavour (Lottici *et al.*, 2006). Thus, research should not only focus on *A. acidoterrestris* and guaiacol production, but should be broadened to include other *Alicyclobacillus* species and taint compounds.

Guaiacol (2-methoxyphenol)

The predominant metabolite associated with spoilage by *Alicyclobacillus* spp. is guaiacol (2-methoxyphenol) (Yamazaki *et al.*, 1996a; Pettipher *et al.*, 1997; Orr *et al.*, 2000; Gocmen *et al.*, 2005). Guaiacol is a well documented flavour compound, as it contributes to the smoky flavour of products such as arabica coffee (Mayer *et al.*, 1999) and smoked salmon (Varlet *et al.*, 2006). However, it is better known for its association with off-flavour spoilage in products such as wine (Simpson *et al.*, 1986; Álvarez-Rodríguez *et al.*, 2003), chocolate milk (Jensen *et al.*, 2001), chocolate ice-cream (Saxby, 1996), vanilla yogurt (Whitfield, 1998) and fruit juices (Cerny *et al.*, 1984; Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996a; Walls & Chuyate, 1998).

Microbial metabolic production pathway of guaiacol

The presence of guaiacol in food products can either be due to heat decomposition of guaiacol precursors, as is the case in roasted products (Mayer *et al.*, 1999), or it can be a product of microbial metabolism (Chang & Kang, 2004). Several microorganisms other than species of *Alicyclobacillus* are able to produce guaiacol, including *Bacillus megaterium* (Crawford & Olson, 1978), *Bacillus subtilis* (Álvarez-Rodríguez *et al.*, 2003), *Streptomyces setonii* and other unidentified *Streptomyces* strains (Crawford & Olson, 1978; Pometto *et al.*, 1981; Álvarez-Rodríguez *et al.*, 2003), *Paecilomyces variotii* (Rahouti *et al.*, 1989), *Rhodotorula rubra* (Huang *et al.*, 1993a) and *Sporotrichum thermophile* (Topakas *et al.*, 2003). *Bacillus megaterium* (Crawford & Olson, 1978), *B. subtilis* (Álvarez-Rodríguez *et al.*, 2003) and the *Streptomyces* strains (Crawford & Olson, 1978; Pometto *et al.*, 1981; Álvarez-Rodríguez *et al.*, 2003) produced guaiacol from vanillic acid, while *P. variotii* (Rahouti *et al.*, 1989), *R. rubra* (Huang *et al.*, 1993a) and *S. thermophile* (Topakas *et al.*, 2003) produced the compound as a product during the metabolism of ferulic acid. In the latter three cases vanillic acid was identified as the immediate precursor to guaiacol in the metabolic pathway.

Although the precise metabolic production pathway for guaiacol in *Alicyclobacillus* spp. has not been completely elucidated, the most common hypothesis is that guaiacol is produced as a product during ferulic acid metabolism. Ferulic acid is ubiquitous in nature and is found in fruits, vegetables, grains, beans, leaves, seeds, nuts, grasses and flowers (Rosazza *et al.*, 1995). It is also a component of the structural plant cell wall polymer, lignin, as it cross-links this compound to plant cell wall polysaccharides (Kirk, 1971; Crawford & Crawford, 1980; Provan *et al.*, 1994; Mathew & Abraham, 2004). The ability to metabolise ferulic acid to various products has been observed in yeasts (Huang *et al.*, 1993b; Donaghy *et al.*, 1999; Mathew *et al.*, 2007), fungi (Nazareth & Mavinkurve, 1986; Rahouti *et al.*, 1989; Topakas *et al.*, 2003) and other bacteria (Karmakar *et al.*, 2000). In most micro-organisms the first step of ferulic acid metabolism is its decarboxylation to 4-vinylguaiacol (Rahouti *et al.*, 1989; Karmakar *et al.*, 2000; Topakas *et al.*, 2003; Mathew *et al.*, 2007), although it can also be directly transformed to vanillin (Peleg *et al.*, 1992) or vanillic acid (Huang *et al.*, 1993a) without production of 4-vinylguaiacol. If it is not metabolised further, 4-vinylguaiacol can cause the unpleasant off-flavour described as “old fruit” or “rotten” (Tatum *et al.*, 1975) in improperly stored citrus products, especially orange juice (Tatum *et al.*, 1975; Naim *et al.*, 1988; Lee & Nagy, 1990; Rouseff *et al.*, 1992). Most micro-organisms metabolise the 4-vinylguaiacol further to vanillin and subsequently vanillic acid (Nazareth & Mavinkurve, 1986; Rahouti *et al.*, 1989; Karmakar *et al.*, 2000), although it can also be converted directly to vanillic acid (Topakas *et al.*, 2003). Vanillin is normally metabolised rapidly through oxidation or reduction to vanillic acid and vanillyl alcohol, respectively, as it has been shown to have a toxic effect on micro-organisms above certain concentrations (Ander *et al.*, 1980; Cerrutti *et al.*, 1997; López-Malo *et al.*, 1998; Alzamora *et al.*, 2003; Fitzgerald *et al.*, 2003; Bahçeci & Acar, 2007a; Ferrante *et al.*, 2007; Char *et al.*, 2009). The vanillic acid that is formed can then be converted to a number of products, including methoxyhydroquinone, protocatechuic acid and guaiacol. Guaiacol is produced from vanillic acid through a non-oxidative decarboxylation reaction (Crawford & Olson, 1978; Pometto *et al.*, 1981; Rahouti *et al.*, 1989; Álvarez-Rodríguez *et al.*, 2003; Topakas *et al.*, 2003) and can subsequently be transformed to other products, most often catechol (Pometto *et al.*, 1981; Rahouti *et al.*, 1989; Álvarez-Rodríguez *et al.*, 2003; Topakas *et al.*, 2003).

Alicyclobacillus acidoterrestris is able to produce guaiacol from vanillin

(Bahçeci *et al.*, 2005a; Bahçeci & Acar, 2007a) and vanillic acid (Niwa & Kuriyama, 2003). The conversion of vanillic acid to guaiacol is more rapid than that of vanillin, which is in agreement with the identification of vanillic acid as the immediate precursor to guaiacol in the metabolic pathway. The ability of *Alicyclobacillus* species to produce guaiacol from other precursors, such as ferulic acid or lignin, has not been investigated. The metabolism of ferulic acid and subsequent formation of guaiacol and other products in micro-organisms is presented in Fig. 1.

Jensen (2000) suggested that the amino acid tyrosine could be another possible precursor for guaiacol formation. Apple and orange juice contains approximately $4.10 \mu\text{g}\cdot\text{mL}^{-1}$ and $3.40\text{-}13.50 \mu\text{g}\cdot\text{mL}^{-1}$ tyrosine, respectively, which should be sufficient in quantity to allow the synthesis of detectable amounts of guaiacol under conditions favourable for growth and taint formation (Jensen, 2000). However, this theory has not been widely investigated and the most widely accepted guaiacol synthetic pathway is that of lignin degradation (Chang & Kang, 2004).

Detection of guaiacol

The presence of guaiacol in beverages can be determined by using sensory, analytical or chemical detection methods. Sensory methods are normally used if only the presence or absence of taint needs to be determined, while analytical and chemical methods can be used for qualitative, as well as quantitative determinations.

Sensory methods

Several studies have determined the sensory odour and taste thresholds for guaiacol in water and apple juice. One of the earliest reports was by Wasserman (1966) who determined taste and odour thresholds of 13.00 ppb and 21.00 ppb, respectively for guaiacol in water. In more recent reports the threshold values were much lower, with Pettipher *et al.* (1997) and Orr *et al.* (2000) reporting best estimated threshold (BET) values of approximately 2.00 ppb for taste in apple, orange and a non-carbonated fruit juice and 2.32 ppb for odour in apple juice. Eisele and Semon (2005) reported even lower values, determining BET values of 0.17 ppb and 0.24 ppb for taste in water and apple juice, respectively and 0.48 ppb and 0.91 ppb for odour in water and apple juice, respectively. Siegmund and Pöllinger-Zierler (2006) lowered the odour threshold for guaiacol in apple juice even further, determining a value of 0.57 ppb.

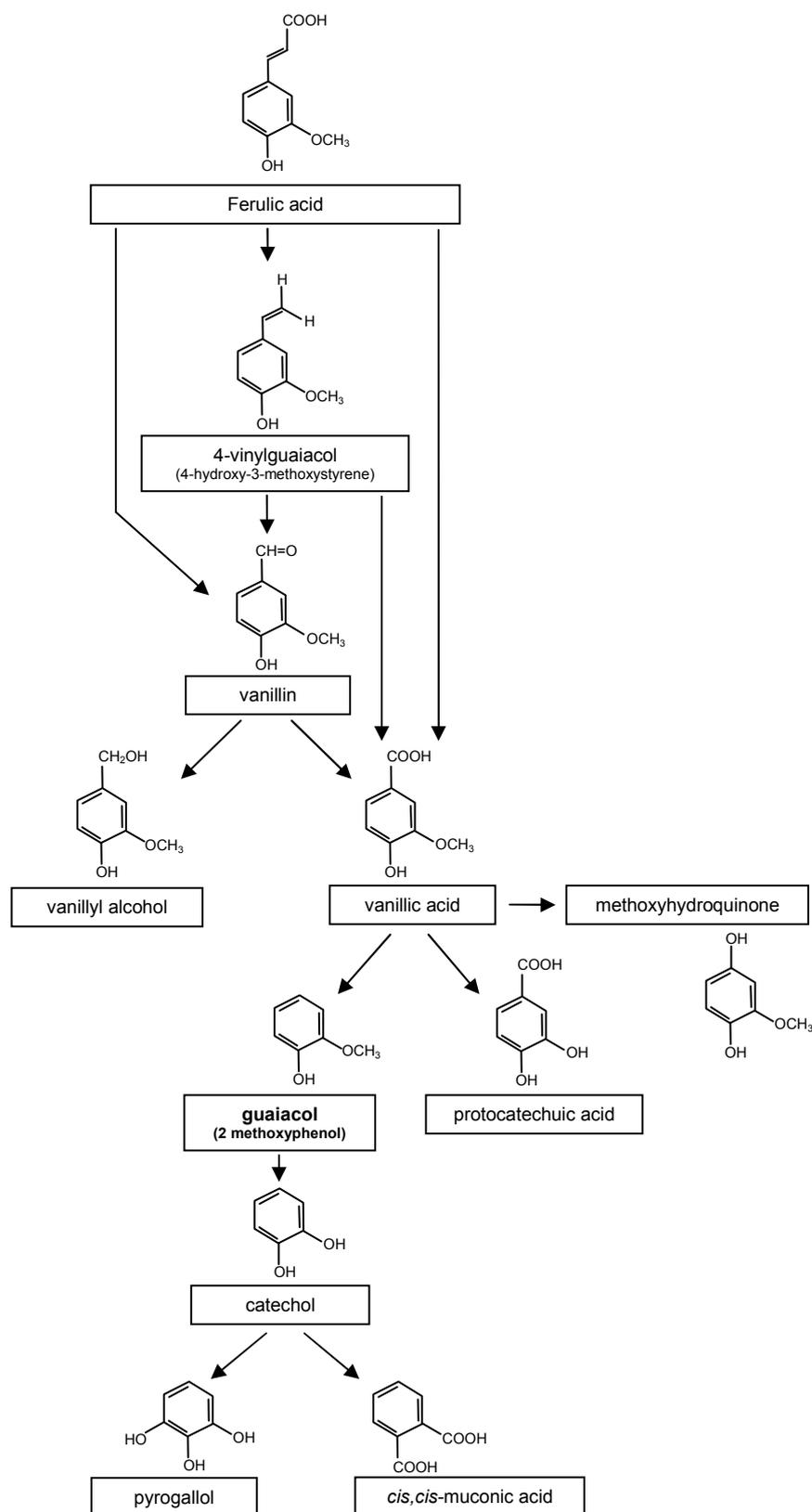


Figure 1 Microbial production pathways of guaiacol and other products through the metabolism of ferulic acid (Crawford & Olson, 1978; Pometto *et al.*, 1981; Rahouti *et al.*, 1989; Huang *et al.*, 1993a; Rosazza *et al.*, 1995; Karmakar *et al.*, 2000; Topakas *et al.*, 2003).

They also determined the odour recognition threshold for guaiacol in apple juice to be 2.00 ppb. The odour threshold for guaiacol has also been determined in other substances. Jensen *et al.* (2001) determined an odour threshold of 43.00 ng.mL⁻¹ for guaiacol in chocolate milk, while the odour threshold for guaiacol in red wine was determined to be 9.50 ug.L⁻¹ (Ferreira *et al.*, 2000).

Variations between studies can be ascribed to differences in the sensitivities and training of the panel members used to conduct the study. In the study conducted by Eisele and Semon (2005) BET odour detection values for individual panelists ranged from 0.06 ppb to 4.71 ppb in water and 0.17 to 4.71 ppb in apple juice. Taste BET values ranged from 0.01 to 4.71 ppb in both water and apple juice. Thus, an approximate 500-fold range existed between panelists within a panel, which is a substantial variation range and could explain the variations in different studies.

Some researchers have found that sensory analysis is more sensitive than analytical methods in identifying the presence of guaiacol. Orr *et al.* (2000) found that their sensory panel was able to detect guaiacol in five samples in which it could not be detected using gas-chromatography mass-spectrometry (GC-MS). In contrast, however, Siegmund and Pöllinger-Zierler (2006) determined a limit of detection of 0.29 µg.L⁻¹ using headspace solid phase microextraction (HS-SPME) GC-MS, which is lower than the lowest sensory detection limit of 0.57 µg.L⁻¹ determined for guaiacol.

Analytical methods

Instrumental analysis has been used for the detection of guaiacol in wine (Ferreira *et al.*, 1998; López *et al.*, 2002; Lee & Noble, 2003; Boutou & Chatonnet, 2007), cork stoppers (Ezquerro & Tena, 2005), oak extracts (Pollnitz *et al.*, 2004), fruit juice (Yamazaki *et al.*, 1996a; Pettipher *et al.*, 1997), urine (Bieniek, 2003), liquid smoke flavouring (Guillén & Ibargoitia, 1998), biomass smoke (Conde *et al.*, 2006), orange essence oil (Högnadóttir & Rouseff, 2003) and smoked salmon (Varlet *et al.*, 2006). Instrumental analysis usually includes three steps, namely extraction/sample preparation/preconcentration, separation and detection/identification. The most commonly used separation procedures for guaiacol detection are high performance liquid chromatography (HPLC) (Bahçeci *et al.*, 2005a; Bahçeci & Acar, 2007a) and GC (Pettipher *et al.*, 1997; Jensen *et al.*, 2001; Pollnitz *et al.*, 2004; Zierler *et al.*, 2004; Gocmen *et al.*, 2005).

Various extraction/sample preparation methods are used in conjunction with GC. In liquid-liquid extraction (LLE), the compound of interest is selectively partitioned into one of two immiscible phases created by appropriate extraction solvents (McDonald, 2001). Dichloromethane (Pettipher *et al.*, 1997; Guillén & Ibargoitia, 1998) and a 1:1 mixture of pentane and diethyl ether (Pollnitz *et al.*, 2004; Gocmen *et al.*, 2005) has been used to extract guaiacol from samples using LLE. However, there are various disadvantages to LLE, including incomplete phase separations, less-than-quantitative recoveries, use of expensive, breakable glassware, disposal of large quantities of organic solvents and time-consuming protocols due to multiple extraction steps needed for higher yield and purity (McDonald, 2001).

Solid phase extraction (SPE) uses cartridges packed with a resin appropriate for either retention of the compound of interest or of impurities in the sample so that the analyte may be purified (Anon., 1998; López *et al.*, 2002; Bieniek, 2003). Bieniek (2003) found that recovery of methoxyphenols, including guaiacol, was much higher when using SPE cartridges containing octyl (C8) material instead of a styrene-divinylbenzene copolymer.

Solid phase microextraction (SPME) is a fairly recently developed method that makes use of fibers containing appropriate material for adsorption of analytes (Shirey & Sidisky, 2000; Wardencki *et al.*, 2004). A number of factors can influence the efficiency of the SPME technique, including the type of fiber, sample volume, temperature and extraction time, salting, mode of extraction, desorption of analytes from the fiber and derivatisation (Wardencki *et al.*, 2004). HS-SPME is a variation of SPME where volatile compounds are collected in the headspace of a container and adsorbed to an appropriate SPME fiber upon exposure of the fiber to the headspace (Orr *et al.*, 2000; Jensen *et al.*, 2001; Zierler *et al.*, 2004; Ezquerro & Tena, 2005; Gocmen *et al.*, 2005; Conde *et al.*, 2006; Siegmund & Pöllinger-Zierler, 2006). HS-SPME is a particularly popular extraction technique as it is simple and easy to carry out, sample manipulation is reduced and the use of hazardous solvents and time-consuming, complicated extraction procedures are eliminated (Zierler *et al.*, 2004; Ezquerro & Tena, 2005).

Gas chromatography is coupled to detection systems such as flame ionisation detection (GC-FID) (Bieniek, 2003), olfactometry (GC-O) (Lee & Noble, 2003; Gocmen *et al.*, 2005) and mass spectrometry (GC-MS) (Pettipher *et al.*, 1997; Orr *et*

al., 2000; Jensen *et al.*, 2001; López *et al.*, 2002; Lee & Noble, 2003; Zierler *et al.*, 2004; Gocmen *et al.*, 2005; Conde *et al.*, 2006; Siegmund and Pöllinger-Zierler, 2006). Mass spectrometry is most often used as it is a very specific, accurate and sensitive detection method. Gocmen *et al.* (2005) found that while the guaiacol, 2,6-DCP and 2,6-DBP peaks on a GC-FID chromatogram were almost undetectable, their corresponding aroma peaks on a GC-O chromatogram were relatively large, indicating a considerable impact on the juice aroma and emphasising the value of GC-O for identifying and characterising aroma compounds in a complex food matrix.

Zierler *et al.* (2004) developed a HS-SPME GC-MS method for the detection of guaiacol and 2,6-DBP produced by *A. acidoterrestris* in apple juice. The influence of parameters such as type and concentration of added salt, type of SPME fibre and thermostating and extraction time and temperature were optimised. The method was fully validated, with limits of detection (LOD) of 0.29 $\mu\text{g.L}^{-1}$ and 0.08 $\mu\text{g.L}^{-1}$ and limits of quantification (LOQ) of 1.06 $\mu\text{g.L}^{-1}$ and 0.27 $\mu\text{g.L}^{-1}$ being determined for guaiacol and 2,6-DBP, respectively (Zierler *et al.*, 2004).

Chemical methods

A third method for the detection of guaiacol in products makes use of a colourimetric assay based on the oxidation of guaiacol by peroxidase enzymes in the presence of H_2O_2 . During this reaction a brown component is formed which has been identified as 3,3'-dimethoxy-4,4'-biphenol (Doerge *et al.*, 1997) and the change in absorbance can be measured spectrophotometrically at 420 nm (Bahçeci *et al.*, 2005a; Bahçeci *et al.*, 2005b) or 470 nm (Doerge *et al.*, 1997; Niwa & Kawamoto, 2003; Niwa & Kuriyama, 2003). This reaction is widely used in assays testing for peroxidase enzyme activity (Doerge *et al.*, 1997; Bahçeci *et al.*, 2005b) and has also formed the basis of the development of a guaiacol detection kit (Niwa & Kawamoto, 2003; Niwa & Kuriyama, 2003; Niwa, 2004). The kit, manufactured by the Kyokuto Pharmaceutical Industrial Company Ltd. from Japan and distributed by Cosmo Bio Company Ltd., (Anon., 2005) consists of vanillic-acid enriched yeast starch glucose (YSG) media, solutions and reagents needed to rapidly carry out the enzymatic assay. This kit can be used to determine the presence of guaiacol or guaiacol producing *Alicyclobacillus* species in a product, as well as the ability of a specific species to produce guaiacol (Niwa & Kawamoto, 2003; Niwa & Kuriyama, 2003; Niwa, 2004). A similar kit has also been developed by the DöhlerGroup in Germany

(Anon., 2006). It differs from the Japanese product in that it makes use of *Bacillus acidoterrestris* (BAT) medium instead of YSG medium for incubation. The chemical method is both qualitative and quantitative, as the guaiacol concentration in a sample can be quantified by comparing the absorbance of the sample to a standard curve of the absorbance of known guaiacol concentrations.

Halophenols

Although guaiacol is the predominant off-flavour compound associated with spoilage by *Alicyclobacillus* species, the halophenols, 2,6-DBP and 2,6-DCP, which have also been described as having a medicinal, antiseptic or disinfectant-like odour and flavour (Jensen, 2000; Gocmen *et al.*, 2005), have also been identified as taint chemicals produced by *Alicyclobacillus* species. The halophenols occur in lower concentrations than guaiacol (Jensen, 2000) and that, as well as the high volatility of guaiacol, is probably the reason for the predominance of guaiacol over the halophenols in taint formation.

Halophenols can be present in food products either due to chemical contamination (Mottram, 1998) or through microbial synthesis (Chang & Kang, 2004). Weak halogen solutions can come into contact with food through residues of cleansing materials used on raw materials and food processing lines and on dilution of juice concentrates (Mottram, 1998; Adams *et al.*, 1999), which can lead to halophenol formation. Microbial synthesis of halophenols is also possible, as there are a number of bacteria that are able to synthesise these compounds (Van Pée, 1996). Therefore, it is possible that *Alicyclobacillus* species also possess enzyme systems capable of synthesising these compounds (Chang & Kang, 2004).

In most cases, production of the halophenols was detected in combination with guaiacol production (Gocmen *et al.*, 2005), but they have also been detected in the absence of guaiacol (Baumgart *et al.*, 1997; Borlinghaus & Engel, 1997; Jensen, 2000). The production of the halophenols also seems to be strain or species specific (Gocmen *et al.*, 2005). Gocmen *et al.* (2005) found that, along with the production of guaiacol by all three strains, *A. cycloheptanicus* was also able to produce both 2,6-DCP and 2,6-DBP, while *A. acidoterrestris* and *A. hesperidum* only produced 2,6-DBP and 2,6-DCP, respectively. In some cases the production of these compounds was time dependent, as *A. cycloheptanicus* had produced only guaiacol and 2,6-DBP

by day 14 of the study, but by the 28th day 2,6-DCP was also present.

Factors influencing taint production

The incidence of *Alicyclobacillus* spp. in acidic products is relatively high. Pinhatti *et al.* (1997) observed that out of 34 commercial fruit juices and -concentrates analysed, 67% contained species of *Alicyclobacillus*. Eiroa *et al.* (1999) also found *Alicyclobacillus* spp. to be prevalent in orange juice concentrate, as 14.7% of the concentrated orange juice samples tested, contained *Alicyclobacillus* spores. Accordingly, *Alicyclobacillus* spp. also showed a high incidence in commercial apple juice concentrate, with 36% of 166 samples testing positive for *Alicyclobacillus* species (Borlinghaus & Engel, 1997). Jensen (2005b) also conducted a study to determine the incidence of *Alicyclobacillus* spp. in Australian fruit juice products. It was found that out of 85 orange juice concentrates, 31% contained *A. acidoterrestris* and 41% contained *A. acidocaldarius*. Out of 64 apple juice concentrates analysed, 12% contained *A. acidoterrestris* and 7% contained *A. acidocaldarius*. In single strength juices *A. acidocaldarius* was more prevalent, as 71% of the 14 orange juices and 55% of the 11 apple juices analysed, contained *A. acidocaldarius*, while *A. acidoterrestris* was not detected. Pettipher *et al.* (1997) found *A. acidoterrestris* to be present in single strength apple juice as well as concentrates.

The presence of species of *Alicyclobacillus* in acidic products will, however, not necessarily always lead to spoilage of the products. Even though cell concentrations as high as 10^3 cfu.mL⁻¹ were recorded in some of the fruit juices and -concentrates analysed by Pinhatti *et al.* (1997), none of the products were spoiled. Pettipher *et al.* (1997) also observed cell concentrations higher than 10^3 cfu.mL⁻¹ in some products without spoilage being observed. These results indicate that there are other factors that play a role in the spoilage of acidic products by *Alicyclobacillus* species. The following factors have been identified.

Cell concentration

Several studies have found that there is a critical *Alicyclobacillus* cell concentration that must be present before taint compounds are produced in detectable concentrations. Pettipher *et al.* (1997) established this cell concentration to be 10^5 cfu.mL⁻¹, while Bahçeci *et al.* (2005a) found that a slightly lower concentration,

10^4 cfu.mL⁻¹, was necessary for detectable guaiacol production to occur. In apple juice inoculated with 10^5 cfu.mL⁻¹ *A. acidoterrestris*, guaiacol production started immediately, while in apple juice inoculated with 10^3 cfu.mL⁻¹, guaiacol production only started after approximately 30 h, once a cell concentration of 10^4 cfu.mL⁻¹ had been reached (Bahçeci *et al.*, 2005a).

Temperature

The rate of taint production seems to increase with an increase in the incubation temperature. Pettipher *et al.* (1997) found that in juice stored at 25°C, guaiacol production started after 6-10 d, while in juice stored at 44°C, guaiacol was produced within 3-6 d. Bahçeci *et al.* (2005a) found that in apple juice inoculated with 10^3 - 10^5 cfu.mL⁻¹ *A. acidoterrestris*, maximum guaiacol concentrations were formed after 75 h in cultures stored at 46°C, while little or no guaiacol was detected in cultures stored at 25°C. In a juice-inoculation study conducted by Jensen (2000) higher concentrations of guaiacol were produced by *A. acidoterrestris* at 46°C than at 37°C. Siegmund and Pöllinger-Zierler (2007) also had similar results, with guaiacol and 2,6-DBP production at concentrations high enough to cause spoilage being observed after 20 d at room temperature (average 21.5°C) compared to only 15 d at 30°C.

Heat shock

Vegetative cells instead of spores must be present for taint compounds to be produced. Thus, dormant spores must be activated and germinate to form vegetative cells. Spore activation can be brought about through exposure to a heat shock treatment. Various heat shock treatments have been suggested for the activation of *Alicyclobacillus* spores, including 60°C for 30 min (Splittstoesser *et al.*, 1998), 70°C for 20 min (Eiora *et al.*, 1999), 80°C for 10 min (Walls & Chuyate, 1998) and 80°C for 20 min (Terano *et al.*, 2005). Terano *et al.* (2005) found that spores were unable to germinate unless they had been exposed to a heat shock treatment. Although other authors have reported that spores may be able to germinate without a heat shock treatment (Pettipher *et al.*, 1997), spore germination will be much accelerated by a heat shock treatment, leading to a higher concentration of vegetative cells and a higher rate of taint production (Chang & Kang, 2004).

Growth medium/susceptible beverages

Not all types of juices are able to support growth and taint production by *Alicyclobacillus* spp. Red grape juice does not support growth, due to the presence of polyphenols that have been shown to inhibit growth (Splittstoesser *et al.*, 1994; Splittstoesser *et al.*, 1998). Splittstoesser *et al.* (1994; 1998) also found that an apple-grape-cherry blend, an apple-raspberry-grape blend, an apple-red grape blend, a cranberry cocktail and prune juice were unable to support growth. Apple juice, an apple-orange-pineapple blend, grapefruit juice, orange juice, pineapple juice, pineapple-orange juice, tomato juice and a tropical fruit blend, on the other hand, were all able to support the growth of *A. acidoterrestris*. Pettipher *et al.* (1997) found that *A. acidoterrestris* was able to grow in apple juice, orange juice and a non-carbonated fruit juice drink and produced guaiacol at concentrations ranging from 1.20-100.80 ppb, depending on the juice and incubation temperature. The highest guaiacol concentration was produced in the non-carbonated fruit juice at 44°C after 3 d. In a study conducted by Jensen (2000), *A. acidoterrestris* isolates were able to grow and produce guaiacol in orange as well as apple juice, with higher guaiacol concentrations being produced in orange juice. Walls and Chuyate (2000b) found that *A. acidoterrestris* was unable to grow in apple-cranberry, pineapple and 10% fruit juice, or salsa. However, growth occurred in grapefruit, apple, orange, pear, white grape and tomato juices, but spoilage only occurred in orange, pear, white grape and tomato juices. The reason for growth and spoilage only occurring in some juices is unclear, but it could be because different juices contain different concentrations of guaiacol precursors or because some juices contain growth inhibitors (Walls & Chuyate, 2000b).

Headspace/oxygen availability

Most species of *Alicyclobacillus* are aerobic and their growth and taint production could, therefore, be influenced by the amount of oxygen that is available in the growth medium. Although a reduced oxygen supply slows the growth rate (Walker & Phillips, 2005; Siegmund and Pöllinger-Zierler, 2007), it does not negatively influence production of taint compounds. Siegmund and Pöllinger-Zierler (2007) found that at a limited oxygen supply *A. acidoterrestris* was still able to produce guaiacol and 2,6-DBP in detectable amounts and concentrations even exceeded those produced

at a free oxygen supply.

D. SOURCES OF CONTAMINATION

Soil is one of the primary isolation sources of *Alicyclobacillus* species (Hippchen *et al.*, 1981; Deinhard *et al.*, 1987a, Deinhard *et al.*, 1987b; Nicolaus *et al.*, 1998; Wisse & Parish, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002c; Tsuruoka *et al.*, 2003; Goto *et al.*, 2007; Goto *et al.*, 2008; Groenewald *et al.*, 2008; Imperio *et al.*, 2008) and thought to be the most important source of contamination of acidic products with these bacteria. Soil can cling to fruit that have fallen on the ground and can also be carried into processing facilities by employees. Groenewald *et al.* (2008) isolated strains of *A. acidoterrestris* and *A. acidocaldarius* from the soil of apple and pear orchards in South Africa. Parish and Goodrich (2005) investigated the occurrence of presumptive *Alicyclobacillus* strains on oranges entering the processing environment and found that the contamination rate was significantly lower at facilities that did not use oranges picked up from the ground.

Water has also been identified as an important source of contamination. Wisse and Parish (1998) found presumptive *Alicyclobacillus* species to be present on the surfaces of unwashed and washed fruit, in condensate water and in juice concentrate. Chen *et al.* (2006) isolated a number of *Alicyclobacillus* strains from the wash water, distilled water, apple juice and apple juice concentrate of an apple juice concentrate-processing facility, while McIntyre *et al.* (1995) found the same strain of *Alicyclobacillus* that was isolated from spoiled products to be present in ingredient water samples from the processing facility. Groenewald *et al.* (2009) found similar strains of *A. acidoterrestris* isolated from soil outside the fruit processing facility and wash water to also be present in the final pear concentrate product, indicating the route of contamination of the product through these sources.

E. ISOLATION AND ENUMERATION

Species of *Alicyclobacillus* have been isolated from thermal acid environments (Uchino & Doi, 1967; Darland & Brock, 1971; Goto *et al.*, 2002c; Simbahan *et al.*, 2004), various types of soil (Hippchen *et al.*, 1981; Deinhard *et al.*, 1987a, Deinhard *et al.*, 1987b; Nicolaus *et al.*, 1998; Wisse & Parish, 1998; Albuquerque *et al.*, 2000;

Goto *et al.*, 2002c; Tsuruoka *et al.*, 2003; Goto *et al.*, 2007; Goto *et al.*, 2008; Groenewald *et al.*, 2008; Imperio *et al.*, 2008), herbal tea (Goto *et al.*, 2002a), iced tea and its ingredients (Duong & Jensen, 2000), fruit juices and acidic beverages (Cerny *et al.*, 1984; Deinhard *et al.*, 1987a; Splittstoesser *et al.*, 1994; McIntyre *et al.*, 1995; Yamazaki *et al.*, 1996a; Pinhatti *et al.*, 1997; Walls & Chuyate, 1998; Wisse & Parish, 1998; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Goto *et al.*, 2006; Goto *et al.*, 2007), fruit juice concentrates (Pinhatti *et al.*, 1997; Wisse & Parish, 1998; Gouws *et al.*, 2005; Chen *et al.*, 2006; Goto *et al.*, 2006), fruit surfaces (Wisse & Parish, 1998; Parish & Goodrich, 2005), fruit juice ingredient water (McIntyre *et al.*, 1995), fruit wash water (Wisse & Parish, 1998; Chen *et al.*, 2006), liquid sugar (Goto *et al.*, 2007) and leaves (Goto *et al.*, 2006) using a variety of different media. Not all media are able to support the growth of *A. acidoterrestris*, including nutrient agar, tryptone soy agar, brain heart infusion agar, standard plate count agar and veal infusion agar, even when these media are acidified to pH 3.50 (Splittstoesser *et al.*, 1994, Pettipher *et al.*, 1997). In contrast, it has been shown that Australian isolates of *A. acidoterrestris* are not as fastidious, as they are able to grow on most media, including nutrient agar, provided the pH is 5.80 or less and the incubation is aerobic (Jensen, 1999; Jensen, 2000).

Uchino and Doi (1967) used a simple medium consisting of 20.00 g peptone, 5.00 g yeast extract and 10.00 g glucose per litre distilled water at pH 4.00 to isolate thermo-acidophilic bacteria, later identified as *Alicyclobacillus* spp., from acid hot springs. Since then a variety of media have been used and new media developed for the isolation and enumeration of *Alicyclobacillus* spp., the compositions of which are presented in Table 5.

Variations of the synthetic salt medium developed by Darland and Brock (1971)

A synthetic salt medium was developed by Darland and Brock in 1971. It has been modified by a number of researchers to yield several media with only slight differences, which are all used for the isolation of *Alicyclobacillus* species.

Bacillus acidocaldarius medium (BAM), also called Bacteriological analytical methods (Pettipher *et al.*, 1997) was originally proposed by Deinhard *et al.* (1987a), who combined the synthetic salts medium used by Darland and Brock (1971) and the trace element solution proposed by Farrand *et al.* (1983). BAM was used to isolate

Table 5 Compositions of media used for the isolation of *Alicyclobacillus* species

Name	Composition	Reference
<i>Bacillus acidocaldarius</i> medium (BAM) / <i>Bacillus acidoterrestris</i> (BAT) medium	Basal medium: 0.25 g CaCl ₂ ·2H ₂ O, 0.50 g MgSO ₄ ·7H ₂ O, 0.20 g (NH ₄)SO ₂ , 3.00 g KH ₂ PO ₄ , 1.00/2.00 g yeast extract, 5.00 g glucose and 1.00 mL trace element solution per litre distilled water. Trace element solution: 0.66 g CaCl ₂ ·2H ₂ O, 0.18 g ZnSO ₄ ·7H ₂ O, 0.16 g CuSO ₄ ·5H ₂ O, 0.15 g MnSO ₄ ·4H ₂ O, 0.18 g CoCl ₂ ·6H ₂ O, 0.10 g H ₃ BO ₃ and 0.30 g Na ₂ MoO ₄ ·2H ₂ O per litre distilled water. Adjusted to pH 4.00 using 1 N H ₂ SO ₄ and/or 1 N NaOH. For solid medium the liquid medium is made up at twice the concentration and mixed with an equal volume of agar (15-20 g agar per litre) after autoclaving.	Deinhard <i>et al.</i> , 1987a; IFU, 2007
<i>Alicyclobacillus acidocaldarius</i> medium (AAM)	0.25 g CaCl ₂ ·2H ₂ O, 0.50 g MgSO ₄ ·7H ₂ O, 0.20 g (NH ₄)SO ₂ , 0.60 g KH ₂ PO ₄ , 1.00 g yeast extract, 1.00 g glucose per litre distilled water. Acidified to pH 4.00.	Yamazaki <i>et al.</i> , 1996a
<i>Alicyclobacillus</i> (ALI) medium	0.25 g CaCl ₂ ·2H ₂ O, 0.50 g MgSO ₄ ·7H ₂ O, 0.20 g (NH ₄)SO ₂ , 3.00 g KH ₂ PO ₄ , 2.00 g yeast extract, 1.00 g glucose and 2.00 g soluble starch per litre distilled water. Acidified to pH 3.50 with 1 N H ₂ SO ₄ prior to autoclaving. For ALI agar ALI broth is prepared at twice the concentration and mixed with an equal volume of 3.50% (m/v) agar aseptically after autoclaving the two solutions separately.	Wisse & Parish, 1998
Yeast starch glucose (YSG) medium	2.00 g yeast extract, 2.00 g soluble starch, 1.00 g glucose and 15.00 g agar (when used as a solid medium) per litre distilled water. Acidified to pH 3.70 using 1 M H ₂ SO ₄ (Goto <i>et al.</i> , 2002a), HCl (Gouws <i>et al.</i> , 2005; IFU, 2007) or 10% (m/v) tartaric acid (Groenewald <i>et al.</i> , 2008).	Goto <i>et al.</i> , 2002a
Hiraishi glucose yeast extract (HGYE) medium	0.40% (m/v) glucose, 0.30% (m/v) (NH ₂)SO ₄ , 0.10% (m/v) trypticase soy broth, 0.05% (m/v) yeast extract, 0.05% (m/v) MgSO ₄ ·7H ₂ O, 0.01% (m/v) K ₂ HPO ₄ and 0.01% (m/v) K ₂ SO ₄ per litre distilled water. Adjusted to pH 3.00 with diluted H ₂ SO ₄ and the medium contains 3% (m/v) agar when used as a solid medium.	Hiraishi <i>et al.</i> , 1997
Potato dextrose agar (PDA)	4.00 g potato extract, 20.00 g dextrose, agar per litre water. Acidified to pH 3.50 through a sterile solution of 10% (m/v) tartaric acid.	Bevilacqua <i>et al.</i> , 2008b
K agar	2.50 g yeast extract, 5.00 g peptone, 1.00 g glucose, 1.00 g Tween 80, 15.00 g agar in 990 mL distilled water. Filter sterilised 25% (m/v) malic acid solution is used to acidify the medium to pH 3.70 after autoclaving.	Walls & Chuyate, 1998
SK agar	2.50 g yeast extract, 5.00 g peptone, 1.00 g glucose, 1.00 mL Tween 80 and 15 g agar in 1 litre distilled water. After autoclaving, filter sterilised 10% (m/v) CaCl ₂ is added to achieve a final concentration of 0.50 g.L ⁻¹ . Filter sterilised 10% (m/v) tartaric acid is used to adjust the pH to 4.00.	Chang & Kang, 2005
Orange serum agar (OSA)	10.00 g tryptone, 3.00 g yeast extract, 4.00 g dextrose, 2.50 g K ₂ HPO ₄ , 200 mL orange juice, agar per litre water. Acidified to pH 3.50 using a sterile solution of 25% (m/v) malic acid.	Bevilacqua <i>et al.</i> , 2008b

A. hesperidum, *Alicyclobacillus* genomic species 1 (in combination with a membrane filtration step) (Albuquerque *et al.*, 2000), *Alicyclobacillus* genomic species 2 (Goto *et al.*, 2002c) and *A. cycloheptanicus* (on SM agar plates, a modification of BAM plates where 3.00 g.L⁻¹, instead of 1.00 g.L⁻¹ yeast extract is added and the pH is adjusted to 4.30) (Deinhard *et al.*, 1987a; Deinhard *et al.*, 1987b). It was also used in growth and characterisation experiments during the isolation of *A. herbarius* (Goto *et al.*, 2002a), *A. acidiphilus* (Matsubara *et al.*, 2002), *A. pomorum* (Goto *et al.*, 2003), *A. sendaiensis* (Tsuruoka *et al.*, 2003), *A. vulcanalis* (Simbahan *et al.*, 2004), *A. contaminans* (Goto *et al.*, 2007), *A. fastidiosus* (Goto *et al.*, 2007), *A. kakegawensis* (Goto *et al.*, 2007), *A. macrosporangiidus* (Goto *et al.*, 2007), *A. sacchari* (Goto *et al.*, 2007), *A. shizuokensis* (Goto *et al.*, 2007) and *A. pohliae* (Imperio *et al.*, 2008).

A standard method developed by the Working Group on Microbiology of the International Federation of Fruit Juice Producers (IFU), the IFU Method No. 12, includes the use of *Bacillus acidoterrestris* medium (BAT), also called *Bacillus acidoterrestris* thermophilic medium (Murray *et al.*, 2007) for the isolation and enumeration of *Alicyclobacillus* spp. (IFU, 2007). The medium described in this method has the same composition as BAM, with the exception of the amount of yeast extract, which is 2.00 g.L⁻¹ instead of 1.00 g.L⁻¹ (IFU, 2007). However, some research papers stating the use of BAM uses the formula for BAT, with 2.00 g.L⁻¹ yeast extract (Silva *et al.*, 1999; Silva *et al.*, 2000). Even though the compositions of the two media are virtually identical, Pacheco (2002) and Murray *et al.* (2007) found that BAT was more efficient than BAM at recovering *Alicyclobacillus* spores. However, Deinhard *et al.* (1987a), who first proposed BAM, are also quoted by Pacheco (2002) as the original developers of BAT. Thus, the reason for the distinction between the two media is unclear.

Yamazaki *et al.* (1996a) made use of *Alicyclobacillus acidocaldarius* medium (AAM) to isolate *A. acidoterrestris* from spoiled acidic juices, isotonic water, lemonade, a fruit juice blend and a fruit-carrot juice blend. It was also used for the cultivation of *A. acidoterrestris* in studies investigating the effect of the bacteriocins enterocin AS-48 (Grande *et al.*, 2005) and bovicin HC5 (De Carvalho *et al.*, 2008) on the survival of *A. acidoterrestris* in fruit products. This medium only differs from BAM in that it contains no trace element solution and 0.60 g.L⁻¹ KH₂PO₄ and 1.00 g.L⁻¹ glucose instead of 3.00 g.L⁻¹ and 5.00 g.L⁻¹, respectively. Murakami *et al.* (1998)

used solidified (by adding 1.50% (m/v) agar) AAM containing 0.05% (m/v) $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ as a sporulation medium for *A. acidoterrestris*.

Wisse and Parish (1998) modified the media used by Darland and Brock (1971) and Cerny *et al.* (1984) to produce *Alicyclobacillus* (ALI) medium and agar. ALI medium has the same composition as BAM, except that it contains no trace element solution, 2.00 g.L⁻¹ yeast extract and 1.00 g.L⁻¹ glucose instead of 1.00 g.L⁻¹ and 5.00 g.L⁻¹, respectively, as well as 2.00 g.L⁻¹ soluble starch. ALI medium and agar were used to isolate presumptive *Alicyclobacillus* species from soil, unwashed and washed fruit surfaces, condensate water, fruit juice concentrates and fruit nectars at 10 different citrus processing plants (Wisse & Parish, 1998). ALI agar performed well when compared to other media for its suitability to isolate *Alicyclobacillus* species. (Parish & Goodrich, 2005; Murray *et al.*, 2007).

Yeast starch glucose (YSG) agar

YSG agar is the medium favoured by Japanese researchers for the isolation of *Alicyclobacillus* species. It is recommended by the Japan Fruit Juice Association for *A. acidoterrestris* detection (Murray *et al.*, 2007) and is also included in the IFU Method No. 12 for the isolation of *Alicyclobacillus* spp. (IFU, 2007). *Alicyclobacillus herbarius* (in combination with membrane filtration) (Goto *et al.*, 2002a), *A. acidiphilus* (Matsubara *et al.*, 2002), *A. pomorum* (Goto *et al.*, 2003) and *A. acidocaldarius* (Gouws *et al.*, 2005) were isolated from herbal tea, an acidic beverage, a mixed fruit juice and spoiled mango concentrate, respectively, using this medium. Goto *et al.* (2006; 2008) also used YSG to isolate a number of *Alicyclobacillus* strains from orange juice, lemon juice, orange-, apple- and watermelon juice concentrate, hyssop leaf, striped bamboo leaf, a soft drink and soil from various fruit orchards (banana, blueberry, chestnut, grape, kiwi, orange, pear, persimmon and strawberry) in Japan. Furthermore, YSG broth has also been used to isolate *Alicyclobacillus* strains from a variety of orchard soils in South Africa (Groenewald *et al.*, 2008).

Hiraishi glucose yeast extract (HGYE) agar

This chemically defined medium was used by Japanese researchers for the cultivation of *Alicyclobacillus* strains that had been isolated from Japanese hot

springs (Hiraishi *et al.*, 1997). However, it performed poorly when compared to other isolation media frequently used for *Alicyclobacillus* spp. (Murray *et al.*, 2007). The authors suggested that the low pH of the medium could have played a role in the poor recovery rates.

Potato dextrose agar (PDA)

A number of studies have used PDA for the enumeration of species of *Alicyclobacillus*. The agar is acidified to pH 3.50 after autoclaving to prevent agar hydrolysis (Chang & Kang, 2004). PDA at pH 3.50 was used to isolate an acidophilic spore-former, later identified as belonging to the genus *Alicyclobacillus*, from water used as an ingredient in fruit juice products that were spoiled (McIntyre *et al.*, 1995, Walls & Chuyate, 1998). Splittstoesser *et al.* (1994) also isolated two strains of *Alicyclobacillus*, VF and WAC, from spoiled apple juice and an apple-cranberry juice blend using PDA at pH 3.50 after membrane filtration. Subsequent characterisation studies conducted on the latter isolates showed better growth and higher colony counts when the pH was adjusted to 5.60 (Splittstoesser *et al.*, 1998).

Orange serum agar (OSA)

OSA is often used for the cultivation and enumeration of micro-organisms associated with citrus product spoilage (Chang & Kang, 2004). Hays and Riester (1952) used OSA (pH 5.50) to study off-flavour spoilage in frozen concentrated orange juice. Pettipher *et al.* (1997) found that spread plating onto OSA gave optimum recovery of *Alicyclobacillus* spp. when compared to PDA and BAM, although the latter two media also strongly supported growth of members of the genus *Alicyclobacillus*. Jensen (1999; 2000) observed improved growth when approximately 0.50% (m/v) sucrose was added to OSA.

K agar

Walls and Chuyate first proposed the use of K agar in 1998. They found that it was superior in its recovery of *Alicyclobacillus* species and that it improved the growth rate of the bacteria when compared to a variety of other media, including OSA, tomato juice agar special (TJAS), PDA (pH 3.50, 4.00, 4.50 and 5.00) and dextrose tryptone agar (DTA) (pH 7.40). When compared to the minimal salts medium (pH

4.00) of Farrand *et al.* (1983), the semi-synthetic medium (pH 4.00) of Darland and Brock (1971) (on which the formulation of BAM, BAT, AAM and ALI agar is based) and OSA (pH 3.50, adjusted with HCl) for isolation of *A. acidoterrestris* from apple juice, orange juice and a fruit juice blend (containing mainly white grape juice), K agar and the semi-synthetic medium had comparable recoveries which were significantly higher than OSA and the minimal salts medium (Walls & Chuyate, 2000a). K agar has also been included in the IFU Method No. 12 for isolating predominantly *A. acidoterrestris* (IFU, 2007).

SK agar

SK agar was developed as a new *Alicyclobacillus* spp. isolation medium for higher recovery rates and sensitivity (Chang & Kang, 2005). K agar was used as basal medium and different components of the medium, including pH, acidulant, Tween 80 concentration and divalent cation concentration, as well as incubation temperature were optimised. Divalent cations other than calcium, namely magnesium, iron and manganese, were also evaluated, but results varied among isolates and supplement concentrations. As $0.50 \text{ g.L}^{-1} \text{ Ca}^{2+}$ consistently increased the recovery of *Alicyclobacillus* spp., only this cation was included in the final formulation of SK agar. An incubation temperature of 43°C led to a higher recovery of *Alicyclobacillus* spp. than 55°C on SK agar. SK agar was significantly more effective than PDA (pH 3.70), OSA (pH 3.70) and K agar (pH 3.70) at recovering *Alicyclobacillus* spp. from apple juice and apple juice concentrate. This medium was more sensitive, allowing a better estimation of the cell concentration of *Alicyclobacillus* spp. present. SK agar was also able to isolate very low numbers of these bacteria (Chang & Kang, 2005).

Comparisons between different isolation media

Several studies have been done to compare different isolation media for members of the genus *Alicyclobacillus*. Some authors (Pettipher *et al.*, 1997; Pettipher, 2000; Pettipher & Osmundson, 2000) found that BAM, PDA and OSA all performed well in supporting the growth of *A. acidoterrestris*, with OSA giving the highest recovery. Spread plating was found to be more effective than pour plating. Orr and Beuchat (2000) found that K agar was most effective at supporting the development of chemically treated *A. acidoterrestris* spores when compared to OSA (pH 5.00) and

acidified PDA, while Parish and Goodrich (2005) found that ALI agar was more effective than K agar (pH 3.70) and PDA (pH 3.70) at recovering presumptive *Alicyclobacillus* spp. from the surfaces of oranges. According to Jensen (2005a) BAT agar was more effective at recovering *A. acidocaldarius*, while the use of K agar gave good results when isolating *A. acidoterrestris*.

Murray *et al.* (2007) evaluated 10 agar media, namely commercial K agar (pH 3.70), prepared K agar (pH 3.70), acidified PDA (pH 3.50), OSA (pH 3.50), YSG (pH 3.70), HGYE agar (pH 3.00), BAM (pH 4.00), ALI medium (pH 4.00), BAT agar (pH 4.00) and AAM (pH 4.00), for their ability to support the growth of six strains of *A. acidoterrestris*, three strains of *A. acidocaldarius* and one strain of *A. cycloheptanicus*. The influence of plating method (spread versus pour plates), incubation temperature (43°C and 50°C) and incubation time (up to 10 d) on colony development was also investigated. Spore recovery was highest when K agar (either commercially purchased or prepared in the laboratory from individual ingredients), ALI medium and BAT agar were used, while OSA and HGYE agar were the least suitable. Surface plating recovered higher numbers than pour plating and, with the exception of one strain of *A. acidocaldarius* which grew better at 50°C, incubation at 43°C or 50°C did not significantly affect spore recovery when using K agar, ALI agar and BAT agar plates. An incubation time of longer than 3 d did not significantly enhance the recovery of *Alicyclobacillus* spores, as all viable spores were detected on media incubated for 3 d at 43°C (Murray *et al.*, 2007).

Witthuhn *et al.* (2007) found that PDA (pH 3.70) and OSA (pH 5.50) plates incubated at 50°C for 3-5 d recovered higher numbers of *Alicyclobacillus* vegetative cells and spores compared to K agar (pH 3.70), YSG agar (pH 3.70) and BAM (pH 4.00). Media pH (pH 3.70 versus pH 5.50 for OSA and 5.60 for PDA) did not significantly influence recovery of *Alicyclobacillus* on PDA and OSA plates, while incubation temperature did have a significant influence, with recoveries being higher at 50°C than at 43°C.

In contrast to the previous studies, Jensen (2000) found that spread and pour plating had similar recoveries when *Alicyclobacillus* species were incubated in orange juice in a high oxygen environment. However, in a reduced oxygen environment pour plating gave higher recoveries than spread plating.

Membrane filtration

Isolation procedures have mostly been performed using plating media, but have also been combined with membrane filtration to isolate *Alicyclobacillus* species (Splittstoesser *et al.*, 1994; Albuquerque *et al.*, 2000; Goto *et al.*, 2002a). Some researchers have also suggested that membrane filtration be used to remove *Alicyclobacillus* spp. from beverages as part of quality control measures (Vieira *et al.*, 2002; Chang & Kang, 2004). To enumerate organisms using membrane filtration, the sample is passed through the filter, placed directly on the agar plate containing the growth medium and incubated (Pettipher, 2000). Filtration is more sensitive and has a lower detection limit than conventional spread plating, as larger samples can be passed through the filter (Chang & Kang, 2004; Lee *et al.*, 2007). However, membrane filtration is not suitable for all products, as many products cannot be filtered (Jensen, 1999).

Lee *et al.* (2007) investigated the ability of different filtration membranes to detect *Alicyclobacillus* spores in apple juice. Filtration membranes with two different pore sizes (0.22 and 0.45 μm) from five different manufacturers were evaluated and compared to conventional spread plating on K agar. Results were varied, with spore recovery differing among filters and isolates. In some cases membrane filtration resulted in higher counts than spread plating on K agar and in other cases membranes failed to recover any spores. Absence of growth when filtrates were plated onto K agar suggested that all *Alicyclobacillus* spores had been retained on the membranes, but that the membranes had not supported growth of the spores. Membranes with a smaller pore size did not result in higher recoveries. Because of the varied results it was recommended that juice manufacturers test the efficacy of their preferred filter membrane before using it in quality control processes (Lee *et al.*, 2007).

Heat shock treatment

Since *Alicyclobacillus* species are spore-formers, isolation procedures are often combined with a heat shock treatment in order to activate dormant spores and encourage germination and enumeration. Cell concentrations are often higher after a heat shock treatment if the bacteria are mostly present as spores. Splittstoesser *et al.* (1998) found that a heat treatment of 60°C for 30 min doubled the viable counts of

a sample containing *A. acidoterrestris*, indicating that about 50% of the cells had been present in the form of endospores. Witthuhn *et al.* (2007) also observed higher cell concentrations after subjecting samples to a heat treatment of 80°C for 10 min.

Various heat shock regimes have been investigated and recommended. Pettipher and others (Pettipher *et al.*, 1997; Pettipher, 2000; Pettipher & Osmundson, 2000) recommended a heat shock treatment of 80°C for 10 min. Walls and Chuyate (2000a) investigated several heat shock regimes and also found that heating at 80°C for 10 min yielded the highest spore recovery and was more effective than a treatment at 60°C for 10 min or 100°C for 5 min. Jensen (2000) found 70°C applied for 10 min to be the most effective treatment for spore germination, while Eiroa *et al.* (1999) found a heat treatment of 70°C for 20 min to be superior when compared to treatments of 60°C for 60 min, 60°C for 30 min, 80°C for 5 min, 80°C for 10 min, 80°C for 30 min and 100°C for 5 min. While the differences between heat treatments are probably minimal, application of some form of a heat treatment is essential to ensure a true reflection of the contamination level in samples.

F. DETECTION AND IDENTIFICATION

The various culture-dependent isolation and identification methods for species of *Alicyclobacillus*, although probably the simplest and least expensive to use, are time consuming and not always very reliable and specific. Although it is a fairly well-known identification method, Pettipher *et al.* (1997) and Jensen (1999; 2000) found that the use of API 50 CHB biochemical test strips for identification purposes was cumbersome and tedious. Results were also inconsistent and unreliable and it was, therefore, considered unsuitable as a routine identification method. A number of studies have developed rapid and sensitive detection/identification methods for *Alicyclobacillus* species.

PCR-based methods

Yamazaki *et al.* (1996b) developed a reverse-transcription-polymerase chain reaction (RT-PCR) method for the detection of *A. acidoterrestris* in acidic beverages. The V2 and V4 regions on the 16S rRNA gene were used as targets for primer design. It has been shown that the 5' end hypervariable region of the 16S rRNA gene sequences is

unique in different species of *Alicyclobacillus*, allowing distinction between species by DNA sequence comparison (Goto *et al.*, 2002b). The primers, named Ba 190F and Ba 490R, amplify a 294 base pair (bp) fragment. The primers were specific for *A. acidoterrestris* strains and no other *Alicyclobacillus* or *Bacillus* species included in the reactions were detected. The detection limit using the primers was 1 cfu.mL⁻¹, while the detection limit for the RT-PCR method was 10⁴-10⁶ cfu.mL⁻¹. The sensitivity could be increased to approximately 2 cfu.mL⁻¹ when samples were subjected to an overnight enrichment procedure and filtered prior to analysis.

The primers designed by Yamazaki *et al.* (1996b) were used to develop a semi-quantitative RT-PCR method for the detection of *A. acidoterrestris* in orange juice (Funes-Huacca *et al.*, 2004). The Agilent 2100 bioanalyser (Agilent Technologies, Waldbronn, Germany) makes use of microchip technology and capillary electrophoresis with laser-induced fluorescence detection to separate, detect and quantify the RT-PCR nucleic acid products. This method has a detection limit of approximately 1 cfu.mL⁻¹ and is able to quantify the cells present, as well as distinguish between viable and dead cells. It is also a rapid method, as results can be obtained within 24 h (Funes-Huacca *et al.*, 2004).

Niwa and Kawamoto (2003) developed an RT-PCR method using primers that amplify the *Vdc* gene, coding for vanillic acid decarboxylase, the enzyme that catalyses the decarboxylation of vanillic acid to guaiacol. The primers were specific for *A. acidoterrestris* and a detection limit of 10² cfu.mL⁻¹ was observed.

A real-time PCR method was developed for the detection of *A. acidoterrestris* and *A. acidocaldarius* vegetative cells in apple juice (Luo *et al.*, 2004). The target used for primer-and-probe development was the squalene-hopene cyclase (*shc*)-encoding gene which encodes for SHC, an enzyme that plays a role in hopanoid biosynthesis. This gene was selected because hopanoids are an important component of *A. acidoterrestris* and *A. acidocaldarius* cell membranes. The developed primer-and-probe set was specific for *A. acidoterrestris* and *A. acidocaldarius* and no cross-reactivity was detected with other organisms commonly found in food or with close relatives of the genus *Alicyclobacillus*. With a detection limit of <10 cells per reaction in either apple juice or saline solution, the sensitivity of the method was comparable to that of conventional culturing methods. Results could be obtained within 3-5 h, compared to 48 h or longer needed by conventional plating methods (Luo *et al.*, 2004). This method was consequently revised to also include

other species of *Alicyclobacillus*. This system targeted the 16S rRNA genes of the organisms, it could be completed within 5 h and it had a detection limit of <100 *Alicyclobacillus* cells (Connor *et al.*, 2005).

Duvenhage *et al.* (2007) also detected *Alicyclobacillus* spp. in apple, pear, white grape and aloe vera juice using a PCR-based denaturing gradient gel electrophoresis (DGGE) identification method. The detection limit of this method in fruit juice concentrate and single strength juice was $1.90\text{-}2.30 \times 10^3$ cfu.mL⁻¹ (Duvenhage *et al.*, 2007).

A randomly amplified polymorphic DNA (RAPD) assay for rapid identification of *A. acidoterrestris* has also been developed (Yamazaki *et al.*, 1997b). The three primers used in the assay were able to accurately distinguish *A. acidoterrestris* from other species of *Alicyclobacillus* as well as other bacteria closely related to the genus. The RAPD assay compared well to a conventional culture method when used for identification of thermo-acidophilic bacteria isolated from various environmental and food samples and was also accurate, cost- and time efficient and easy to perform (Yamazaki *et al.*, 1997b).

Fourier transform infrared spectroscopy (FT-IR)

FT-IR analyses the biochemical constituents of the bacterial cell wall and membrane (phospholipid bilayer, peptidoglycan and lipopolysaccharides), as well as the cell cytoplasm (water, fatty acids, proteins, polysaccharides and nucleic acids). This method has been used to distinguish and identify *Alicyclobacillus* spp. from other bacterial species in mixed cultures (Al-Qadiri *et al.*, 2006), as well as to discriminate between different strains of *Alicyclobacillus* (Lin *et al.*, 2005). It is also able to distinguish between guaiacol producing and non-guaiacol producing *Alicyclobacillus* strains with a reasonable degree of accuracy (Lin *et al.*, 2005).

VIT-Alicyclobacillus

A rapid detection method based on DNA probe technology, called VIT-Alicyclobacillus, was developed by Vermicon AG (Thelen *et al.*, 2003). Gene probes that are complimentary to *Alicyclobacillus* gene sequences are combined with fluorescent dyes and allowed to anneal to *Alicyclobacillus* DNA. When viewed under a fluorescent microscope the dye becomes visible where it has bound, indicating the

presence of *Alicyclobacillus* spp. The method is able to distinguish bacteria belonging to the genus *Alicyclobacillus* from other species (fluoresces green) and can also distinguish *A. acidoterrestris* from other *Alicyclobacillus* species (fluoresces red). Further advantages include that only viable cells are detected, that results can be obtained rapidly (after a pre-enrichment procedure of 2 d the complete analysis can be performed within 3 h), that staff do not need to be trained in molecular biology and that the equipment required is routine in most laboratories (Thelen *et al.*, 2003).

Sedlmair and Thelen (2006) compared the VIT-*Alicyclobacillus* method with conventional cultivation on K agar and BAM agar with and without pre-enrichment. Nine *Alicyclobacillus* strains belonging to five different species (three strains of *A. acidoterrestris*, three strains of *A. acidocaldarius* and one strain of *A. acidiphilus*, *A. cycloheptanicus* and *A. herbarius*, respectively), as well as five bacterial species not belonging to the genus *Alicyclobacillus*, were analysed. The VIT-*Alicyclobacillus* method was able to detect all nine *Alicyclobacillus* strains with a strong signal, as well as distinguish the three *A. acidoterrestris* strains from the rest of the *Alicyclobacillus* strains. None of the non-*Alicyclobacillus* species were detected, showing the specificity of the method.

Flow cytometry

Flow cytometry also makes use of fluorescent dyes to detect micro-organisms. Cells take up the dye and are then introduced to the flow cytometer, where a laser beam is directed onto the sample. The cell scatters light according to its size and DNA density, resulting in a specific signal for a particular cell type. Thus, different organisms can be distinguished based on the specific signal obtained. This method can also distinguish between dead and viable cells and spores qualitatively and quantitatively to 1 cell per 100 mL. The method is more rapid than conventional plating methods, as the procedure can be completed within 10 h instead of 5-10 d (Borlinghaus & Engel, 1997). Borlinghaus and Engel (1997) partially validated a flow cytometry method for *Alicyclobacillus* spp. using inoculated apple juice concentrate and parallel testing with well known cultural plating methods. They were able to qualitatively and quantitatively detect *Alicyclobacillus* spp. in the inoculated concentrate using flow cytometry and also detected *Alicyclobacillus* spp. in a number of commercially available apple juice concentrates (Borlinghaus & Engel, 1997).

Determination of guaiacol-producing ability

Apart from the various rapid molecular and analytical detection methods developed for *Alicyclobacillus* spp., a rapid detection kit based on the detection of guaiacol using its reaction with peroxidase enzyme in the presence of H₂O₂ has also been developed (Niwa & Kawamoto, 2003; Niwa & Kuriyama, 2003; Niwa, 2004; Anon., 2005; Anon., 2006). The kit consists of YSG or BAT broth containing vanillic acid and the peroxidase enzyme, phosphate buffer and H₂O₂ solutions. To determine the presence of guaiacol producing *Alicyclobacillus*, the medium containing vanillic acid is inoculated with the suspect organism and incubated at 45°C for 1-3 h. When detecting directly from a product, such as fruit juice concentrate, the product must be diluted with the medium and incubated at 45°C for 2-3 d. A sample of the culture is then combined with the enzyme, buffer and H₂O₂. If a brown colour develops which can be detected visually or spectrophotometrically at 470 nm (Niwa & Kuriyama, 2003) or 420 nm (Bahçeci & Acar, 2007a), it is an indication of the presence of guaiacol producing *Alicyclobacillus* spp., as well as the potential for spoilage to occur.

Ribotyping

Pettipher *et al.* (1997) and Walls & Chuyate (1998) have suggested using ribotyping for the identification of *Alicyclobacillus* spp. The DuPont RiboPrinter was used in both studies and *Alicyclobacillus* spp. could be distinguished from other bacterial species. However, this method was reported to be too expensive for routine laboratory use (Pettipher *et al.*, 1997).

G. CONTROL

The high heat resistance of *Alicyclobacillus* spores allows them to survive commercial hot-fill-hold pasteurisation processes. In fact, the pasteurisation treatment imitates a heat shock treatment (Jensen, 1999; Gouws *et al.*, 2005), which activates the spores and, since the acidic environment is favourable for their growth, they can easily multiply to numbers at which guaiacol is produced and spoilage occurs. *Alicyclobacillus* spp. are mostly a problem in fresh (not heat treated) and pasteurised (but not ultra high temperature (UHT) treated) fruit juices, as these

products are stored unpreserved at ambient temperatures (Pettipher *et al.*, 1997, Orr *et al.*, 2000).

Treatment at UHT is able to eliminate *Alicyclobacillus* spp., as no UHT treated juices have been found to contain *Alicyclobacillus* spp. (Pettipher, 2000). However, UHT treatment is not suitable for all products. Higher pasteurisation temperatures are also not feasible, as this can change the organoleptic and nutritional properties of the juice products. Cooling to below 4°C, which inhibits the growth of *Alicyclobacillus* spp., is problematic, as it presents a major additional cost factor to manufacturers. Thus, a variety of other possible control measures for *Alicyclobacillus* spp. in fruit juices and the processing environment have been investigated.

Bacteriocins

Several studies have been conducted to investigate the efficacy of bacteriocins against *Alicyclobacillus* spp. Bacteriocins are antimicrobial compounds that are produced naturally by many bacteria (Komitopoulou *et al.*, 1999; Walker & Phillips, 2008).

Nisin is by far the most widely researched bacteriocin for the control of *Alicyclobacillus* spp. Nisin is a polypeptide produced by strains of *Lactococcus lactis* subsp. *lactis* and is active against a broad range of gram-positive bacteria, particularly spore-formers, through the inhibition of spore germination (Komitopoulou *et al.*, 1999; Walker & Phillips, 2008). Peña and De Massaguer (2006) observed nisin to be effective with regards to inhibiting the growth of *A. acidoterrestris*. Nisin at concentrations ranging from 17.50-52.50 International Units (IU).mL⁻¹ increased the lag phase of growth to varying degrees, compared to a culture with 0.00 IU.mL⁻¹ nisin where growth commenced immediately. A concentration of 52.50 IU.mL⁻¹ nisin was able to inhibit growth at 45.5°C for up to 47 d. Komitopoulou *et al.* (1999) found that the addition of 50.00 IU.mL⁻¹ nisin increased the heat sensitivity of *A. acidoterrestris* spores in apple juice, with reductions in D-values of up to 40%. When spores of *A. acidoterrestris* were grown at different temperatures in a variety of juices, sensitivity to nisin increased with decreasing temperature. At 25°C, representing ambient storage, a nisin concentration of only 5.00 IU.mL⁻¹ was sufficient to inhibit growth of spores in apple (pH 3.51), orange (pH 3.42) and grapefruit (pH 3.90) juices, while 100 IU.mL⁻¹ was required to inhibit growth at 44°C in apple and orange juice. In

grapefruit juice at 44°C, 5.00 IU.mL⁻¹ nisin inhibited growth, illustrating an increased sensitivity of the spores in grapefruit juice compared to apple and orange juice. Furthermore, spores were more sensitive to nisin than cells in the vegetative state (Komitopoulou *et al.*, 1999). A study done by Yamazaki *et al.* (2000) showed comparable results. When vegetative cells and spores of several strains of *A. acidoterrestris* were incubated at 46°C on modified yeast peptone glucose (YPG) agar plates at pH 3.40 and 4.20, respectively, the minimum inhibitory concentrations (MIC) of nisin for spores ranged from <0.78-12.50 IU.mL⁻¹ and 25.00-100.00 IU.mL⁻¹ at pH 3.40 and 4.20, respectively. For vegetative cells it ranged from 1.56-50.00 IU.mL⁻¹ and 25.00-100.00 IU.mL⁻¹ at pH 3.40 and 4.20, respectively. Thus, the results also showed an increased sensitivity of spores compared to vegetative cells. The addition of 200.00 IU.mL⁻¹ nisin reduced the thermal resistance of *A. acidoterrestris* spores by 71% and 76% in clear apple juice and an orange drink, respectively, and growth at 40°C was inhibited by 25.00 IU.mL⁻¹ and 50.00 IU.mL⁻¹ nisin in orange and mixed fruit drinks, respectively. However, as observed by Komitopoulou *et al.* (1999), the type of juice seemed to influence the sensitivity of the organism, as even 600.00 IU.mL⁻¹ nisin was unable to inhibit growth in a clear apple drink (Yamazaki *et al.*, 2000). A third study, conducted by Walker and Phillips (2008), found that nisin at 5.00-10.00 IU.mL⁻¹ was able to prevent the growth of *A. acidoterrestris* cells and spores in apple juice at 30°C.

Other bacteriocins have also been studied to determine their efficacy against *Alicyclobacillus* spp. *Alicyclobacillus acidocaldarius* and *A. acidoterrestris* were sensitive to enterocin AS-48 from *Enterococcus faecalis* (Grande *et al.*, 2005). A concentration of 2.50 µg.mL⁻¹ enterocin AS-48 was able to reduce vegetative cells of *A. acidoterrestris* to below the detection limit after 24 h of incubation in AAM at 37°C. The same observations were made when it was incubated in freshly-made orange (pH 3.86) and apple (pH 3.55) juices containing 2.50 µg.mL⁻¹ enterocin AS-48. Vegetative cells and spores of *A. acidoterrestris* were also inoculated into five commercial fruit juices (orange, apple, pineapple, peach and grapefruit) containing 2.50 µg.mL⁻¹ enterocin AS-48 and incubated at 37°C, 15°C and 4°C. Cells were inactivated as soon as 15 min after inoculation and growth was inhibited for up to 90 d in orange and pineapple juices, 60 d in apple, peach and grapefruit juices at 37°C and for the entire incubation period in all the juices at 15°C and 4°C. Electron microscopy performed after addition of enterocin AS-48 to *A. acidoterrestris* cells

revealed cell wall damage, leakage of cytoplasmic contents, cell disorganisation and degraded endospores (Grande *et al.*, 2005).

Bovicin HC5 from *Streptococcus bovis* also showed antimicrobial activity against *A. acidoterrestris* (De Carvalho *et al.*, 2008). At concentrations of 40.00-160.00 AU.mL⁻¹ it was able to completely inhibit growth in AAM broth (pH 4.00) at 40°C for as long as 15 d. The MIC of bovicin HC5 was determined to be 5.00 AU.mL⁻¹ and 2.50 AU.mL⁻¹ for vegetative cells and spores, respectively. When mango pulp with a pH ranging from 4.00-7.00 inoculated with *A. acidoterrestris* cells or spores at a level of 10⁵-10⁶ cfu.mL⁻¹ was treated with 80.00-100.00 AU.mL⁻¹ bovicin HC5, a bactericidal and sporicidal effect was observed. When bovicin HC5 was added to mango pulp (pH 4.00) at a concentration of 80.00 AU.mL⁻¹ the D₈₀₋₉₅-values of *A. acidoterrestris* spores decreased with 77-95% and the z-value with 48.7% (De Carvalho *et al.*, 2008).

Chemical disinfectants

Orr and Beuchat (2000) investigated the efficacy of sodium hypochlorite (NaOCl) (free chlorine in phosphate buffer), acidified sodium chlorite (NaClO₂), trisodium phosphate (Na₃PO₄), H₂O₂ and Tsunami when it came to killing spores of *A. acidoterrestris* in aqueous suspensions. NaOCl and H₂O₂ were the most effective at killing spores and the sporicidal effect was significantly strengthened with each increase in the concentration of treatment solutions. Exposure to 200-1000 ppm NaOCl for 10 min at 23±2°C resulted in a 2.3-6 log reduction in spores, while spores were reduced by approximately 5.00 logs when treated with 2-4% H₂O₂ in the same manner. Treatment with NaClO₂ (1200 ppm), Na₃PO₄ [12% (v/v)] and Tsunami (160 ppm) was not very effective, with only 1.50, 0.20 and 0.20 log reductions being achieved, respectively. Individual strains of *A. acidoterrestris* did not differ dramatically in their sensitivity to the different chemicals. The chemical treatments were considerably less effective at killing spores when used to treat the surfaces of apples containing *A. acidoterrestris* spores. Treatment with 500 ppm NaOCl or 1200 ppm NaClO₂ for 1 min achieved a less than 1.00 log reduction, while 2% H₂O₂ was even less effective. This could be due to the micro-organisms adhering to grooves, cracks and cuts on the apple surface, making them less accessible to the chemicals (Orr & Beuchat, 2000).

Free chlorine dioxide (ClO₂) has approximately 3.5 times the oxidation capacity of chlorine. Lee *et al.* (2004) investigated the effect of this chemical against *A. acidoterrestris* spores in aqueous suspensions and on apple surfaces. In aqueous suspensions at 22±2°C, treatment with 40 ppm ClO₂ reduced spore numbers by more than 4 logs after 5 min. Spores were reduced by 2.8 and 4.8 logs after only 1 min of treatment with 80 ppm and 120 ppm ClO₂, while treatment for 5 min resulted in spore reductions of more than 5 logs, to below the detection limit of 0.70 cfu.mL⁻¹. This is comparable to the treatment of 1000 ppm NaClO or 4% (v/v) H₂O₂ for 10 min reported by Orr and Beuchat (2000), showing that ClO₂ achieved comparable results at a lower concentration and in a shorter time. ClO₂ was also more effective at killing *A. acidoterrestris* spores on apple surfaces, with treatment of 40 ppm or 120 ppm for 4 or 1 min, respectively, resulting in a more than 5 log reduction to below the detection limit of 2 cfu.mL⁻¹. Thus, in contrast to the observations made by Orr and Beuchat (2000), ClO₂ was more effective at killing spores on apple surfaces than in aqueous suspensions (Lee *et al.*, 2004).

Gaseous ClO₂ was also tested for its efficacy against *A. acidoterrestris* spores on apple surfaces. Apples were exposed to the gas using high, medium or low release ClO₂ gas sachets. High and medium release sachets were able to bring about a 5 log reduction in spores after 1 h, while the low release sachet took 3 h to reduce spores by 4.5 logs. However, the high and medium release sachets had a negative effect on the visual quality of the apples, as small black spots developed on the skin after 3 d of storage at 4°C. This problem did not arise when using the low release sachets. Thus, use of high and/or medium release sachets was recommended for apples to be used in juice production, where the visual quality of the apples would be less important. Low release sachets, which had comparable spore reduction effects but did not have a negative impact on apple appearance, were recommended for apples to be sold on the fresh produce market (Lee *et al.*, 2006b).

Natural compounds

Current consumer trends in food and beverage products are inclined towards products that are healthier, minimally processed and contain less chemical food additives (Lee *et al.*, 2002). The current trend of “greener living” also demands

products with a smaller impact on the environment, as well as more natural methods of food preservation (Bevilacqua *et al.*, 2008a).

This trend has resulted in some natural compounds being investigated for their ability to inhibit the growth of *A. acidoterrestris*. Extracts from the leaves of eight species of eucalyptus, *Eucalyptus bridgesiana*, *E. caley*, *E. drepanophylla*, *E. globulus*, *E. maculata*, *E. maidenii*, *E. robusta* and *E. viminalis* were able to significantly inhibit the growth of *A. acidoterrestris*. Extracts from the first seven species all had an MIC of 7.80 mg.L⁻¹, while *E. viminalis* had an MIC of 15.60 mg.L⁻¹ (Takahashi *et al.*, 2004).

Essential oils are aromatic liquids used primarily as components of flavouring agents and fragrances (Bevilacqua *et al.*, 2008a). Eugenol, cinnamaldehyde and limonene are major compounds in essential oils and were investigated for their antimicrobial activity against *A. acidoterrestris* c8 and γ 4 spores (Bevilacqua *et al.*, 2008a). Cinnamaldehyde at a concentration of 500 ppm was able to inhibit the growth of *A. acidoterrestris* γ 4 spores for the duration of the study (13 d). At 100 ppm this compound was able to extend the lag phase of growth of *A. acidoterrestris* γ 4 spores to 8 d but was unable to inhibit growth completely. *Alicyclobacillus acidoterrestris* c8 spores were more resistant to the antimicrobial activity of the compounds and only cinnamaldehyde at 500 ppm was able to inhibit spore germination. Limonene had no antimicrobial activity against the spores of either of the investigated strains (Bevilacqua *et al.*, 2008a).

Heat-stable peptides extractable from barley and wheat seeds called α - and β -hordothionins and α - and β -purothionins, respectively, have also been investigated for possible antimicrobial activity against *A. acidoterrestris* (Oita, 2002). The MIC of α -hordothionin, β -hordothionin and α -purothionin ranged from 5.00-10.00 μ g.mL⁻¹ in YPG broth and was 15.00 μ g.mL⁻¹ in satsuma mandarin juice. The α -purothionin at a concentration of 20.00 μ g.mL⁻¹ was able to decrease a population of *A. acidoterrestris* spores at 37°C from 10⁵ cfu.mL⁻¹ to 10³ cfu.mL⁻¹ and 10² cfu.mL⁻¹, in a fruit-vegetable juice mixture and satsuma mandarin juice, respectively. Addition of citric acid extracts from barley flour containing α - and β -hordothionins at a concentration of 3% (v/v) to satsuma mandarin juice, prevented spoilage of the juice by inhibiting growth of *A. acidoterrestris* for 10 d (Oita, 2002).

Preservatives

Sodium benzoate and potassium sorbate have been investigated with regards to their suitability as control mediums for *A. acidoterrestris*. Walker and Phillips (2008) found that concentrations of 0.10-0.50 mg.mL⁻¹ sodium benzoate and potassium sorbate were able to inhibit the growth of 10¹ cfu.mL⁻¹ *A. acidoterrestris* vegetative cells and spores in apple juice at 30°C for 12 d. Slightly higher concentrations of 0.50-1.50 mg.mL⁻¹ of the preservatives were able to inhibit the growth of 10⁴ cfu.mL⁻¹ *A. acidoterrestris* vegetative cells and spores in apple juice at 30°C for 30 d. Similarly, Bevilacqua *et al.* (2008a) found that 500 ppm sodium benzoate effectively inhibited the growth of *A. acidoterrestris* in MEB at 44°C for approximately 8 d, while 100 ppm of the compound could only inhibit growth for 3 d. Pettipher and Osmundson (2000) found that 300 mg.L⁻¹ sorbic acid, 150 mg.L⁻¹ benzoic acid, or a combination of the two prevented spoilage of fruit juice drinks by *Alicyclobacillus* spores and vegetative cells. Addition of the preservatives kept vegetative cells and spores below a level of 150 cfu.mL⁻¹ for up to three months. The action of the preservatives was determined to be sporostatic rather than sporocidal (Bevilacqua *et al.*, 2008a; Walker & Phillips, 2008).

High pressure technology

High hydrostatic pressure (HHP) is able to kill vegetative microbial cells by damaging the cell membrane, causing leakage of cytoplasmic content, as well as disrupting the structure of the nucleus, cell organelles and cell proteins. HHP is normally used in combination with other treatments such as heat, since the pressure levels that can be applied to food without altering the texture, aroma and colour are ineffective when used alone (Farr, 1990).

When used in combination with a heat treatment of 50°C it was found that pressurisation at 350 MPa for 20 min in BAM broth (pH 3.00) was able to bring about a log reduction of 4.7 in *A. acidoterrestris* vegetative cells, in contrast to thermal treatment alone, which only caused a reduction of 1.13 logs (Alpas *et al.*, 2003). The viability loss was time, temperature and pressure dependent, with an increase in viability loss being observed as time, temperature and pressure increased. The D₅₀-values were reduced by 23% when heat treatment was accompanied by a pressurisation treatment of 350 MPa. *Alicyclobacillus acidoterrestris* cells inoculated

into orange (pH 2.44, 11.40°Brix), apple (pH 3.01, 10.60°Brix) and tomato (pH 4.16, 5.60°Brix) juices were reduced by more than 4 logs in all juices after pressurisation at 350 MPa at 50°C for 20 min and treated cells increased only by approximately 1 log during three weeks incubation at 30°C (Alpas *et al.*, 2003).

Bacterial spores are more resistant to high pressure treatment than vegetative cells and much higher pressures (above 700 MPa) or longer treatment times at lower pressures are needed for this type of treatment to be effective. However, such high pressures could lead to undesirable organoleptic changes occurring in products (Alpas *et al.*, 2003). Lee *et al.* (2002; 2006a) found that high pressure of up to 621 MPa at room temperature for 10 min or a heat treatment of 90°C for 1 min alone was ineffective against *A. acidoterrestris* spores. Thus, they emphasised that the employment of a hurdle concept, where HHP is combined with heat or other treatments, is vital when bacterial spores, instead of vegetative cells, are treated. Pressures of 207-621 MPa combined with temperatures of 45°C, 71°C or 90°C all resulted in decreases in spore viability. However, a higher pressure and temperature combination required a much shorter treatment time to achieve the same viability loss as a treatment at a lower pressure and temperature. For instance, a treatment of 207 MPa at 45°C resulted in a >3.5 log reduction in the number of spores after 10 min, while a treatment of 414 or 621 MPa at 71°C took only 1 min to achieve the same viability loss. For a given pressure at 45°C or 71°C, an increase in treatment time resulted in a higher viability loss, but there was no significant difference between the effects of pressurisation at 207, 414 or 621 MPa on spore viability. Treatment combinations that were able to decrease spores to undetectable levels were 414 or 621 MPa at 71°C for 10 min and 90°C for 1 min, as well as a treatment of 207 MPa at 90°C for 5 min (Lee *et al.*, 2002; Lee *et al.*, 2006a).

The efficacy of high pressure treatment is dependent on the SS content of the juice being treated. Lee *et al.* (2006a) found that in apple juice with a SS content of 17.50°Brix, all combinations of high pressure (207, 414 and 621 MPa) and heat (45°C, 71°C and 90°C) treatments resulted in a reduction of *A. acidoterrestris* spores ranging from 2-5 logs depending on the treatment. In 30.00°Brix apple juice, however, high pressure treatments at 45°C were ineffective and spore reduction was only observed when high pressure treatments were combined with heat treatments of 71°C or 90°C. In apple juice concentrate (70.00°Brix) none of the treatments were able to reduce the concentration of *Alicyclobacillus* spores.

High-pressure homogenisation

High-pressure homogenisation (HPH) is another high-pressure-based method with the potential to destroy *Alicyclobacillus* cells and spores. This technique most probably exerts its antimicrobial activity by disrupting the cell wall and outer membrane of micro-organisms (Bevilacqua *et al.*, 2007). Bevilacqua *et al.* (2007) investigated the efficacy of HPH (500-1700 bar) against three strains of *A. acidoterrestris* cells and spores in MEB at pH 4.50. The efficacy of the HPH treatment was strain-dependent, with some strains being more resistant than others, and cells were more susceptible than spores. In the least resistant strain, HPH treatment at 1700 bar resulted in a cell reduction of 1.67-1.97 logs (Bevilacqua *et al.*, 2007).

Irradiation

Irradiation involves exposure of foods to low levels of radiant energy such as gamma-rays, x-rays or electrons in order to sterilise or preserve it (Mahapatra *et al.*, 2005). Electron-beam and gamma-ray irradiation were investigated for their ability to inactivate *A. acidoterrestris* spores (Nakauma *et al.*, 2004). D-values for *Alicyclobacillus* spores on filter paper using gamma-ray or electron-beam irradiation were determined to be 1.02 ± 0.12 and 1.10 ± 0.07 kGy, respectively. Combination of either gamma-ray or electron-beam radiation at levels from 0.50-2.00 kGy with a heat treatment of 95°C resulted in a significantly higher level of spore reduction when compared to a heat treatment alone. The duration of the heat treatment needed to achieve significant reductions in spore levels was also shortened by prior irradiation treatment. The combination of low doses of irradiation with heat is advantageous as it reduces the duration of the heat treatment required for inactivation of spores and, therefore, reduces the possibility of damage to treated food products (Nakauma *et al.*, 2004).

Antimicrobial packaging

An active film consisting of a silver (Ag^{2+})-containing polyethylenoxide-like coating on a polyethylene layer was tested for its ability to inhibit the growth of *A. acidoterrestris* (Del Nobile *et al.*, 2004). The film was able to either inhibit or reduce the growth of *A. acidoterrestris* in acidified MEB and apple juice at 44°C, but its effectiveness

depended on the type of medium in which it was used (Del Nobile *et al.*, 2004).

Lysozyme, a natural antibacterial agent which is able to damage the structure of bacterial cell wall peptidoglycan, has also been used as the active compound in the development of an active polyvinylalcohol-based antimicrobial film (Conte *et al.*, 2006). Incubation of either a single strain or a five-strain culture cocktail of *A. acidoterrestris* in acidified MEB at 44°C in the presence of the film led to a loss of viable cells, and the film maintained its efficacy even if the medium volume was reduced. The film was even more effective against viable *A. acidoterrestris* spores and was able to reduce the viable spore count in acidified MEB and in apple juice, demonstrating its ability to inhibit the germination of the spores (Conte *et al.*, 2006).

Calcium lactate fortification

Juices are often fortified with calcium to increase their nutritional value, but calcium salts also possess antimicrobial properties and thus have the potential to aid in the control of spoilage micro-organisms in juice. Yeh *et al.* (2004) investigated the effect of calcium lactate at concentrations ranging from 0-30% dietary reference intake (DRI) on the survival and growth of *A. acidoterrestris* spores in orange juice at pH 3.60 and 4.10 and temperatures of 10°C or 4°C. Growth was inhibited at a temperature of 4°C, regardless of the pH or presence of calcium lactate, for up to seven weeks. Similar results were seen in orange juice at pH 3.60 stored at 10°C. However, in orange juice at pH 4.10 stored at 10°C, growth was only inhibited in juices containing a minimum calcium lactate concentration of 10% DRI. The spores were able to grow in the juices with no calcium fortification and a calcium lactate concentration of 5% DRI. The calcium lactate was only able to inhibit growth of *A. acidoterrestris*, but was not able to reduce the cell population (Yeh *et al.*, 2004).

Alternative approaches

An alternative approach to the above-mentioned preventative measures has been to investigate possible damage control measures that can be applied to eliminate spoilage compounds after a spoilage incident has occurred. Laccase enzymes were identified to have the potential to eliminate off-flavours often found in apple juice due to contaminating organisms such as *Alicyclobacillus* spp., as they are able to oxidise a wide range of compounds, including polyphenols and methoxy-substituted phenols

such as guaiacol (Schroeder *et al.*, 2008).

A laccase enzyme purified from the fungus *Trametes hirsuta* was tested for its ability to eliminate guaiacol and other off-flavours from apple juice (Schroeder *et al.*, 2008). The highest reductions were seen for guaiacol, with a 99% reduction, and 2,6-DBP, with a 52% reduction. The oxidation effect of the enzyme was enhanced by addition of the mediators ABTS or 1-hydroxybenzotriazole (HOBt) and, in the case of 2,6-DBP, the performance of the enzyme improved when other compounds, such as guaiacol, were present (Schroeder *et al.*, 2008).

Sensory analysis clearly demonstrated the ability of the enzyme to remove the off-flavours (Schroeder *et al.*, 2008). Sensory analysis was conducted on samples containing guaiacol and 2,6-DBP, respectively after treatment with laccase. With regards to 2,6-DBP, two of the seven panel members were able to detect the typical medicinal flavour of the 2,6-DBP in the 2,6-DBP-containing samples after laccase treatment, while the rest of the panel could detect no difference between the laccase treated 2,6-DBP-containing and reference samples. When samples containing guaiacol were treated with laccase, none of the panel members could distinguish between the guaiacol-containing and reference samples, indicating the higher efficacy of the enzyme against guaiacol (Schroeder *et al.*, 2008).

A disadvantage of this method is that treatment of apple juice with the laccase enzyme also resulted in a change in the flavour characteristic of apple juice. This could either be because the enzyme incubation temperature of 50°C resulted in organoleptic changes in the product, or because the excessive removal of phenolic compounds by the enzyme negatively affected the flavour of the juice (Schroeder *et al.*, 2008).

H. CONCLUSION

Alicyclobacillus species have become an increasing threat to the fresh and pasteurised fruit juice industry. Spoilage incidents can be very costly for the manufacturer and manufacturers risk financial losses and loss of consumer confidence when spoilage incidents result in product recalls. Numerous isolation, identification and control methods for *Alicyclobacillus* spp. have been investigated, but a standardised isolation and identification method has still not been established.

Although research has focussed on *A. acidoterrestris* as the *Alicyclobacillus*

species primarily responsible for spoilage incidents, other species have also been implicated. It is, therefore, important to establish the taint producing abilities and spoilage potential of other *Alicyclobacillus* isolates. This is essential for quality control procedures, as non-taint producing *Alicyclobacillus* spp. are not able to spoil products and thus their presence in products does not pose a spoilage risk. The influence of storage conditions, such as temperature, and the ability of *Alicyclobacillus* spp. to grow in juices need to be investigated in order to establish which conditions are favourable for spoilage to occur so that exposure of products to such conditions can be avoided.

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CHAPTER 3

COMPARISON OF ISOLATION METHODS AND GROWTH CURVES OF DIFFERENT STRAINS OF *ALICYCLOBACILLUS* SPECIES FROM SOUTH AFRICA

ABSTRACT

Alicyclobacillus species are thermo-acidophilic, spore-forming bacteria that are able to survive pasteurisation and have been implicated in a number of spoilage incidents involving acidic foods and beverages. The aim of this study was to compare three isolation methods used for the detection of species of *Alicyclobacillus*, to investigate the growth patterns under optimum conditions of strains of *Alicyclobacillus* species and finally to investigate the influence of incubation temperature on the growth of a selection of these strains. The method comparison included inoculation of diluted peach juice concentrate samples with *A. acidoterrestris* K47, followed by a heat shock treatment at 80°C for 10 min. The heat shocked sample was then analysed using either the International Federation of Fruit Juice Producers (IFU) Method No. 12, which involved spread plating onto *Bacillus acidoterrestris* (BAT) agar at pH 4.00; Method 2, which involved pour plating using potato dextrose agar (PDA) at pH 3.70; or Method 3, which made use of membrane filtration followed by incubation on K agar at pH 3.70. The performance of the three methods differed significantly, with the IFU Method No. 12 recovering the highest percentage of cells at 75.97%, followed by Method 2 at 66.79% and Method 3 at 3.43%. These findings strengthen the proposal of the IFU for the use of the IFU Method No. 12 as a standard international method for the detection of species of *Alicyclobacillus*. To determine growth patterns under optimum conditions, BAT media, supplemented with 100 mg.L⁻¹ vanillin, was inoculated with either *A. acidoterrestris* (five different strains), or *A. acidocaldarius* (two different strains), to a final cell concentration of 10²-10³ cfu.mL⁻¹. Cultures were incubated at 45°C for 7 d and samples analysed every 12 h. The absorbance at 540 nm, pH and cell concentration before and after heat shock (using PDA pour plates at pH 4.00) was determined. All strains showed a rapid increase in cell concentrations during the first 24 h, after which cell concentrations

fluctuated around maximum values of 10^5 - 10^7 cfu.mL⁻¹. Cell concentrations after heat shock, measured as an indication of spore formation, also increased rapidly to maximum values of 10^5 - 10^7 cfu.mL⁻¹, indicating an increase in spore formation as cell density and competition increased. To investigate the effect of different incubation temperatures on the growth patterns of strains of *Alicyclobacillus*, either *A. acidoterrestris* (three strains) or *A. acidocaldarius* (two strains) were incubated at either 45°C or 25°C. Growth at 25°C was slower and maximum cell concentrations were lower (10^5 - 10^6 cfu.mL⁻¹ compared to 10^7 - 10^8 cfu.mL⁻¹) than at 45°C for the *A. acidoterrestris* strains. The *A. acidocaldarius* strains were unable to grow at 25°C and cell concentrations decreased by 1-2 logs. Since a growth temperature of 25°C could not inhibit growth of *A. acidoterrestris*, cooling to room temperature (20°-25°C) is not an effective control measure for *A. acidoterrestris*.

INTRODUCTION

Species of *Alicyclobacillus* are thermo-acidophilic, spore-forming, non-pathogenic bacteria that pose a problem to the food industry as they are able to survive pasteurisation temperatures (Splittstoesser *et al.*, 1998; Eiroa *et al.*, 1999; Vieira *et al.*, 2002). These bacteria can multiply in acidic products such as fruit juice to cell concentrations high enough to produce off-flavour and odour taints, leading to product spoilage (Pettipher *et al.*, 1997; Orr *et al.*, 2000; Gocmen *et al.*, 2005).

Species of *Alicyclobacillus* have been isolated from a variety of environments, including thermal acid environments such as hot springs (Darland & Brock, 1971; Wisotzkey *et al.*, 1992; Goto *et al.*, 2002; Simbahan *et al.*, 2004), various soils (Goto *et al.*, 2007; Imperio *et al.*, 2008; Groenewald *et al.*, 2008; Goto *et al.*, 2008) and acidic food- and beverage products such as fruit concentrate and fruit juice (Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996; Matsubara *et al.*, 2002), iced tea (Duong & Jensen, 2000) and canned diced tomatoes (Walls & Chuyate, 1998). A number of agar media and growth conditions for the isolation of species of *Alicyclobacillus* have been proposed, developed and compared (Murray *et al.*, 2007), but no standard method has thus far been accepted.

Fruit processors in South Africa primarily make use of three methods for the isolation of *Alicyclobacillus* species, namely the International Federation of Fruit Juice Producers (IFU) Method No. 12, Method 2 and Method 3. The IFU Method No. 12

for the isolation of species of *Alicyclobacillus* was developed in 2004 and revised in 2007. This method was proposed as a standard method for the isolation of species of *Alicyclobacillus* (IFU, 2007). Method 2 makes use of pour plates with acidified potato dextrose agar (PDA). Several studies have also made use of this agar for the isolation of *Alicyclobacillus* spp. (Splittstoesser *et al.*, 1994; McIntyre *et al.*, 1995; Walls & Chuyate, 1998; Witthuhn *et al.*, 2007). Method 3 makes use of membrane filtration, followed by incubation on K agar. K agar was first suggested for the isolation of species of *Alicyclobacillus* in 1998 by Walls and Chuyate.

The growth temperature range for the genus *Alicyclobacillus* is 20°C-et al., 2005), with an optimum range of et al., 1992; Karavaiko *et al.*, 2005) The pH range for growth is 0.50-7.50, with an optimum range of 1.50-5.50 (Wisotzkey *et al.*, 1992; Nicolaus *et al.*, 1998; Walls & Chuyate, 1998; Albuquerque *et al.*, 2000; Goto *et al.*, 2002a; Goto *et al.*, 2002c; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Karavaiko *et al.*, 2005; Goto *et al.*, 2007; Imperio *et al.*, 2008; Jiang *et al.*, 2008). Most researchers use incubation temperatures between et al., 1987; Yamazaki *et al.*, 1996; Splittstoesser *et al.*, 1998; Walls & Chuyate, 1998; Wisse & Parish, 1998; Goto *et al.*, 2002a; Chang & Kang, 2005). Some studies have found that *A. acidoterrestris* is not able to grow at et al., 2005), while others found that growth is only inhibited at temperatures below

The aim of this study was to compare the three isolation methods primarily used by South African fruit processors for the isolation of members of the genus *Alicyclobacillus* and to characterise the growth patterns of species of *Alicyclobacillus* isolated from the South African fruit processing environment under different conditions.

MATERIALS AND METHODS

Bacterial strains

Alicyclobacillus acidoterrestris K47 (Department of Food Science Culture Collection, Stellenbosch University) was isolated from white grape juice concentrate obtained from a manufacturer in South Africa (Witthuhn *et al.*, 2007). *Alicyclobacillus acidoterrestris* FB2, FB14, FB32 and FB38, as well as *A. acidocaldarius* FB19, were isolated from fruit concentrate, wash water, evaporator water, flume water and vinegar flies, respectively, at a fruit processing plant in the Western Cape province of South Africa (Groenewald *et al.*, 2009). The type strains *A. acidoterrestris* DSM 3922^T and *A. acidocaldarius* DSM 446^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

Comparison of three isolation methods

Three isolation methods primarily used by South African fruit processors for the isolation of members of the genus *Alicyclobacillus* were compared with regards to their ability to effectively isolate a strain of *A. acidoterrestris* from diluted peach juice concentrate.

Bacterial inoculation culture

An isolate of *A. acidoterrestris* K47 was inoculated into yeast starch glucose (YSG) (Goto *et al.*, 2002b) broth [2.00 g.L⁻¹ yeast extract (Biolab, supplied by Merck, Cape Town, South Africa), 1.00 g.L⁻¹ glucose (AnalAR, supplied by Merck) and 2.00 g.L⁻¹ soluble starch (Pro Analyti, supplied by Merck)], acidified to pH 4.00 with 1 M H₂SO₄ (AnalAR, supplied by Merck), and incubated at 45°C for 5 d.

A volume of 100 µL of the above culture was spread onto BAT agar plates (IFU, 2007) and incubated at 45°C for 4 d. BAT agar was prepared by mixing equal volumes of BAT broth and a 3-4% (m/v) agar (Biolab, supplied by Merck) solution after autoclaving. BAT broth consists of 0.25 g.L⁻¹ CaCl₂·H₂O (Saarchem, supplied by Merck), 0.50 g.L⁻¹ MgSO₄·7H₂O (Saarchem, supplied by Merck), 0.20 g.L⁻¹ (NH₄)₂SO₄ (Pro Analyti, supplied by Merck), 3.00 g.L⁻¹ KH₂PO₄ (AnalAR, supplied by Merck), 2.00 g.L⁻¹ yeast extract (Biolab, supplied by Merck), 5.00 g.L⁻¹ glucose (AnalAR, supplied by Merck) and 1.00 mL trace element solution, consisting of 0.66

g.L^{-1} $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (Saarchem, supplied by Merck), 0.18 g.L^{-1} $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck), 0.16 g.L^{-1} $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Merck), 0.15 g.L^{-1} $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (Merck), 0.18 g.L^{-1} $\text{CoCl}_2 \cdot 5\text{H}_2\text{O}$ (Merck), 0.10 g.L^{-1} H_3BO_3 (Merck) and 0.30 g.L^{-1} $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (Merck). The trace element solution was prepared separately and sterilised by autoclaving. The broth was adjusted to pH 4.00 using 1 M H_2SO_4 (AnalAR, supplied by Merck) and sterilised by autoclaving.

Colonies from the BAT agar plates were aseptically removed and streaked onto fresh BAT agar plates, followed by incubation at 45°C for 4 d, to ensure a pure culture. BAT broth at pH 4.00 was then inoculated with a single colony aseptically removed from one of the BAT agar plates and incubated at 45°C for 5 d.

A volume of 30 mL of the inoculated broth was centrifuged in a Beckman Coulter TJ-25 centrifuge (Beckman Coulter Inc., Fullerton, California, USA) at 5500 g for 6 min. The supernatant was discarded and the pellet resuspended in 30 mL sterile saline solution (SSS) [0.85% (m/v) NaCl (Saarchem, supplied by Merck)]. This process was repeated and after resuspension of the pellet in SSS the optical density (OD) of the culture was measured at 540 nm using a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA). The approximate cell concentration was determined using a standard curve of OD versus cell concentration. The cell concentration of the culture was also confirmed by preparing serial dilutions of the culture (10^0 - 10^{-6}) in SSS and plating out in duplicate on PDA (Biolab, supplied by Merck) adjusted to pH 4.00 after autoclaving, using the pour plate technique and incubating the plates at 45°C for 4 d.

Method comparison

Volumes of 10 mL peach concentrate were diluted 1:10 in SSS to final volumes of 100 mL to obtain single strength juice. The samples were placed in a water bath at 80°C and the temperature monitored using an identical control sample containing a thermometer. Once the samples had reached a temperature of 80°C they were inoculated with approximately 10^6 *A. acidoterrestris* K47 cells. The samples were heat shocked at 80°C for 10 min, followed by cooling on ice. Subsequently, one of the following three procedures was carried out.

Method 1 - IFU Method No. 12 (IFU, 2007)

The samples were serially diluted (10^0 - 10^{-6}) in SSS to final volumes of 10 mL and 100 μ L of each sample was spread in duplicate onto BAT agar plates at pH 4.00, followed by incubated at 45°C for 5 d. The method was repeated three times.

Method 2

Fifty mL volumes of 1.5xPDA (Biolab, Merck) was prepared and acidified after autoclaving to pH 3.70 using 9.50% (m/v) tartaric acid (Saarchem, supplied by Merck). The heat shocked samples were serially diluted (10^0 - 10^{-6}) in duplicate in SSS to final volumes of 100 mL and 50 mL PDA was added to each 100 mL dilution. The samples were mixed and divided into four to five petri-dishes using the pour plate technique. The plates were incubated at 46°C for 4 d. The method was repeated three times.

Method 3

The samples were serially diluted (10^{-2} - 10^{-6}) in duplicate in SSS to final volumes of 100 mL and each dilution was vacuum filtered through a 0.45 μ m membrane filter (S-Pak, Millipore, USA). Each filter was placed onto a plate of K agar (Walls & Chuyate, 1998) [2.50 g.L⁻¹ yeast extract (Biolab, supplied by Merck), 5.00 g.L⁻¹ peptone (Biolab, supplied by Merck), 15.00 g.L⁻¹ agar (Biolab, supplied by Merck), 1.00 g.L⁻¹ glucose (AnalAR, supplied by Merck) and 1.00 mL Tween 80 (Pro Analysis, supplied by Merck), acidified to pH 3.70 using 10 mL 12.5% (m/v) filter sterilised malic acid (Merck)]. The filter was tapped several times to remove air bubbles and to ensure contact with the medium. The plates were incubated at 40°C for 5 d. The method was repeated three times.

Growth curves under optimum conditions

Bacterial inoculation cultures

Isolates of *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 were cultured as previously mentioned, except that the single colony aseptically removed from the BAT agar plates was inoculated into YSG broth acidified to pH 4.00 with 1 M H₂SO₄ (AnalAR, supplied by Merck). After incubation at 45°C for 4-5 d the approximate cell concentration was determined

by measuring the OD at 540 nm. The cell concentration of the inoculum was also confirmed by serially diluting the culture (10^0 - 10^{-6}) in SSS and plating out in duplicate on PDA (Biolab, supplied by Merck) adjusted to pH 4.00 after autoclaving, using the pour plate technique, followed by incubation at 45°C for 4 d.

Growth curves

Three hundred mL BAT broth supplemented with 100 mg.L⁻¹ vanillin (Merck), a guaiacol precursor, was inoculated with one of either *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32, FB38, *A. acidocaldarius* DSM 446^T or FB19 to a final cell concentration of 10^1 - 10^3 cfu.mL⁻¹. Cultures were incubated at 45°C for 7 d and samples were analysed every 12 h. The OD, cell concentration before and after heat shock and pH was measured and the experiment was performed in triplicate.

OD

One mL of the sample was used to measure the OD at 540 nm using a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA).

Cell concentration before and after heat shock

The cell concentration was measured by serially diluting the sample (10^0 - 10^{-6}) in SSS and using the pour plate technique with PDA (Biolab, supplied by Merck) adjusted to pH 4.00 after autoclaving with 1 M H₂SO₄ (AnalAR, supplied by Merck). Plates were poured in duplicate and incubated at 45°C for 4 d. The rest of the sample was subjected to a heat shock treatment in a water bath at 80°C for 10 min. A control sample with a thermometer was used to ensure that the samples remained at 80°C for the required time. The cell concentration after heat shock was determined in the same way as the cell concentration before heat shock.

pH

Four mL of the sample was used to determine the pH using a Mettler Toledo 320 pH meter (Mettler-Toledo Ltd., Leicester, England).

Effect of temperature on growth and spore development

Three hundred mL BAT broth supplemented with 100 mg.L⁻¹ vanillin (Merck), was inoculated with one of either *A. acidoterrestris* DSM 3922^T, FB2, FB38, *A. acidocaldarius* DSM 446^T or FB19 to a final cell concentration of 10¹-10³ cfu.mL⁻¹. Cultures of each strain were incubated at both 25°C and 45°C for 6 d and samples were analysed every 24 h. The OD, pH and cell concentration before and after heat shock of every sample was measured as described. The experiment was performed in triplicate.

Statistical analysis

Differences in percentage recovery between the three different methods were tested using one-way analysis of variance (ANOVA). Species/time effects on absorbance, cell concentrations before and after heat shock and pH were tested using mixed model repeated measures ANOVA. Correlations between different measurements were calculated using non-parametric Spearman correlation. Effects of temperature on growth and spore development were analysed using mixed model repeated measures ANOVA. All analyses were performed using Statistica 8. A 5% significance level was used as guideline for indicating significant results.

RESULTS AND DISCUSSION

Comparison of three different isolation methods

Three methods frequently used by South African fruit processors for the isolation of *Alicyclobacillus* species from fruit products were compared with regards to their recovery of inoculated *A. acidoterrestris* K47 cells from peach juice concentrate diluted to single strength juice. A graphic representation of the results is given in Fig. 1. All three methods differ significantly ($p \leq 0.05$) with regards to their ability to recover *A. acidoterrestris* K47. The IFU Method No. 12 (IFU, 2007) is the most effective with an average recovery of 75.97%, followed closely by Method 2 with an average recovery of 66.79%, while Method 3 is significantly the least effective with an average recovery of 3.43%.

The IFU Method No. 12 and Method 2 differ primarily in the agar medium used for recovery, as well as the plating technique. The IFU Method No. 12 makes use of

BAT agar and spread plating. A study by Pacheco (2002) found BAT agar to be superior to PDA with regards to the recovery of *Alicyclobacillus* spp. Murray *et al.* (2007) compared the performance of K agar, acidified PDA, orange serum agar (OSA), YSG, Hiraishi glucose yeast extract (HGYE) agar, *Bacillus acidocaldarius* medium (BAM), *Alicyclobacillus* (ALI) agar, BAT agar and *Alicyclobacillus acidocaldarius* medium (AAM) for the recovery of *Alicyclobacillus* spp. Although the recoveries were not significantly different between K agar, PDA, YSG, BAM, ALI agar, AAM and BAT, there was a trend towards the better performance of BAT agar for the recovery of *Alicyclobacillus* spp. Spread plating onto BAT agar followed by incubation of plates at 43°C for 3 d was identified as the most effective method for enumerating 10 strains of three species of *Alicyclobacillus* most frequently involved in the spoilage of beverages (Murray *et al.*, 2007). The Working Group on Microbiology of the IFU developed the IFU Method No. 12 and recommended it as an internationally acceptable standard method for the isolation and detection of taint producing *Alicyclobacillus* spp. from fruit juices and concentrates (IFU, 2007).

Method 2 makes use of PDA and the pour plating technique. The use of PDA as an isolation medium has been reported by several researchers (Splittstoesser *et al.*, 1994; McIntyre *et al.*, 1995; Walls & Chuyate, 1998). Splittstoesser *et al.* (1998) recommended using PDA acidified to a pH of 3.50 as a selective medium for the detection of *Alicyclobacillus* spp. in foods. Witthuhn *et al.* (2007) found that PDA acidified to pH 3.70 was more effective than YSG agar, BAM and K agar at recovering *Alicyclobacillus* spp.

There is conflict in the literature regarding the influence of plating technique on the recovery of *Alicyclobacillus* spp. Some researchers found that spread plating produces better results (Pettipher *et al.*, 1997; Murray *et al.*, 2007), while others found that under certain conditions pour plating performs better (Jensen, 2000). Jensen (2000) found that spread and pour plating had similar recoveries when *Alicyclobacillus* strains were incubated in orange juice in a high oxygen environment. However, in a reduced oxygen environment pour plating gave higher recoveries than spread plating.

A variety of factors can play a role in the successful recovery of *Alicyclobacillus* spp., including the agar medium and plating technique as previously mentioned, but also the pH, incubation temperature, oxygen content and pretreatments such as heat shock or membrane filtration. Murray *et al.* (2007)

reported that in some cases there seems to be an interaction between the isolation medium and plating technique, as some agars performed better in combination with a specific plating method. For example, in six out of the eight cases where pour plating performed better than spread plating, AAM was involved, while none involved BAT or ALI agar (Murray *et al.*, 2007).

Method 3 makes use of K agar at pH 3.70 as a growth medium. The use of K agar was first proposed by Walls and Chuyate in 1998. When compared to a minimum salts medium (pH 4.00) suggested by Farrand *et al.* (1983), the semi-synthetic medium (pH 4.00) of Darland and Brock (1971) (on which the formulation of BAM, BAT, AAM and ALI agars are based) and OSA (pH 3.50) for isolation of *A. acidoterrestris*, K agar and the semi-synthetic medium had comparable recoveries, which were significantly higher than OSA and the minimal salts medium (Walls & Chuyate, 2000). Orr and Beuchat (2000) also found K agar (pH 3.70) to be a better isolation medium for *Alicyclobacillus* spp. when compared to OSA (pH 5.00) and PDA (pH 3.50) and it also performed well in a comparative study by Murray *et al.* (2007). In contrast, Witthuhn *et al.* (2007) found that K agar was not able to recover any inoculated *A. acidoterrestris* cells from diluted pear juice concentrate. Even though K agar generally seems to be an effective isolation medium, recovery was poor using Method 3. The reason for the poor performance of this method can possibly be ascribed to the fact that this method makes use of membrane filtration. Although the use of membrane filtration has a number of advantages, including the ability to analyse large sample volumes, which gives it a low detection limit (Chang & Kang, 2004; Lee *et al.*, 2007), there are also disadvantages and complications involved with the use of this method. The membrane filter has a limited number of pores through which the juice is passed, which could cause the cells to become concentrated around the pores as the juice passes through. If the cell concentration in the sample to be filtered is large it could result in colonies developing on top of each other, giving the impression of the presence of one large colony, while in reality it is a number of cells clustered together. This would result in a lower colony count. This was certainly typical of the colonies observed in this study, as there were a number of large colonies clustered around the membrane pores. Low recovery could also be due to ungerminated spores passing through or lodging in the membrane, as the spores are smaller than the vegetative cells and cannot always be retained by the membrane. Outgrowth of spores lodged in the membrane will be hindered, leading

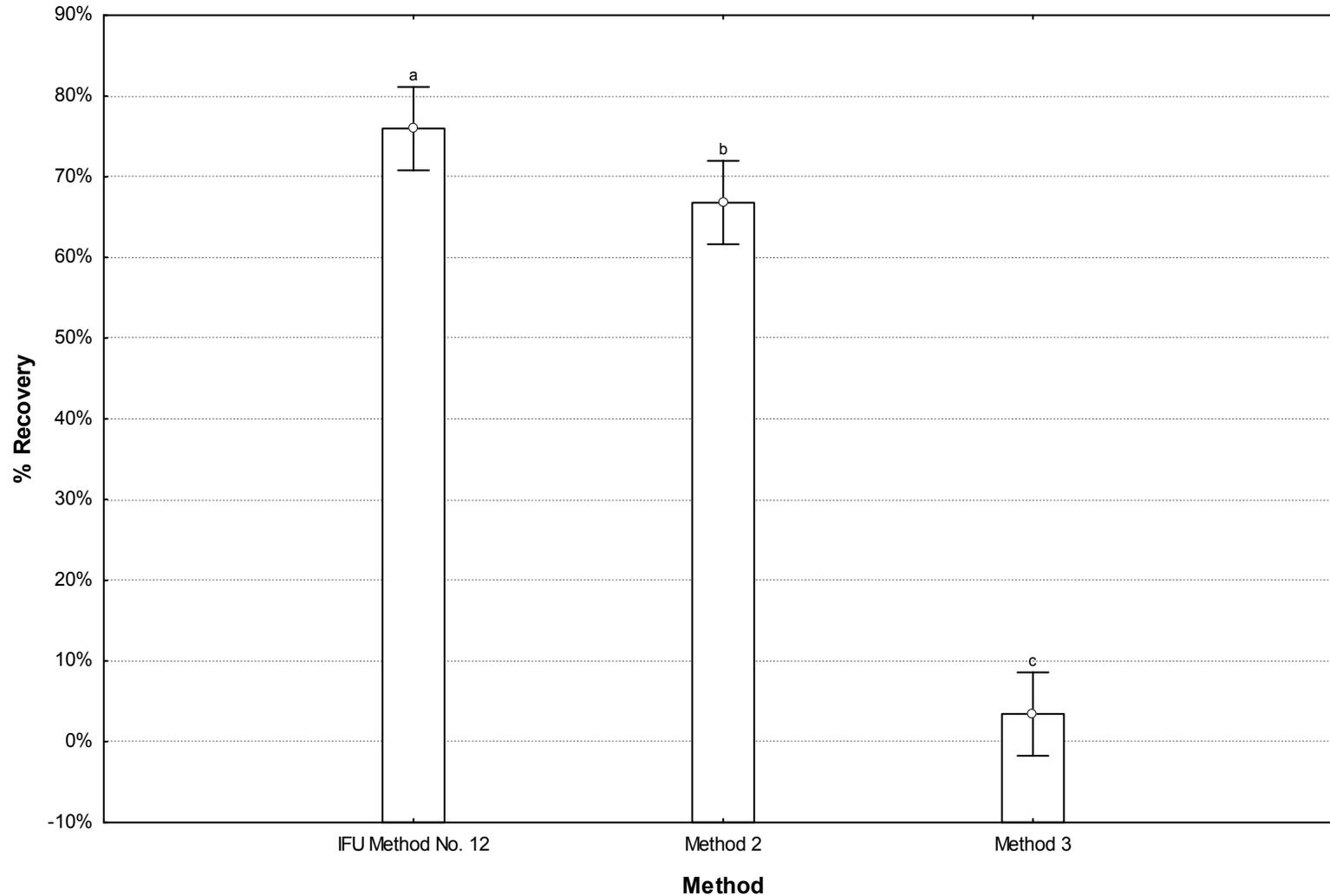


Figure 1 Recovery of *A. acidoterrestris* K47 from 1:10 diluted peach juice concentrate using the IFU Method No. 12, Method 2 and Method 3. Vertical bars denote 0.95 confidence intervals. Statistically significant differences ($p \leq 0.05$) between recoveries are indicated by non-identical subscript letters.

to lower numbers of colonies forming (Lee *et al.*, 2007). The composition of the specific membrane may also affect recovery. Lee *et al.* (2007) observed that membranes with the same pore size but from different manufacturers gave significantly different results, as some membranes, although successfully retaining the spores, were unable to support growth and colony development. Therefore, a number of factors could play a role in the success of a membrane filtration method and processors should test different filter membranes to determine which one gives the best results before they include such a method as part of their quality control procedures.

Growth under optimum conditions

The absorbance of samples was measured as an indication of an increase in cell density and thus cell proliferation (Fig. 2). With all seven strains the absorbance increased little from 0-12 h, followed by a fairly sharp increase from 12-24 h. After 24 h the increase in absorbance was more gradual, with some strains increasing to higher absorbance levels than others. The absorbance of all the strains reached a plateau at approximately 96 h and fluctuated around maximum absorbance values ranging from 0.39-0.66. Absorbance values of control samples, which consisted of uninoculated growth medium, remained constant throughout the experiment, indicating a lack of growth.

The pattern of absorbance increase is consistent with the increase in cell concentration (Fig. 3). The cell concentration of all the strains increased sharply, indicating exponential growth, from 0-24 h and then stabilised around maximum values ranging from 10^5 - 10^7 cfu.mL⁻¹. Cell concentrations in control samples remained constant, with small fluctuations at 24 and 144 h.

As bacteria in the genus *Alicyclobacillus* are spore-formers, the formation of spores during growth was also monitored (Fig. 4). Vegetative cells were killed and spores encouraged to germinate by subjecting the sample to a heat shock treatment at 80°C for 10 min. The cell concentrations observed in Fig. 4 were only from colonies that developed after the heat shock treatment, due to spore germination. The pattern of spore development was similar to that of vegetative cell growth, as a sharp increase in cell concentrations after heat shock was observed for all strains from 0-24 h. The cell concentrations after heat shock for *A. acidoterrestris* FB14 and

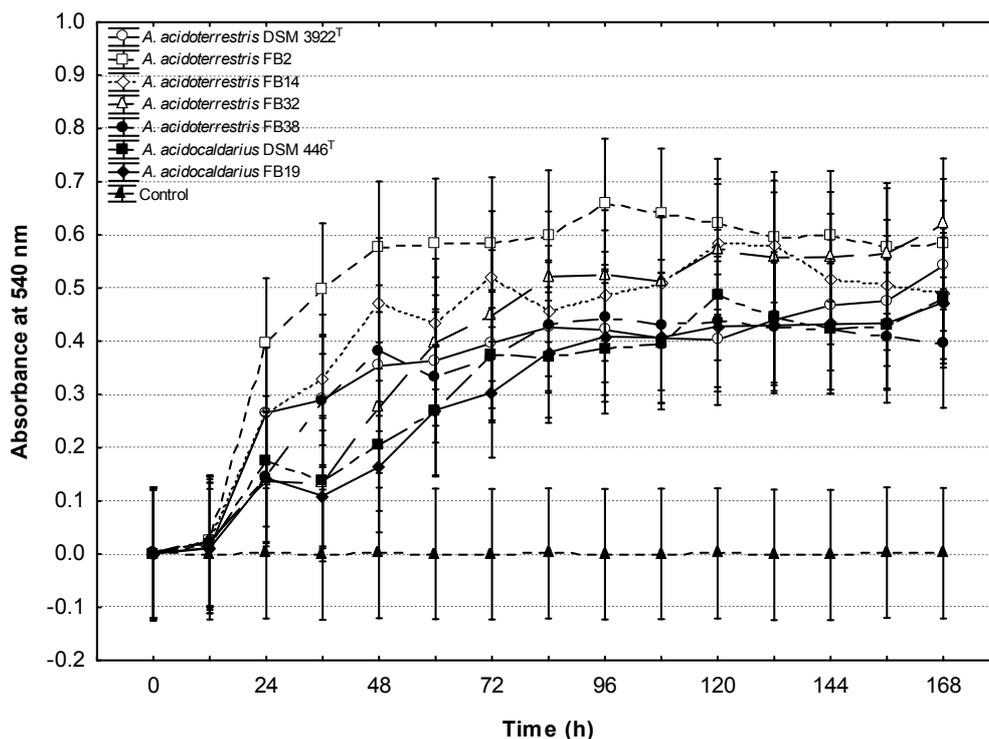


Figure 2 Change in absorbance of *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 45°C over a period of 7 d. Vertical bars denote 0.95 confidence intervals.

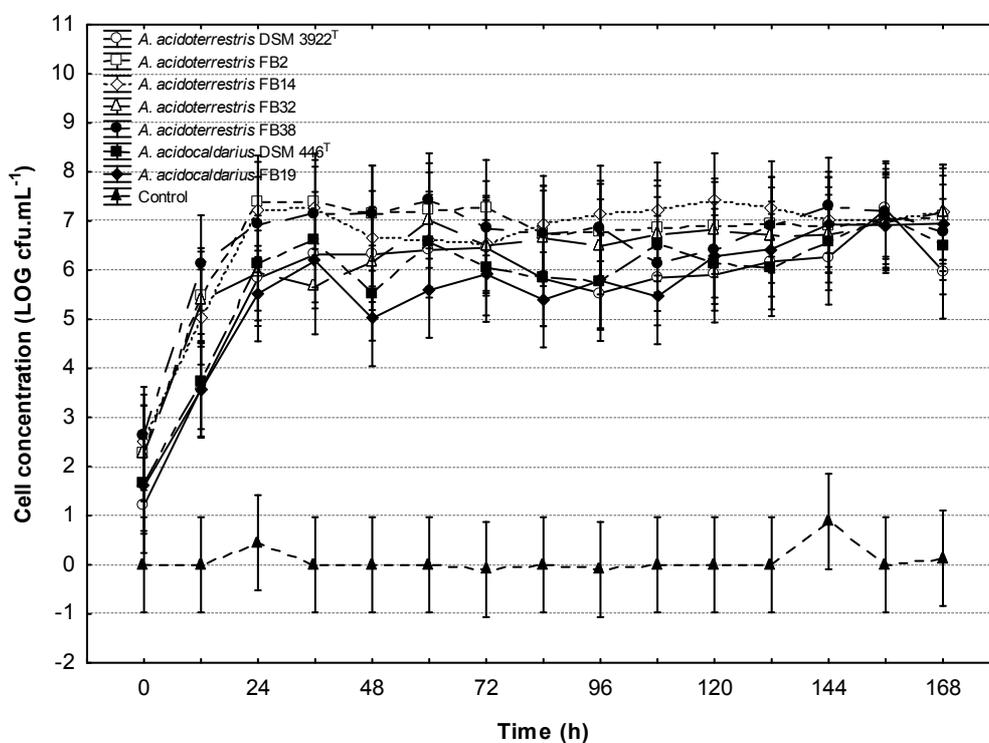


Figure 3 Change in cell concentrations of *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 45°C over a period of 7 d. Vertical bars denote 0.95 confidence intervals.

FB2 reached a plateau after 24 h and fluctuated around a maximum of 10^6 - 10^7 cfu.mL⁻¹, while the cell concentrations after heat shock of the other strains still increased gradually up to 96 h, after which they stabilised and fluctuated around maximums of 10^5 - 10^6 cfu.mL⁻¹. Cell concentrations after heat shock in control samples also remained constant.

There were strong positive correlations between absorbance, cell concentration and cell concentration after heat shock. Absorbance and cell concentration had a correlation of 0.70, while the correlation between absorbance and cell concentration after heat shock was 0.81. The correlation between cell concentration and cell concentration after heat shock was 0.74. These strong positive correlations indicated that absorbance and cell concentration before and after heat shock will increase as the other increases. This is to be expected, as absorbance, an indication of cell density, should increase with cell concentration. As cell concentration increases, spores will also increase as competition for resources increases.

The change in pH observed during growth varied between strains, but also between repetitions with the same strain, which resulted in large confidence intervals (Fig. 5). However, it seemed like the pH tended to increase during growth of the two *A. acidocaldarius* strains, with the pH for *A. acidocaldarius* DSM 446^T and FB19 increasing from 4.00 to 4.24 and 4.23, respectively, while in the five *A. acidoterrestris* strains the pH tended to decrease during growth, with the pH for *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32 and FB38 decreasing from 4.00 to 3.61, 3.55, 3.87, 3.81 and 3.83, respectively. The correlations between pH and the other three analyses were weakly negative, with correlations of -0.38, -0.19 and -0.23 being observed between pH and absorbance, cell concentration before heat shock and cell concentration after heat shock, respectively. These values were very small, indicating that no clear correlation exists between pH and the rest of the analyses.

Effect of temperature on growth and spore development

Alicyclobacillus acidoterrestris DSM 3922^T, FB2, FB38 and *A. acidocaldarius* DSM 446^T and FB19 were selected from the previous strains for this experiment. Strains were inoculated into BAT growth medium, incubated at 25°C and 45°C and the absorbance, cell concentration before and after heat shock and pH was measured daily.

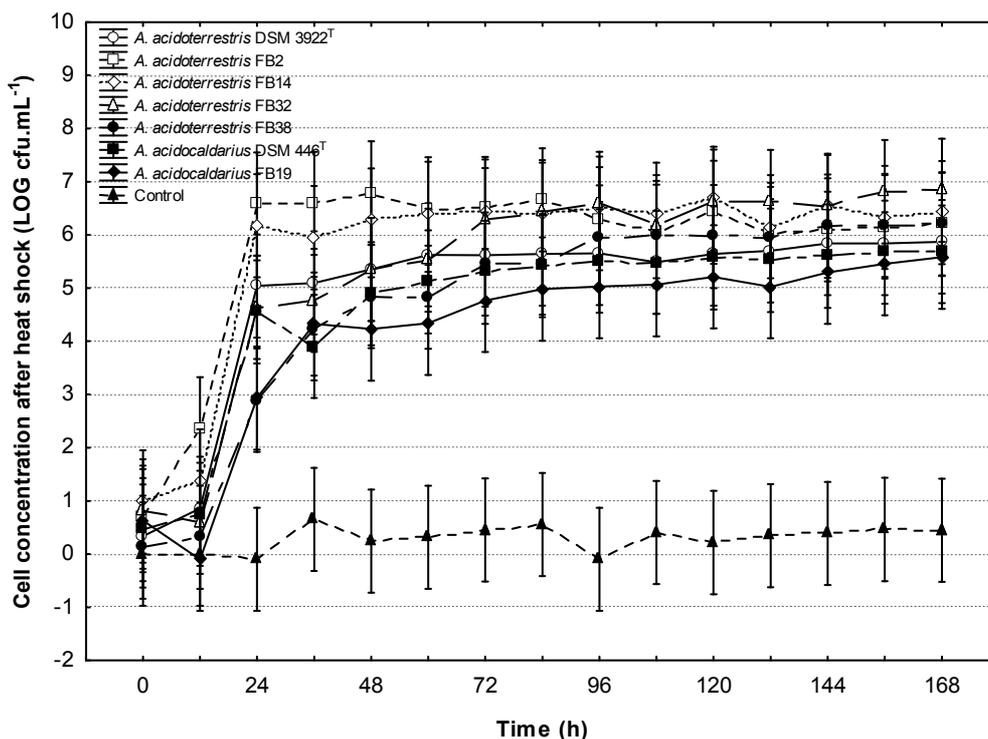


Figure 4 Change in cell concentrations after heat shock of *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 45°C over a period of 7 d. Vertical bars denote 0.95 confidence intervals.

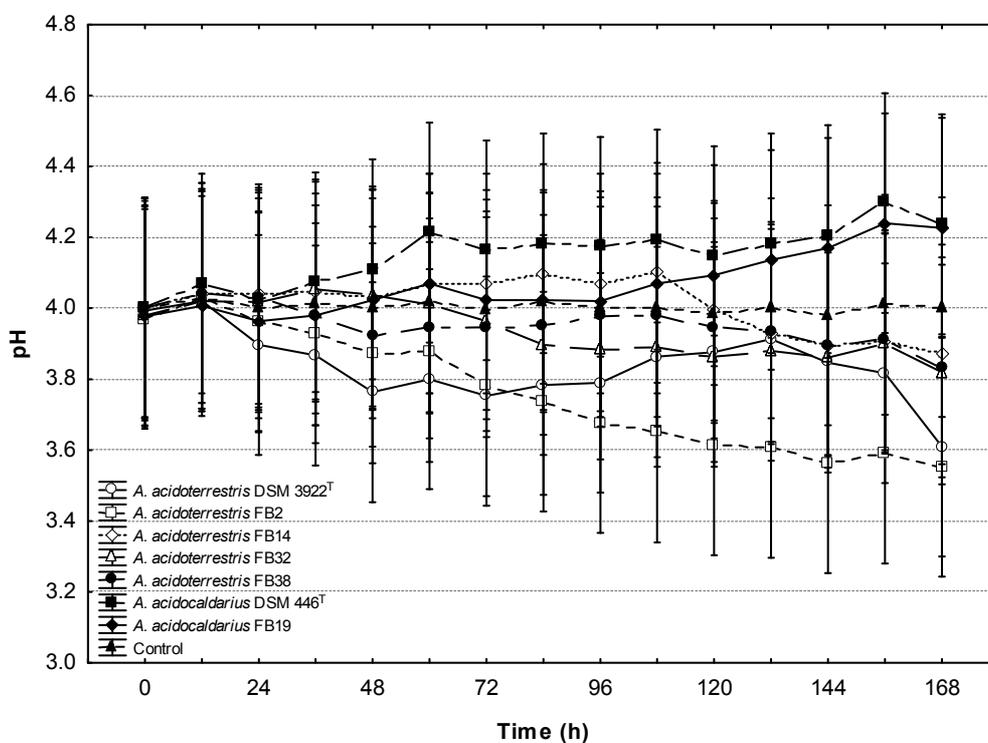


Figure 5 Change in pH of *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 45°C over a period of 7 d. Vertical bars denote 0.95 confidence intervals.

Alicyclobacillus acidoterrestris DSM 3922^T, FB2 and FB38 all show similar patterns of absorbance change at 25°C and at 45°C (Figs. 6-8). At 45°C the absorbance increases gradually from day 0 to day 6, with *A. acidoterrestris* DSM 3922^T, FB2 and FB38 reaching maximum absorbances of 0.59, 0.75 and 0.86, respectively. At 25°C the growth rate is slower, indicated by a longer lag phase of growth, with 2 d needed to observe an increase in the absorbance. This is in contrast to the absorbances at 45°C where an increase was observed immediately. The maximum absorbances reached at 25°C are significantly lower than those at 45°C, especially with *A. acidoterrestris* FB2 and FB38. At 25°C *A. acidoterrestris* DSM 3922^T reached a maximum absorbance of 0.34, while *A. acidoterrestris* FB2 and *A. acidoterrestris* FB38 reached maximum absorbances of 0.14 and 0.15, respectively. The pattern of absorbance change of the two *A. acidocaldarius* strains is very similar at both temperatures, but different from that of the *A. acidoterrestris* strains (Figs. 9 and 10). Absorbances of the *A. acidocaldarius* strains at 45°C only started increasing after 1 d and instead of increasing gradually up to a maximum, increased sharply up to day 4, reaching maximum absorbances of 0.71 and 0.63, followed by a stabilisation. In contrast, hardly any increase in absorbance was observed at 25°C.

Fig. 11 shows the change in the absorbance of all five strains at 25°C and at 45°C. It is clear that the rate of absorbance increase is higher at 45°C, indicating more rapid growth, and that the maximum absorbances are also higher and reached sooner, indicating a higher cell density and a higher growth rate. Control values, from uninoculated BAT media, remained constant over the 6 d at both temperatures.

In correspondence with the absorbance changes, the cell concentrations of the three *A. acidoterrestris* strains also increased gradually from 0-6 d at both 25°C and 45°C (Figs. 12-14). However, as indicated by the absorbance, the cell concentrations are approximately 2 logs higher at 45°C than at 25°C and the rate of growth is also more rapid as maximum cell concentrations are reached sooner at 45°C than at 25°C. *Alicyclobacillus acidoterrestris* DSM 3922^T, FB2 and FB38 reached maximum cell concentrations of 10^7 - 10^8 cfu.mL⁻¹ at 45°C, compared to 10^5 - 10^6 cfu.mL⁻¹ at 25°C. As indicated by the absorbance, the two *A. acidocaldarius* strains grew well at 45°C, with both strains reaching maximum cell concentrations of 10^8 cfu.mL⁻¹. The change in cell concentrations observed for *A. acidocaldarius* DSM 446^T and FB19 at 25°C also explain the lack of change in absorbance at 25°C, as

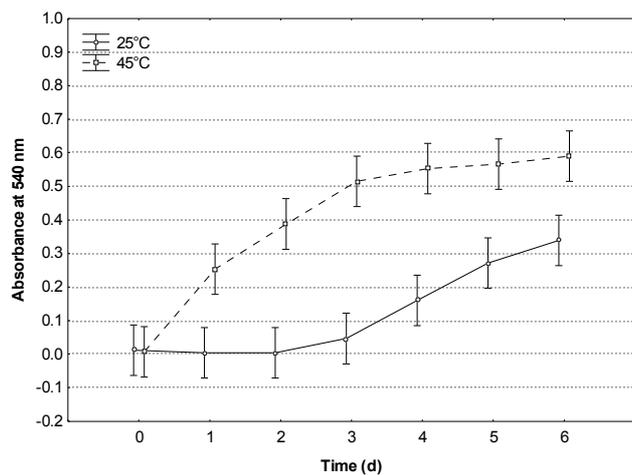


Figure 6 Change in absorbance of *A. acidoterrestris* DSM 3922^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

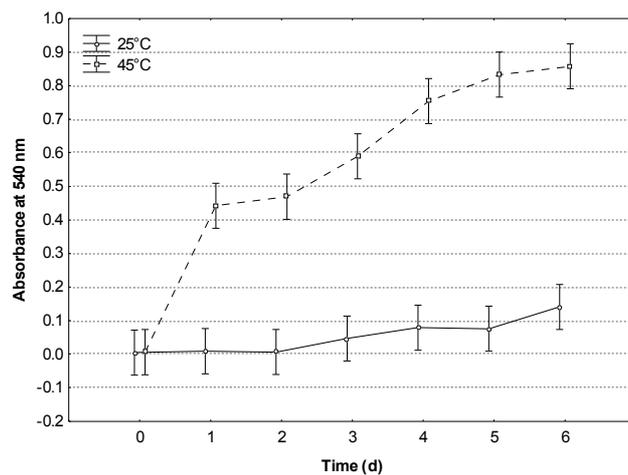


Figure 7 Change in absorbance of *A. acidoterrestris* FB2 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

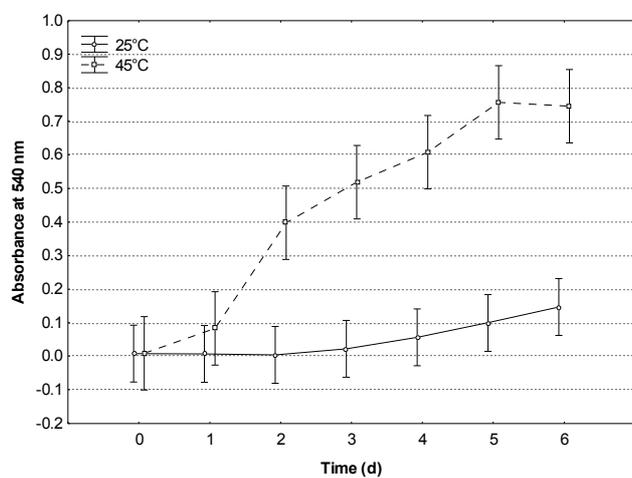


Figure 8 Change in absorbance of *A. acidoterrestris* FB38 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

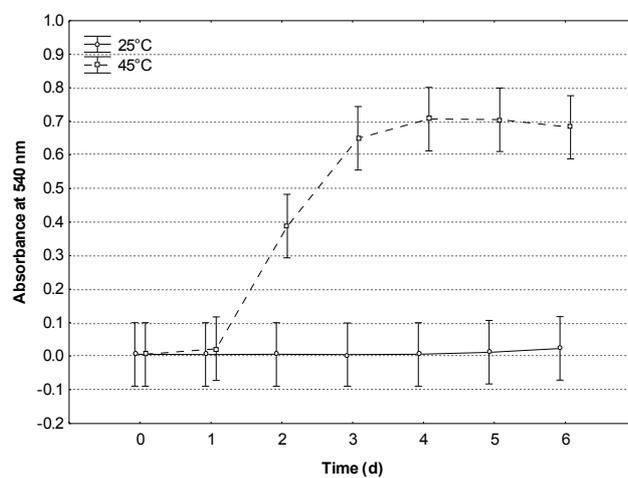


Figure 9 Change in absorbance of *A. acidocaldarius* DSM 446^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

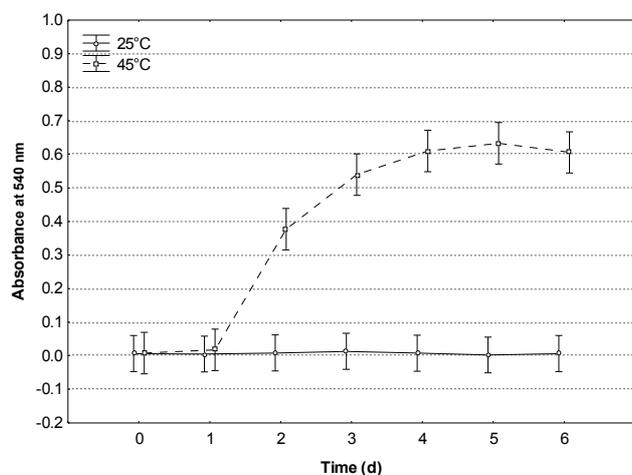


Figure 10 Change in absorbance of *A. acidocaldarius* FB19^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

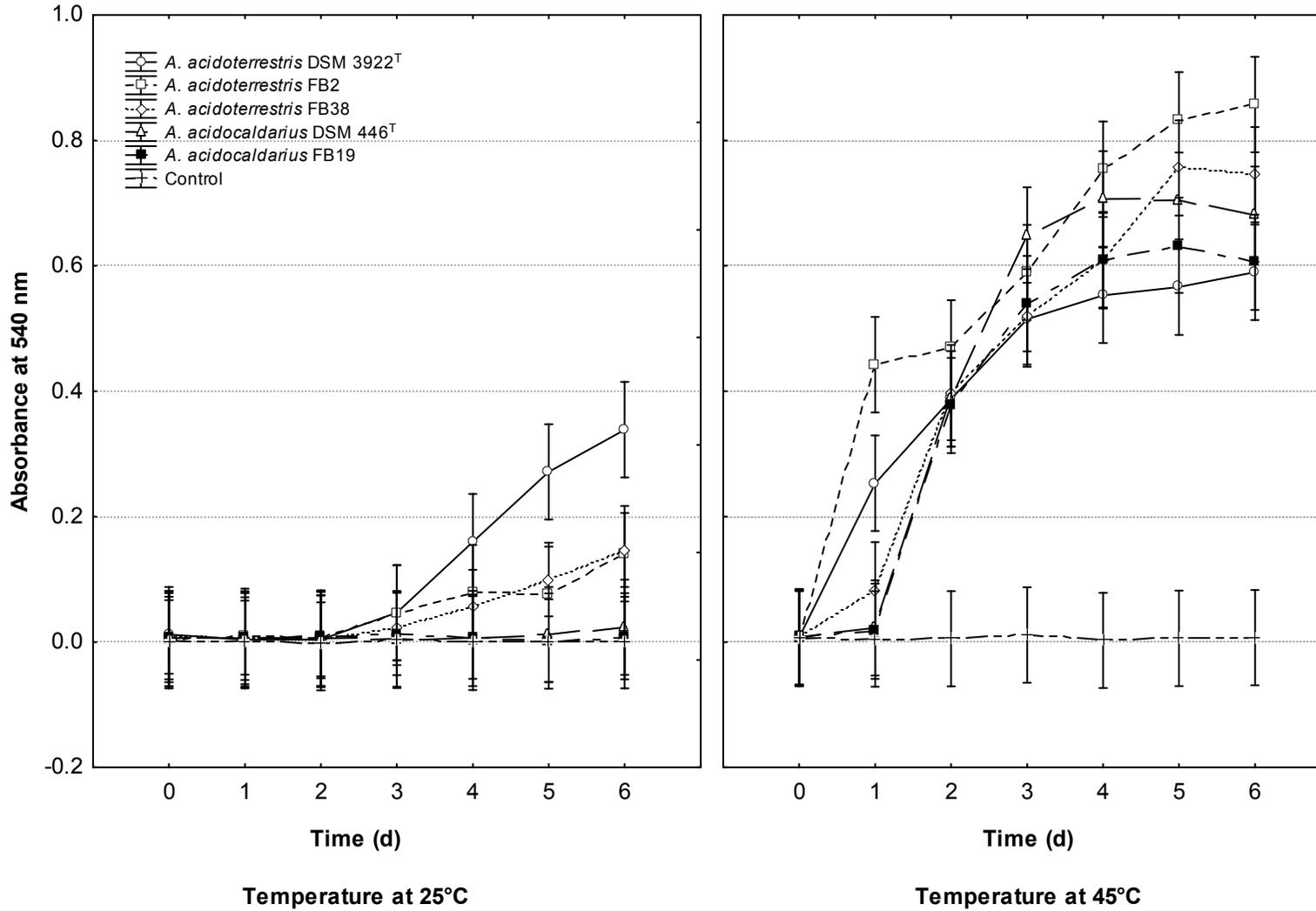


Figure 11 Change in absorbance of *A. acidoterrestriis* DSM 3922^T, FB2 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

cell concentrations decreased by approximately 1 log, from 10^4 - 10^3 cfu.mL⁻¹ and 2 logs, from 10^3 - 10^1 cfu.mL⁻¹, for *A. acidocaldarius* DSM 446^T and FB19, respectively over the 6 d (Figs. 15 and 16).

As species belonging to the genus *Alicyclobacillus* are classified as mildly to highly thermophilic, it would be expected that growth would be slower at lower temperatures, especially in a highly thermophilic species such as *A. acidocaldarius*. *Alicyclobacillus acidoterrestris* is less thermophilic than *A. acidocaldarius*, with a growth temperature range of <35°C to >55°C (Hippchen *et al.*, 1981; Deinhard *et al.*, 1987; Wisotzkey *et al.*, 1992). The growth temperature range of *A. acidocaldarius* is higher, ranging from 45°-70°C (Uchino & Doi, 1967; Darland & Brock, 1971; Wisotzkey *et al.*, 1992). The lower growth temperature range of *A. acidoterrestris* enables it to grow at 25°C, albeit slower than at 45°C. In agreement with these results, Pettipher *et al.* (1997) observed that *A. acidoterrestris* was able to grow at 25°C in apple and orange juice, but also at a slower rate and reaching lower maximum cell concentrations after 6 d than at 44°C. Siegmund and Pöllinger-Zierler (2007) also observed that *A. acidoterrestris* was able to grow at room temperature (20°-23°C) and Jensen & Whitfield (2003) observed growth of *A. acidoterrestris* at 19.5°-20.6°C. In contrast to these results, Bahçeci *et al.* (2005) found that *A. acidoterrestris* was not able to grow at 25°C. Since a temperature of 25°C falls outside the growth temperature range of *A. acidocaldarius*, the two strains used in this study were unable to grow at this temperature.

Fig. 17 shows the change in the cell concentrations of all five *Alicyclobacillus* strains at 25°C and at 45°C. Where an increase in cell concentrations was observed, the maximum cell concentrations were higher at 45°C than at 25°C. The lower cell concentrations of the three *A. acidoterrestris* strains and the decrease in cell concentrations of the two *A. acidocaldarius* strains at 25°C compared to the increase in cell concentrations and higher cell concentrations in all strains at 45°C is a clear indication of slower or lack of growth at the lower temperature. Control values, from uninoculated BAT media, remained constant over the 6 d at both temperatures. At 45°C the cell concentrations after heat shock increased in all five strains, reaching maximum values ranging from approximately 10^6 - 10^7 cfu.mL⁻¹ (Figs. 18-22). At an incubation temperature of 45°C growth is fairly rapid, causing more competition between cells and thus more spore formation.

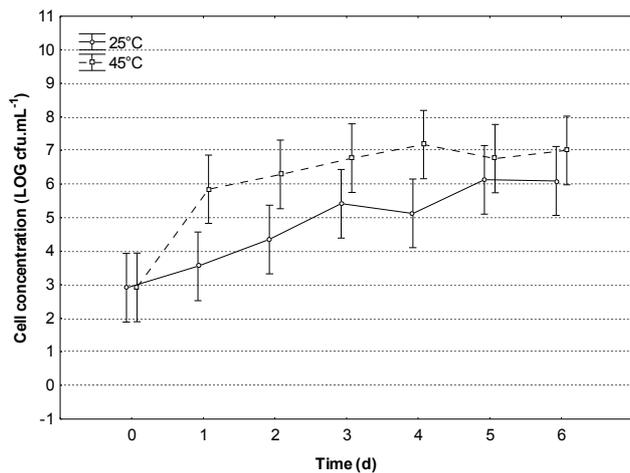


Figure 12 Change in cell concentration of *A. acidoterrestris* DSM 3922^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

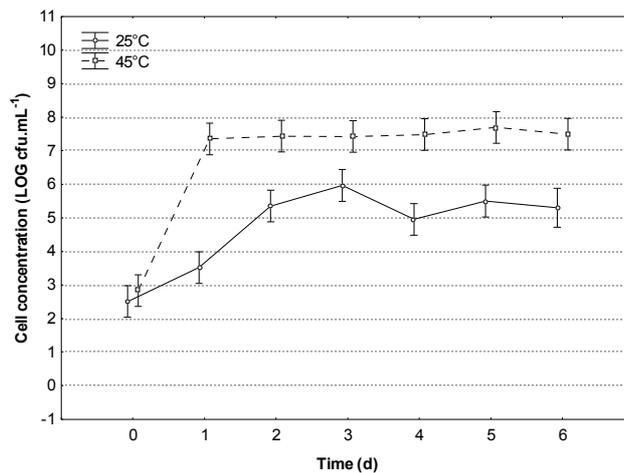


Figure 13 Change in cell concentration of *A. acidoterrestris* FB2 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

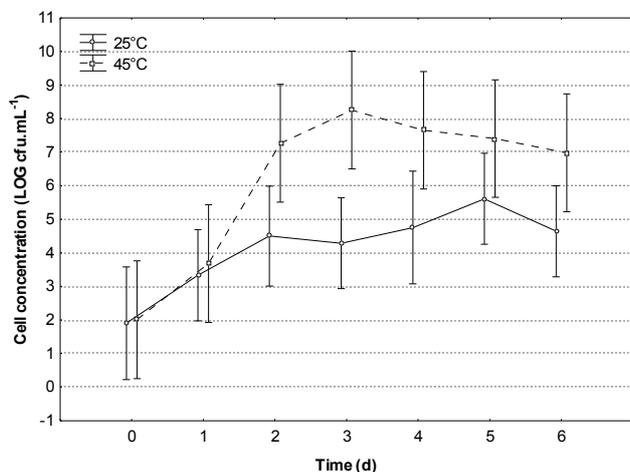


Figure 14 Change in cell concentration of *A. acidoterrestris* FB38 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

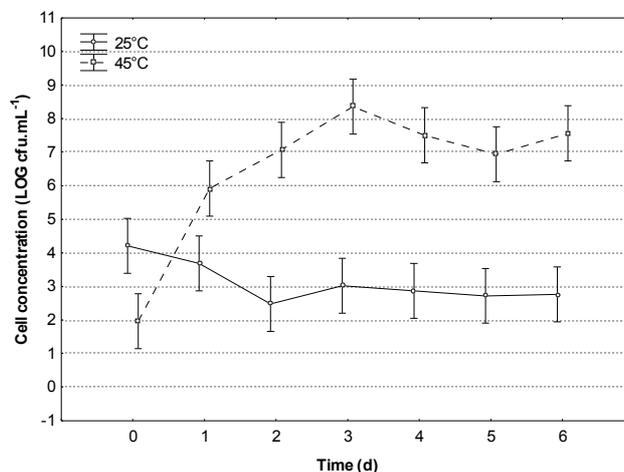


Figure 15 Change in cell concentration of *A. acidocaldarius* DSM 446^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

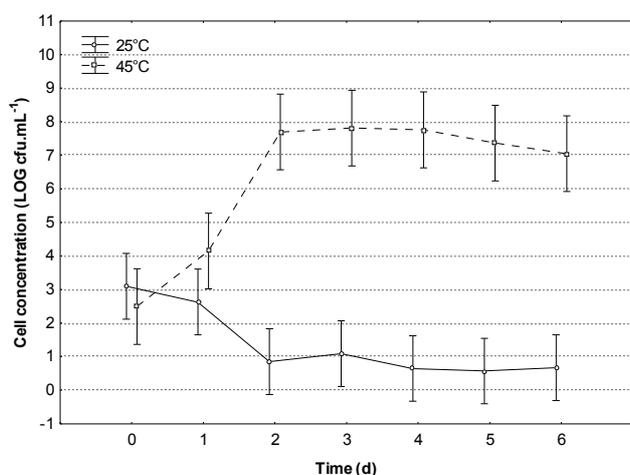


Figure 16 Change in cell concentration of *A. acidocaldarius* FB19 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

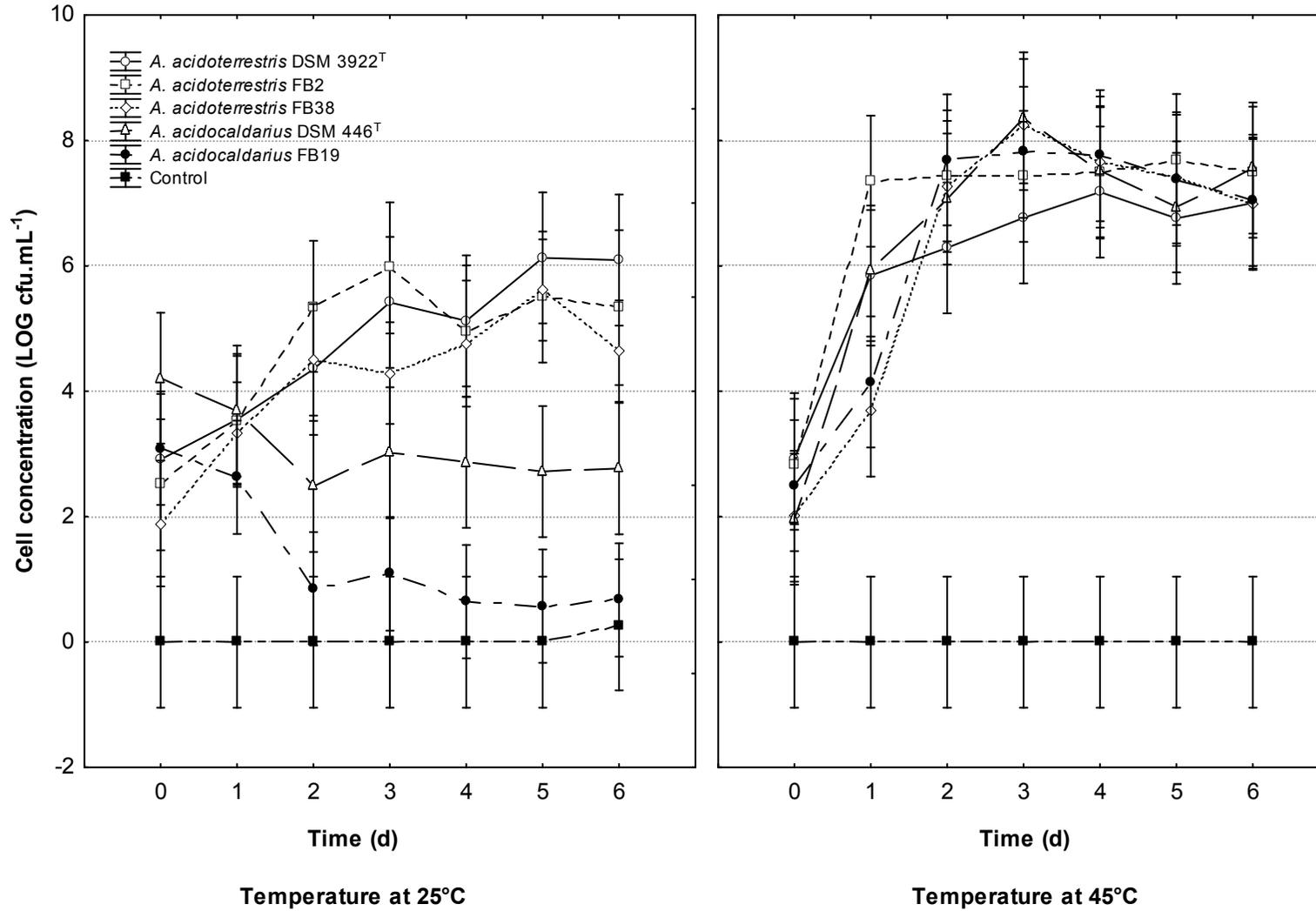


Figure 17 Change in cell concentration of *A. acidoterrestris* DSM 3922^T, FB2 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

At 25°C the cell concentrations after heat shock increased for *A. acidoterrestris* DSM 3922^T and FB2, reaching maximums of 10³ and 10⁵ cfu.mL⁻¹ after 6 d (Figs. 18 and 19). However, these maximum cell concentrations are significantly lower than the maximums of 10⁶ and 10⁷ cfu.mL⁻¹ reached at 45°C, indicating less spore formation at 25°C. Since the cell concentrations before heat shock are also lower at 25°C, it would lead to a lower cell density and less competition, and, therefore, less spore formation.

It would appear as if a high cell density and nutrient depletion is a stronger driver for spore formation than other unfavourable conditions, in this case sub-optimal growth temperature, as the sub-optimal growth temperature of 25°C did not lead to more spore formation. This is in agreement with the fact that it is well known that sporulation is generally induced by starvation conditions (Errington, 1993; Bogdonova *et al.*, 2002; Setlow & Johnson, 2007). In the laboratory sporulation is induced by one of three methods, of which two involve starvation of cells. Nutrient exhaustion involves growing cells in a medium until one or more of the components become limiting and the resuspension method involves transfer of cells from a rich to a starvation medium. Finally, sporulation can also be induced by the addition of the antibiotic decoyinine (Grossman & Losick, 1988; Errington, 1993). The initiation of sporulation is governed by at least three inputs, namely a nutritional signal, the population density and the cell cycle (Errington, 1993). The nutritional signal involves sending of information regarding the availability of essential nutrients such as carbon, nitrogen or phosphorous and the depletion of these nutrients would result in a signal for spore formation to be induced (Errington, 1993). The population density plays an important role in initiation of sporulation as it has been observed that sporulation could not be induced efficiently in cultures maintained at a low cell density. Furthermore, it has been found that *B. subtilis*, a classic example in the study of spore formation in gram-positive bacteria, produces an extracellular differentiation factor(s) in response to a high cell density which was found to be required additionally to starvation conditions for sporulation to be efficiently induced. *Bacillus subtilis* cells will, therefore, sporulate more efficiently at high cell densities (Grossman & Losick, 1988) and it would appear as if this is also the case with *Alicyclobacillus* spp.

The cell concentrations after heat shock for *A. acidoterrestris* FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 25°C remain constant over the 6 d and no

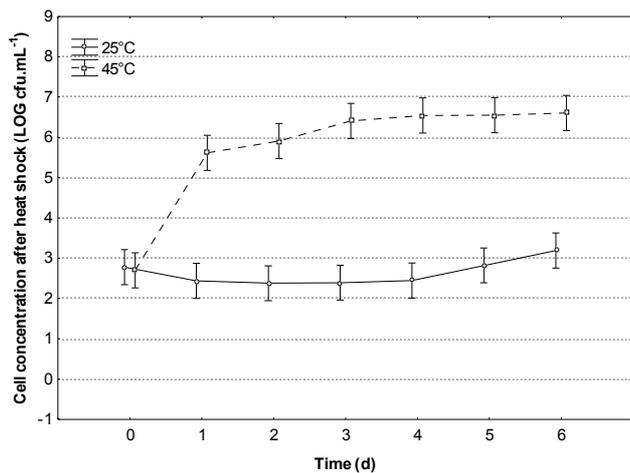


Figure 18 Change in cell concentration after heat shock of *A. acidoterrestris* DSM 3922^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

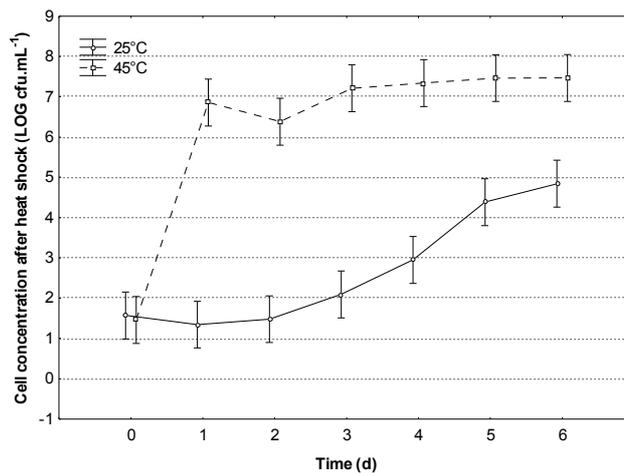


Figure 19 Change in cell concentration after heat shock of *A. acidoterrestris* FB2 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

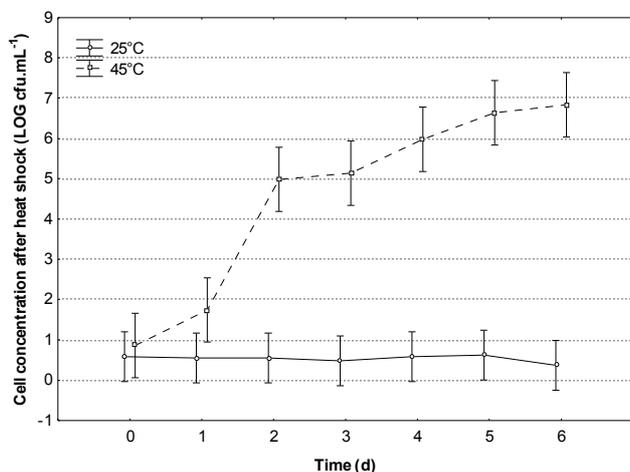


Figure 20 Change in cell concentration after heat shock of *A. acidoterrestris* FB38 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

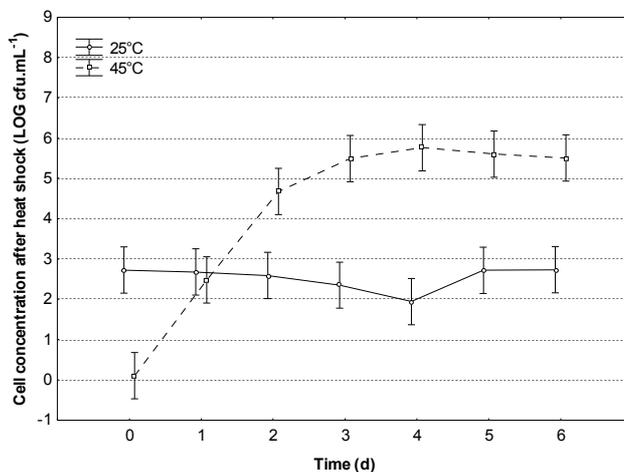


Figure 21 Change in cell concentration after heat shock of *A. acidocaldarius* DSM 446^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

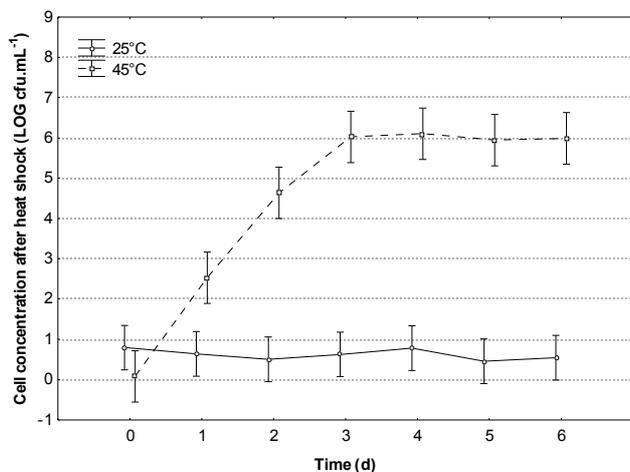


Figure 22 Change in cell concentration after heat shock of *A. acidocaldarius* FB19 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

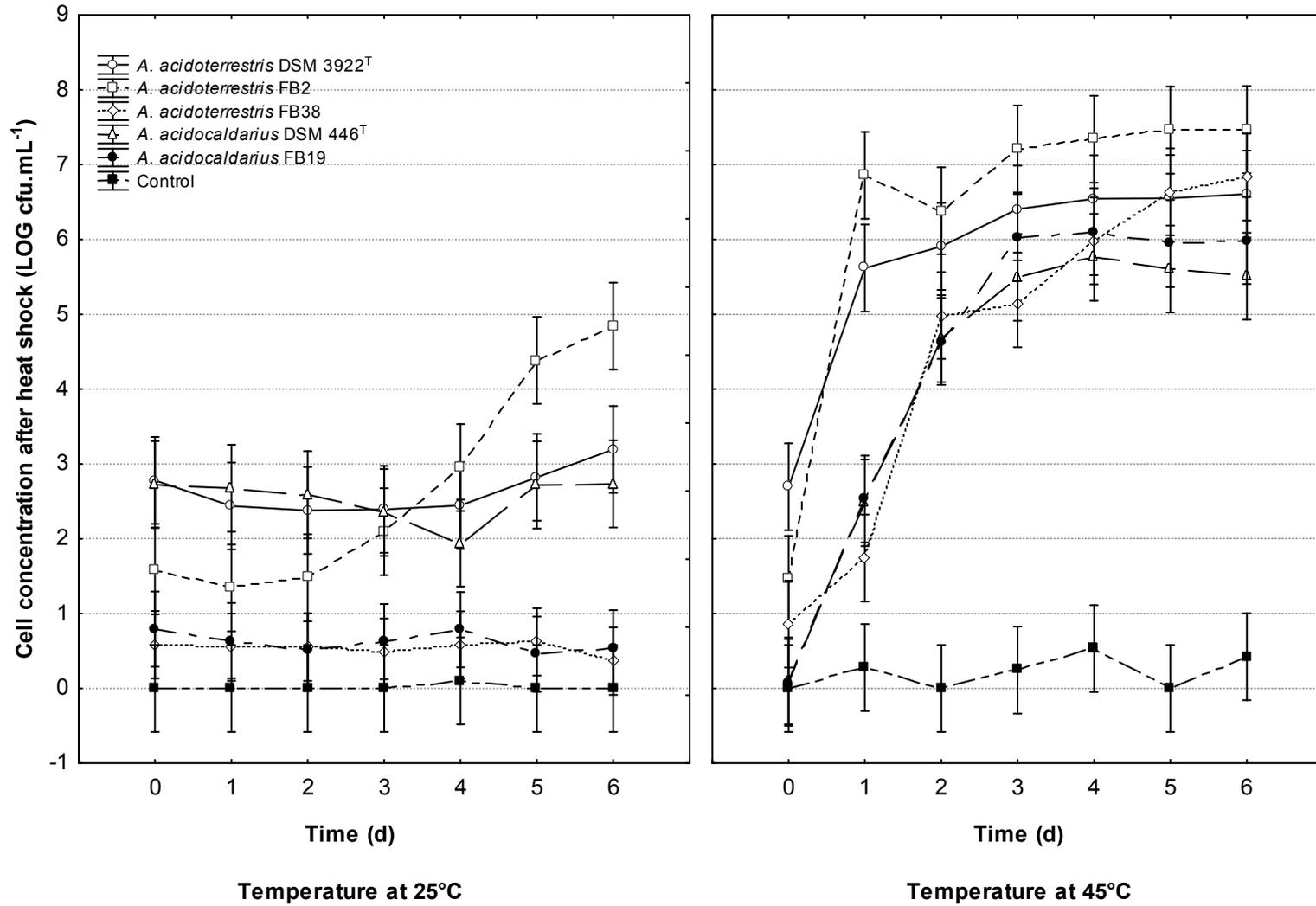


Figure 23 Change in cell concentrations after heat shock of *A. acidoterrestris* DSM 3922^T, FB2 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

increase is observed, indicating little or no spore formation other than the spores that were present in the inoculum (Figs. 20-22). The cell concentrations before heat shock of the two *A. acidocaldarius* strains decreased and, therefore, without cell growth, there would not be a need for spore formation as there would be minimal competition between cells. Fig. 23 shows the change in the cell concentrations after heat shock in all five *Alicyclobacillus* strains at 25°C and at 45°C. Control values, from uninoculated BAT media, fluctuated slightly at 45°C over the 6 d.

There are once again strong positive correlations between the absorbance and cell concentrations before and after heat shock. Absorbance and cell concentration before heat shock had a correlation of 0.82, while the correlation between absorbance and cell concentration after heat shock was 0.76. The correlation between cell concentration before heat shock and cell concentration after heat shock was 0.82. These results are to be expected, as the absorbance is an indication of cell growth and thus would increase as cell growth increases. The cell concentration after heat shock is an indication of spore formation in the culture and one would expect it to increase along with the cell concentration before heat shock, as competition and spore formation increases along with an increase in cell density.

Incubation temperature has a significant ($p \leq 0.05$) influence on the pH change during growth (Figs. 24-28). Much variation in the pH change at 45°C was observed previously in Fig. 5, although the pH in the *A. acidoterrestris* samples tended to decrease, while those of the *A. acidocaldarius* samples tended to increase. These tendencies were more prominent in the present study, with the pH clearly decreasing to varying degrees at 45°C for the three *A. acidoterrestris* strains (Figs. 24-26) and increasing for *A. acidocaldarius* FB19 (Fig. 28). The minimum pH values reached in the *A. acidoterrestris* DSM 3922^T, FB2 and FB38 strains were 3.45, 3.70 and 3.30, respectively and in *A. acidocaldarius* FB19 the maximum pH was 4.26. In *A. acidocaldarius* DSM 446^T no clear pattern could be observed as the pH initially increases, but then decreases again after 4 d (Fig. 27). However, the increase was very slight, as the maximum pH reached was 4.10, only 0.10 pH units more than the starting pH of 4.00.

At 25°C the pH remained constant in the three *A. acidoterrestris* strains, with slight increases (Figs. 24-26). In the two *A. acidocaldarius* strains the pH at 25°C fluctuated around the initial pH of 4.00, first rising, followed by a decrease and another increase, but never more than 0.10 pH units from the initial pH (Figs. 27 and

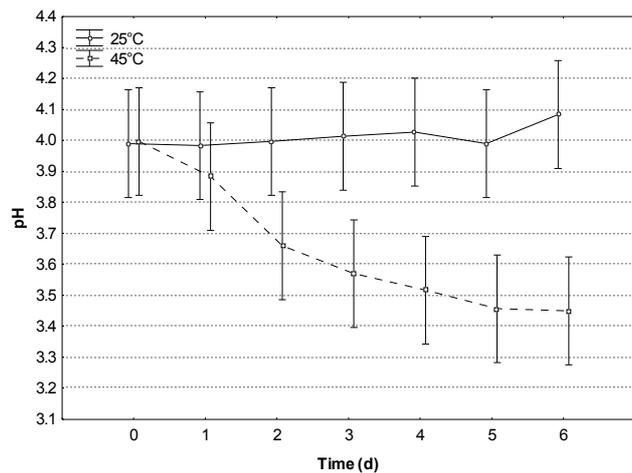


Figure 24 Change in pH of *A. acidoterrestris* DSM 3922^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

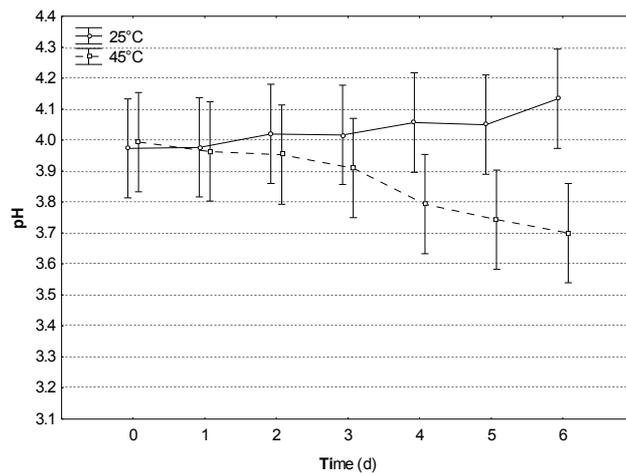


Figure 25 Change in pH of *A. acidoterrestris* FB2 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

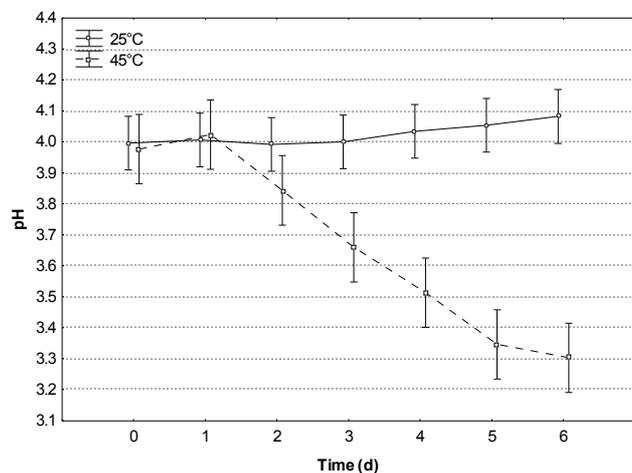


Figure 26 Change in pH of *A. acidoterrestris* FB38 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

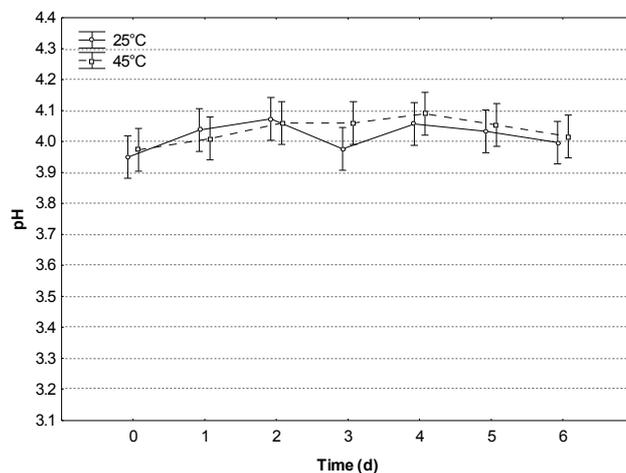


Figure 27 Change in pH of *A. acidocaldarius* DSM 446^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

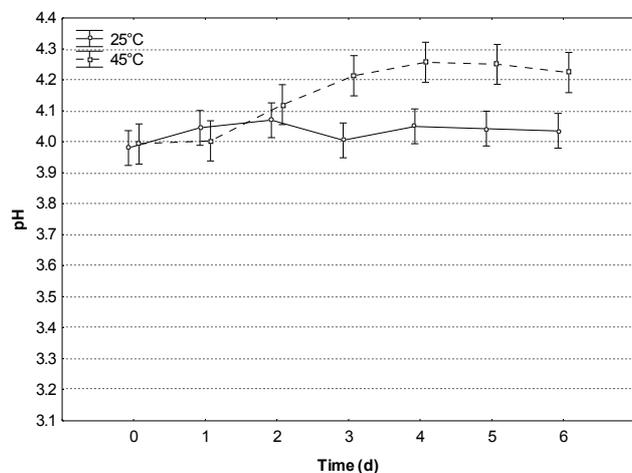


Figure 28 Change in pH of *A. acidocaldarius* FB19 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

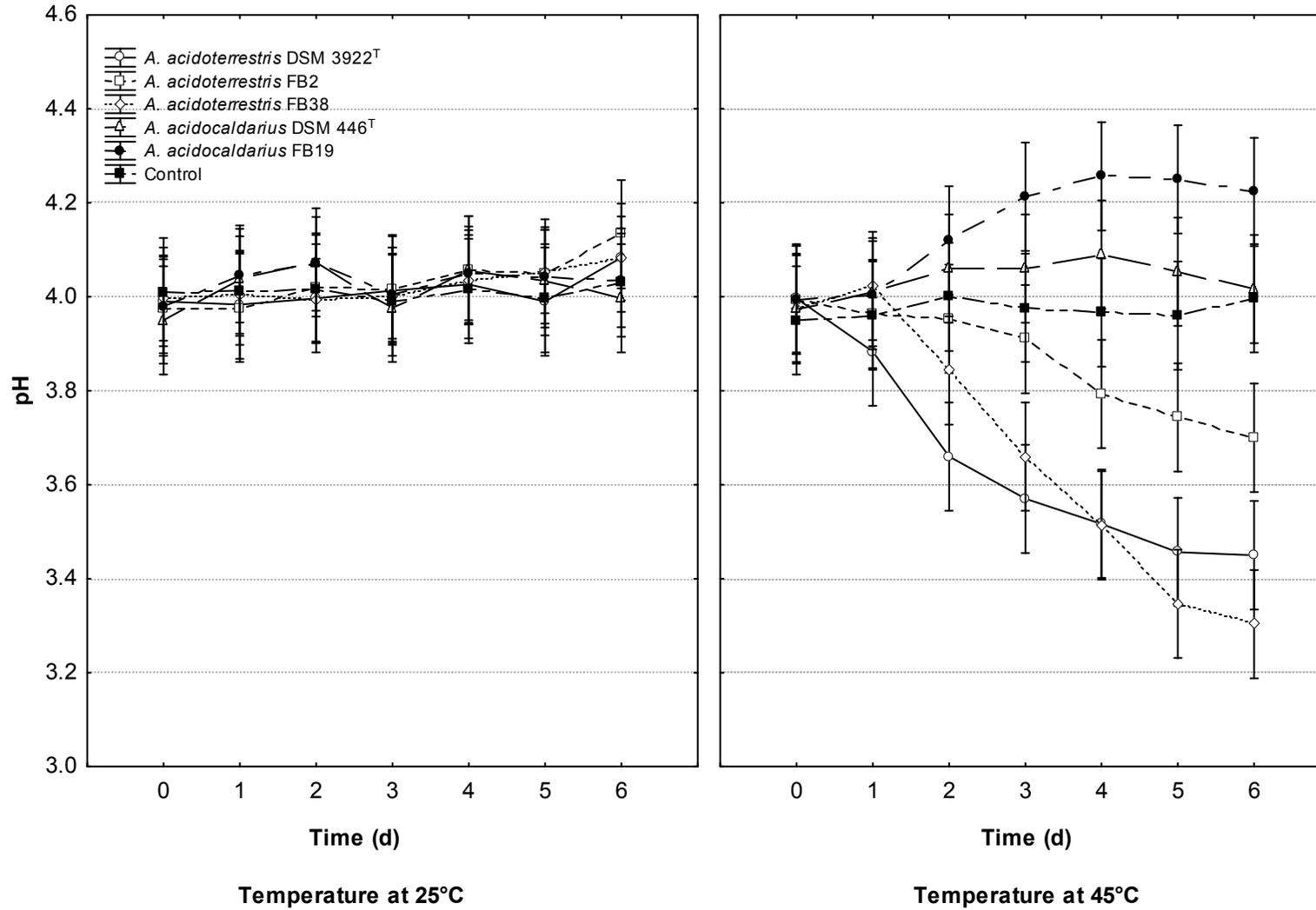


Figure 29 Change in pH of *A. acidoterrestri* DSM 3922^T, FB2 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

28). When considering Fig. 29, which shows the change in pH of all five strains at 25°C and at 45°C, the changes in pH at 25°C is very slight when compared to the corresponding changes at 45°C. The pH values of control samples remained constant over the 6 d at both temperatures. There is little information available in the literature regarding the change in pH in cultures containing *Alicyclobacillus* spp. Jensen (2000) observed an increase in the medium pH during growth of *A. acidoterrestris*, which is in contrast to the observations made in this study.

The pH was again slightly negatively correlated with the other three analyses, with correlations of -0.14, -0.07 and -0.19 being observed between pH and absorbance, cell concentration before heat shock and cell concentration after heat shock, respectively. However, these correlations were too small to be significant, indicating that no clear correlation exists between pH and the rest of the analyses.

CONCLUSION

Although various methods have been developed for the isolation of *Alicyclobacillus* spp., not all of them are equally effective. The type of agar, plating method and the use of additional procedures such as membrane filtration, can all play a role in the efficacy of the method for the isolation and detection of *Alicyclobacillus* spp. from fruit products. The method developed by the IFU for the isolation of *Alicyclobacillus* spp. is a good candidate for an international standard method of isolation for these bacteria, as several studies have shown its superiority over other methods of isolation.

Temperature has a significant effect on the growth of *Alicyclobacillus* spp. Temperature control can be used as a control measure to prevent or slow down growth of unwanted organisms in food and has long been applied in refrigeration and freezing of food products. From this study it would appear as though cooling of products to room temperature (20°-25°C) would only be effective in controlling the more thermophilic *Alicyclobacillus* species, such as *A. acidocaldarius*. Since *A. acidoterrestris* was able to grow at 25°C, only cooling to this temperature would be inefficient for control. Refrigeration temperatures would probably be effective (Siegmond & Pöllinger-Zierler, 2007), but the institution of such a control measure would present a major additional cost factor in production and distribution.

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CHAPTER 4

GUAIACOL PRODUCTION BY SPECIES OF *ALICYCLOBACILLUS* FROM SOUTH AFRICA AND COMPARISON OF TWO GUAIACOL DETECTION METHODS

ABSTRACT

Alicyclobacillus species are able to cause spoilage of acidic products through the production of the chemical compound guaiacol, which causes a medicinal or disinfectant-like off-flavour and/or odour in the products. The aim of this study was to determine the guaiacol producing abilities under different growth conditions of *A. acidoterrestris* and *A. acidocaldarius* strains isolated from the South African fruit processing environment and to compare two methods for guaiacol detection. To determine guaiacol production under optimum conditions, *Bacillus acidoterrestris* (BAT) media, supplemented with 100 mg.L⁻¹ vanillin, was inoculated with either *A. acidoterrestris* (five different strains), or *A. acidocaldarius* (two different strains), to a final cell concentration of 10²-10³ cfu.mL⁻¹. Cultures were incubated at 45°C for 7 d and samples analysed every 12 h. The guaiacol concentrations were determined using the peroxidase enzyme colourimetric assay (PECA). Guaiacol concentration increased along with the cell concentrations in all the samples to maximum concentrations ranging from 0.17 to 0.29 mM. Surprisingly, the two *A. acidocaldarius* strains were also able to produce guaiacol in fairly high concentrations, although the concentrations were slightly less than those produced by the *A. acidoterrestris* strains. Guaiacol was only detected once a cell concentration of 10⁴-10⁶ cfu.mL⁻¹ had been reached. The guaiacol production rate of *A. acidoterrestris* FB38 was more rapid than the other *Alicyclobacillus* strains and it was unable to accumulate guaiacol in the medium like the other strains, as the guaiacol concentration decreased steadily after reaching a maximum. These results indicate that the sources from which these strains were isolated can all act as possible routes of contamination of final products by *Alicyclobacillus* spp. In order to investigate guaiacol production by strains of *Alicyclobacillus* species at different incubation temperatures, *A. acidoterrestris* (three strains) or *A. acidocaldarius* (two strains) were incubated at either 25°C or 45°C and

guaiacol concentration measured using the PECA, as well as headspace gas-chromatography mass-spectrometry (HS GC-MS). In this experiment guaiacol concentrations detected in the *A. acidocaldarius* samples at 45°C were significantly ($p \leq 0.05$) lower than those detected in the *A. acidoterrestris* samples. The three *A. acidoterrestris* strains were all able to produce guaiacol at 25°C. According to the PECA, *A. acidoterrestris* DSM 3922^T and FB38 produced higher maximum guaiacol concentrations at 25°C than at 45°C, indicating that they are able to cause spoilage even when products are stored at sub-optimum growth temperatures. The two *A. acidocaldarius* strains were unable to produce guaiacol at 25°C as this temperature is below their growth temperature range. A fairly strong positive correlation of 0.76 was observed between the two guaiacol detection methods, although the sensitivity of the methods differed. Use of the PECA is recommended for determining the presence/absence of guaiacol, while HS GC-MS is recommended where accurate quantification of guaiacol is required.

INTRODUCTION

Spoilage caused by species of *Alicyclobacillus* has been described as a phenolic, medicinal or disinfectant-like flavour and/or odour and is attributed primarily to the microbial production of guaiacol (2-methoxyphenol) (Yamazaki *et al.*, 1996; Splittstoesser *et al.*, 1998; Jensen, 2000; Walls & Chuyate, 2000; Gocmen *et al.*, 2005; Siegmund & Pöllinger-Zierler, 2006), although the halophenols 2,6-dichlorophenol (2,6-DCP) (Jensen, 2000; Jensen & Whitfield, 2003; Gocmen *et al.*, 2005) and 2,6-dibromophenol (2,6-DBP) (Borlinghaus & Engel, 1997; Jensen, 1999; Jensen, 2000; Jensen & Whitfield, 2003; Gocmen *et al.*, 2005; Siegmund & Pöllinger-Zierler, 2006; Siegmund & Pöllinger-Zierler, 2007) have also been identified as possible sources of the taint produced by *Alicyclobacillus* spp. Guaiacol is the predominant metabolite associated with spoilage, as production of the halophenols occurs in much lower concentrations (Gocmen *et al.*, 2005) and appears to be species specific (Gocmen *et al.*, 2005). Various factors can influence the production of guaiacol, including the cell concentration, incubation temperature, heat shock treatment and growth medium (Chang & Kang, 2004).

Since the first spoilage incident by *Alicyclobacillus* spp. was reported in 1984 (Cerny *et al.*, 1984), a number of spoilage incidents involving various low pH

products have been reported worldwide (Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996; Walls & Chuyate, 1998; Jensen, 2000; Pettipher & Osmundson, 2000; Gouws *et al.*, 2005). The impact on the industry is substantial, as a recent survey by the European Fruit Juice Association (AIJN) found that out of a total of 68 participants involved in various areas of the fruit processing industry, 45% of the respondents had experienced *Alicyclobacillus* spp. related problems in the three years preceding the survey, with 33% of these experiencing more than three incidents. Of those that had experienced spoilage problems, 35% of the incidents were reported as being intermediately to majorly severe. Problems occurred primarily in apple raw materials and the product that was contaminated was mostly concentrates (Howard, 2006).

To date, the genus *Alicyclobacillus* includes 19 species, two subspecies and two genomic species. However, only *A. acidoterrestris* has consistently been associated with product spoilage, while only six other species, including *A. acidiphilus* (Matsubara *et al.*, 2002; Goto *et al.*, 2008), *A. pomorum* (Goto *et al.*, 2003), *A. hesperidum* (Niwa & Kawamoto, 2003; Gocmen *et al.*, 2005; Goto *et al.*, 2008), *A. cycloheptanicus* (Gocmen *et al.*, 2005), *A. herbarius* (Goto *et al.*, 2008) and *A. acidocaldarius* (Gouws *et al.*, 2005) have been implicated as potential spoilage species due to their isolation from spoiled products and/or their ability to produce taint chemicals.

Guaiacol can be detected in products using sensory, chemical or analytical methods. The sensory method, which involves smelling and/or tasting of the product by a sensory panel trained to be able to detect guaiacol, is the easiest and simplest to use, but can be subjective and is not quantitative (Eisele & Semon, 2005; Siegmund & Pöllinger-Zierler, 2006). Chemical and analytical methods are more accurate and quantitative, but are more time consuming and sometimes make use of complicated methodology and expensive instrumentation.

The chemical method for guaiacol detection is based on the principle that peroxidase enzyme, in the presence of H₂O₂, reacts with guaiacol to form a brown coloured component, 3,3'-dimethoxy-4,4'-biphenylquinone (Doerge *et al.*, 1997), which can be detected spectrophotometrically at a wavelength of 420 nm (Sheu & Chen, 1991; Bahçeci & Acar, 2007) or 470 nm (Doerge *et al.*, 1997; Niwa & Kuriyama, 2003). The guaiacol can be quantified using a standard curve of absorbance versus known guaiacol concentrations. This method is easy to perform and does not require expensive instrumentation. The development of commercially

available guaiacol detection kits (Anon., 2005; Anon., 2006) based on this reaction has further simplified the use of this method, as all the required chemicals are supplied. Detection only requires mixing of the sample and the provided chemicals, followed by a visual detection or a spectrophotometric measurement if quantification is required (Niwa & Kawamoto, 2003; Niwa & Kuriyama, 2003).

Analytical methods consist of three steps, namely sample preparation, separation and detection. Separation instruments such as gas chromatographs (GCs) or high pressure liquid chromatographs (HPLCs) are most often used and are combined with different sample preparation and detection methods. Headspace gas-chromatography mass-spectrometry (HS GC-MS) has become a popular method for the detection of volatile substances such as guaiacol, as minimal sample preparation is required because samples are simply heated to expel volatiles into the headspace (Zierler *et al.*, 2004; Ezquerro & Tena, 2005). Mass spectrometry for detection of compounds is a very sensitive and accurate method and is the method of choice in most research papers on this subject (Pettipher *et al.*, 1997; Orr *et al.*, 2000; Jensen *et al.*, 2001; López *et al.*, 2002; Lee & Noble, 2003; Gocmen *et al.*, 2005; Conde *et al.*, 2006; Siegmund and Pöllinger-Zierler, 2006).

The aim of this study was to determine the guaiacol producing abilities of strains of *Alicyclobacillus* isolated from the South African fruit processing environment and to establish the influence of incubation temperature on guaiacol production by these strains. The peroxidase enzyme colourimetric assay (PECA) was also compared with HS GC-MS with regards to the detection of guaiacol.

MATERIALS AND METHODS

Bacterial strains

Alicyclobacillus acidoterrestris FB2, FB14, FB32 and FB38, as well as *A. acidocaldarius* FB19 were isolated from fruit concentrate, wash water, evaporator water, flume water and vinegar flies, respectively at a fruit processing plant in the Western Cape Province of South Africa (Groenewald *et al.*, 2009). The type strains *A. acidoterrestris* DSM 3922^T and *A. acidocaldarius* DSM 446^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

Bacterial inoculation cultures

Isolates of *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 were inoculated into yeast starch glucose (YSG) broth (Goto *et al.*, 2002) [2.00 g.L⁻¹ yeast extract (Biolab, Merck, Cape Town, South Africa), 1.00 g.L⁻¹ glucose (AnalAR, supplied by Merck) and 2.00 g.L⁻¹ soluble starch (Pro Analyti, supplied by Merck)] acidified to pH 4.00 with 1 M H₂SO₄ (AnalAR, supplied by Merck), and incubated at 45°C for 5 d.

A volume of 100 µL of the above culture was spread onto *Bacillus acidoterrestris* (BAT) agar plates (IFU, 2007) and incubated at 45°C for 4 d. BAT agar was prepared by mixing equal volumes of BAT broth and a 3-4% (m/v) agar (Biolab, supplied by Merck) solution after autoclaving them separately. BAT broth consists of 0.25 g.L⁻¹ CaCl₂·H₂O (Saarchem, supplied by Merck), 0.50 g.L⁻¹ MgSO₄·7H₂O (Saarchem, supplied by Merck), 0.20 g.L⁻¹ (NH₄)₂SO₄ (Pro Analyti, supplied by Merck), 3.00 g.L⁻¹ KH₂PO₄ (AnalAR, supplied by Merck), 2.00 g.L⁻¹ yeast extract (Biolab, supplied by Merck), 5.00 g.L⁻¹ glucose (AnalAR, supplied by Merck) and 1.00 mL trace element solution, consisting of 0.66 g.L⁻¹ CaCl₂·H₂O (Saarchem, supplied by Merck), 0.18 g.L⁻¹ ZnSO₄·7H₂O (Merck), 0.16 g.L⁻¹ CuSO₄·5H₂O (Merck), 0.15 g.L⁻¹ MnSO₄·H₂O (Merck), 0.18 g.L⁻¹ CoCl₂·5H₂O (Merck), 0.10 g.L⁻¹ H₃BO₃ (Merck) and 0.30 g.L⁻¹ Na₂MoO₄·2H₂O (Merck). The trace element solution was prepared separately and sterilised by autoclaving. The broth was adjusted to pH 4.00 using 1 M H₂SO₄ (AnalAR, supplied by Merck).

Colonies from the BAT agar plates were aseptically removed and streaked onto fresh BAT agar plates at pH 4.00 and incubated at 45°C for 4 d to ensure pure colonies. A single colony from the streak plate was inoculated into YSG broth acidified to pH 4.00 with 1 M H₂SO₄ (AnalAR, supplied by Merck). After incubation at 45°C for 4-5 d the approximate cell concentration was determined by measuring the optical density (OD) at 540 nm using a Beckman Coulter DU 530 Life Science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA). The cell concentration of the inoculum was also confirmed by serially diluting the culture (10⁰-10⁻⁶) in sterile saline solution (SSS) [0.85% (m/v) NaCl (Merck)] and plating out in duplicate on PDA (Biolab, supplied by Merck) adjusted to pH 4.00 after autoclaving, using the pour plate technique, followed by incubation at 45°C for 4 d.

Guaiacol production under optimum growth conditions

Three hundred mL BAT broth supplemented with 100 mg.L⁻¹ vanillin (Merck), a guaiacol precursor, was inoculated with one of either *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32, FB38, *A. acidocaldarius* DSM 446^T or FB19 to a final cell concentration of 10¹-10³ cfu.mL⁻¹. Cultures were incubated at 45°C for 7 d. Samples were analysed every 12 h and the guaiacol concentration and cell concentration was determined.

Peroxidase enzyme colourimetric assay (PECA)

The guaiacol concentration was determined using the PECA as described by Sheu & Chen (1991) and Bahçeci & Acar (2007). One mL of the sample was centrifuged for 10 min at 2300 g using an Eppendorf 5415 D centrifuge (Hamburg, Germany). A volume of 300 µL of the supernatant of the centrifuged sample was vortexed with 2.10 mL 0.20 M potassium phosphate buffer [4.18 g.L⁻¹ K₂HPO₄ (Merck), 23.95 g.L⁻¹ KH₂PO₄ (Merck), calculated using the Henderson-Hasselbalch equation (Segel, 1976) adjusted to pH 6.00 with 1 M H₂SO₄ (AnalAR, supplied by Merck) or 1 M NaOH (AnalAR, supplied by Merck)], 300 µL (5 U) peroxidase enzyme (Merck) and 300 µL 0.50% (m/v) H₂O₂ (AnalAR, supplied by Merck). A blank was prepared in the same way using 300 µL potassium phosphate buffer instead of the sample. Colour changes were detected by measuring the OD at 420 nm using a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA). The guaiacol concentrations were quantified using a standard curve of OD versus guaiacol (Sigma-Aldrich, USA) concentrations. The experiment was carried out in triplicate.

Cell concentration

The cell concentration was measured by serially diluting the sample (10⁰-10⁻⁶) in SSS and using the pour plate technique with PDA (Biolab, supplied by Merck) adjusted to pH 4.00 after autoclaving with 1 M H₂SO₄ (AnalAR, supplied by Merck). Plates were poured in duplicate and incubated at 45°C for 4 d.

Effect of incubation temperature

Three hundred mL BAT broth supplemented with 100 mg.L⁻¹ vanillin (Merck), a

known guaiacol precursor, was inoculated in duplicate with one of either *A. acidoterrestris* DSM 3922^T, FB2, FB38, *A. acidocaldarius* DSM 446^T or FB19 to a final cell concentration of 10^1 - 10^3 cfu.mL⁻¹. Cultures of each strain were incubated at both 25°C and 45°C for 6 d. Samples were analysed every 24 h and the guaiacol concentration measured using the PECA, as well as HS GC-MS. The cell concentration was determined as described previously. The experiment was carried out in triplicate.

Headspace gas-chromatography mass-spectrometry (HS GC-MS)

For HS GC-MS analysis each sample was conditioned at 60°C for 20 min with intermittent shaking to expel volatiles into the headspace. The GC separation was carried out on a ThermoFinnigan Focus Dual Stage Quadruple (DSQ) gas-chromatograph equipped with a headspace autosampler and a 30 m x 0.25 mm ZB-1 (Separations SA) glass capillary column containing 100% dimethyl polysiloxane (0.25 µm). Helium was used as carrier gas (constant flow - 1.0 mL.min⁻¹) and the instrument was operated in a split-less mode of injection. The temperature program is summarised as follows: 40°-270°C at a rate of 8°C.min⁻¹ followed by a ramp from 90°-280°C at 10°C.min⁻¹. The column was coupled to a Finnigan Focus DSQ mass spectrometer for mass detection of fragments with a m/z ranging from 50.00-650.00. Mass analysis was performed at 70 eV with an ion source temperature of 200°C. Integration of the peaks was performed on the total ion chromatogram (TIC) using Xcalibur 1.4 software (Finnigan) and the Nist 2.0 database.

Statistical analysis

Repeated measure ANOVA using the mixed model approach was used to investigate the effect of different treatments on the various measurements over time. Correlation analyses were done to determine the strength of relationships between measurements. All analyses were performed using Statistica 8. A 5% significance level was used as guideline for indicating significant results.

RESULTS AND DISCUSSION

Guaiacol production under optimum growth conditions

Initial experiments were carried out in order to determine the guaiacol producing abilities of different strains of *Alicyclobacillus* spp. The PECA was used for guaiacol detection in these experiments as it is a rapid, inexpensive method which is easy to use and does not require expensive and complicated equipment or chemicals. Unknown guaiacol concentrations can be quantified with this method using a standard curve of absorption versus guaiacol concentration. Guaiacol concentrations produced by the seven *Alicyclobacillus* strains all followed the same pattern, except in the case of *A. acidoterrestris* FB38 (Fig. 1). *Alicyclobacillus acidoterrestris* DSM 3922^T, FB2, FB14 and FB32 had similar guaiacol production patterns, with guaiacol concentrations increasing steadily over the 7 d and stabilising around maximum concentrations of 0.24, 0.27, 0.29 and 0.28 mM, respectively, after 144 h. Guaiacol concentrations remained stable after reaching maximum levels, indicating that guaiacol did not undergo further degradation and that the strains were able to accumulate guaiacol in the medium. The same pattern of guaiacol production by *A. acidoterrestris* was observed in studies by Jensen (2000) and Bahçeci *et al.* (2005). Bahçeci *et al.* (2005) found that *A. acidoterrestris* at an inoculation concentration of 10^3 cfu.mL⁻¹ was able to produce guaiacol to a maximum amount of approximately 0.36 mM after 10 d in apple juice supplemented with 100 mg.L⁻¹ vanillin and incubated at 46°C. Similar guaiacol production patterns have also been observed in other bacteria, including *B. subtilis* and strains of *Streptomyces*, with guaiacol concentrations increasing steadily and stabilising around maximum values after 24 h (Álvarez-Rodríguez *et al.*, 2003).

In contrast to reports in the literature (Niwa, 2004; Jensen, 2005; Goto *et al.*, 2008), the two *A. acidocaldarius* strains were also able to produce guaiacol, but at slightly lower concentrations than the *A. acidoterrestris* strains. Guaiacol concentrations produced by the *A. acidocaldarius* strains followed the same pattern as the *A. acidoterrestris* strains mentioned previously. Maximum guaiacol concentrations of 0.17 and 0.22 mM were detected in *A. acidocaldarius* DSM 446^T and FB19, respectively, after 144 h. The potential of *A. acidocaldarius* to spoil products has also been recognised in other studies. Gouws *et al.*, (2005) isolated

A. acidocaldarius from spoiled mango juice, which implicated the organism as the cause of the spoilage, while Lottici *et al.* (2006) found that *A. acidocaldarius* was responsible for the spoilage of tomato products due to the production of an off-flavour compound called 2-methyltetrahydrothiophene-3-one. These results emphasise the need for research to not only focus on *A. acidoterrestris*, which has long been considered to be the only *Alicyclobacillus* species that presents a potential spoilage threat due to its frequent isolation from spoiled products (Cerny *et al.*, 1984; Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996; Walls & Chuyate, 1998), but to also include other *Alicyclobacillus* species with the potential to cause product spoilage due to their ability to produce taint chemicals. These species include *A. hesperidum* (Gocmen *et al.*, 2005; Goto *et al.*, 2008), *A. cycloheptanicus* (Gocmen *et al.*, 2005), *A. acidiphilus* (Matsubara *et al.*, 2002; Goto *et al.*, 2008) and *A. herbarius* (Goto *et al.*, 2008).

Alicyclobacillus acidoterrestris FB38 had a different guaiacol production pattern than the rest of the strains. The rate of guaiacol production was faster, with a maximum guaiacol concentration of 0.28 mM detected after only 84 h. Instead of stabilising after reaching a maximum, as was observed in the other strains, the guaiacol concentration decreased steadily, reaching a concentration of 0.13 mM by 168 h, which marked the end of the experiment. A similar pattern of guaiacol production was observed in the thermophilic fungus *Sporotrichum thermophile* when the latter was incubated in the presence of ferulic acid, with the guaiacol concentration reaching a maximum after 12 h, followed by a decrease in the guaiacol concentration. This decrease was attributed to the guaiacol being metabolised to catechol (Topakas *et al.*, 2003). In the proposed microbial metabolic pathway of guaiacol production guaiacol can indeed be further metabolised to catechol, which can, in turn, be further degraded (Pometto *et al.*, 1981; Rahouti *et al.*, 1989; Álvarez-Rodríguez *et al.*, 2003). Therefore, it is possible that, where the pathway seems to be truncated in the other *A. acidoterrestris* strains, *A. acidoterrestris* FB38 may be able to continue the metabolic pathway and convert guaiacol to other products.

Control values, from uninoculated BAT media supplemented with 100 mg.L⁻¹ vanillin, remained constant over the 7 d. Therefore, guaiacol production could mainly be attributed to the presence of the *Alicyclobacillus* strains in the media and not to other factors such as possible thermal decomposition of vanillin.

A moderate positive correlation of 0.61 was observed between guaiacol

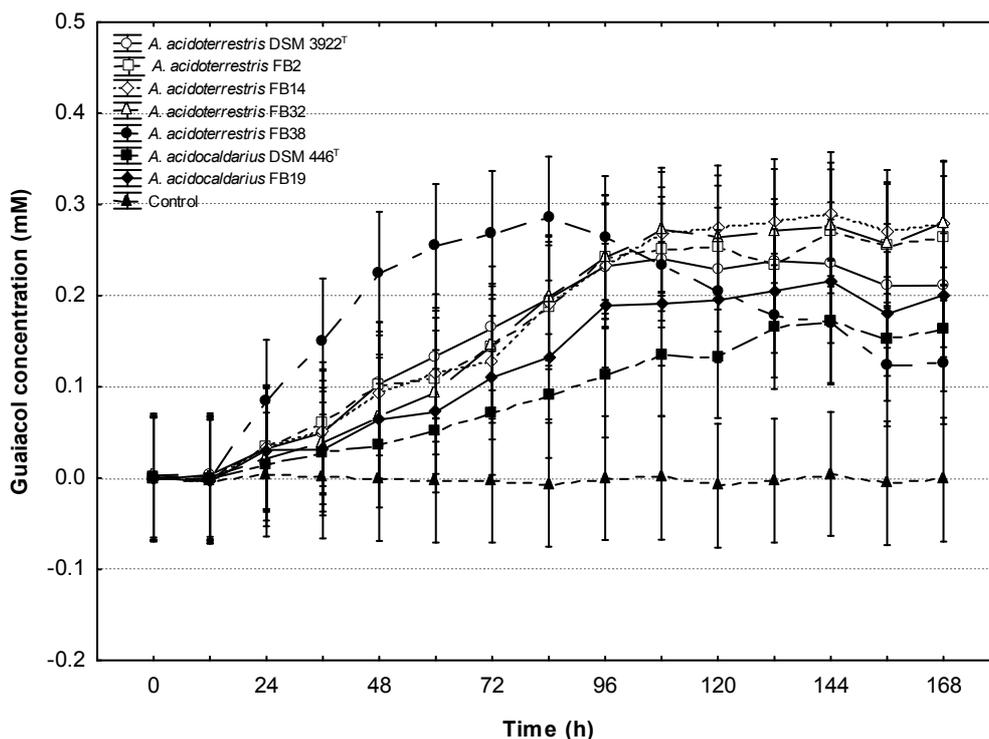


Figure 1 Guaiacol production by *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 45°C over 168 h (7 d) as determined by the PECA. Vertical bars denote 0.95 confidence intervals.

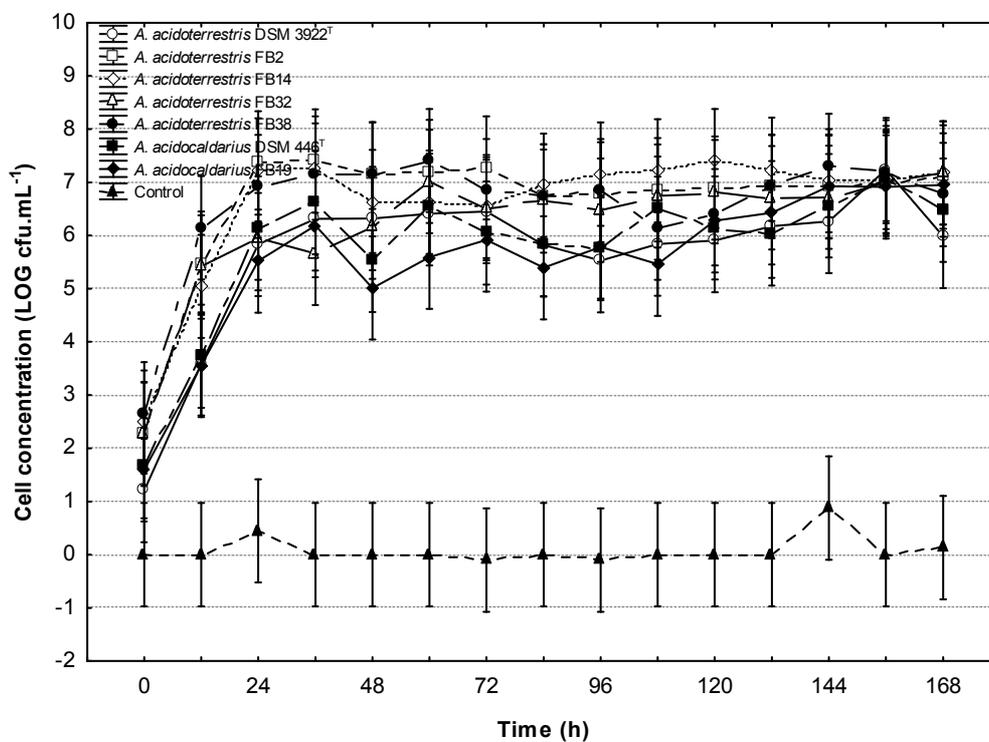


Figure 2 Change in cell concentrations of *A. acidoterrestris* DSM 3922^T, FB2, FB14, FB32 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 at 45°C over a period of 168 h (7 d). Vertical bars denote 0.95 confidence intervals.

concentration and cell concentration (cfu.mL⁻¹). Therefore, an increase in cell concentration should lead to an increase in guaiacol production. Guaiacol was only detected once a critical cell concentration ranging from 10⁴-10⁶ cfu.mL⁻¹, depending on the *Alicyclobacillus* strain, had been reached (Fig. 2, as discussed in Chapter 3). These results are in agreement with other researchers who have also reported that a critical cell concentration of 10⁴ cfu.ml⁻¹ (Bahçeci *et al.*, 2005) to 10⁵ cfu.ml⁻¹ (Pettipher *et al.*, 1997) needs to be present before guaiacol is detected.

Effect of incubation temperature on guaiacol production

Alicyclobacillus acidoterrestris DSM 3922^T, FB2 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 were selected from the previous seven strains, inoculated into BAT growth medium and incubated at 25°C and 45°C for 6 d. The guaiacol concentration was quantified daily using the PECA, as well as HS GC-MS, and the cell concentrations were determined as described.

Guaiacol production at different temperatures as measured by the PECA

At 45°C guaiacol concentrations detected in the *A. acidoterrestris* DSM 3922^T and FB2 samples followed a linear pattern, reaching maximum concentrations of 0.19 and 0.31 mM, respectively, after 5 d (Figs. 3 and 4). At 45°C *A. acidoterrestris* FB38 again had a more rapid rate of guaiacol production than the other strains, with a maximum guaiacol concentration of 0.22 mM being detected after only 2 d, followed by a decrease in the guaiacol concentration to almost undetectable levels after 6 d (Fig. 5).

Guaiacol was detected in all three *A. acidoterrestris* samples at 25°C. In agreement with this, Pettipher *et al.* (1997) also observed guaiacol production in detectable amounts by *A. acidoterrestris* in orange and apple juice at 25°C after 6-10 d. Siegmund and Pöllinger-Zierler (2007) detected guaiacol production by *A. acidoterrestris* at room temperature (20°-23°C) and Bahçeci *et al.* (2005) found that *A. acidoterrestris* started producing detectable guaiacol concentrations after approximately 6 d at 25°C in apple juice supplemented with 100 mg.L⁻¹ vanillin. In the present study a lag phase of 2 d was observed at 25°C in all three *A. acidoterrestris* strains before guaiacol was detected (Figs. 3-5). Other researchers have reported that a minimum *Alicyclobacillus* cell concentration of 10⁴-10⁵ cfu.mL⁻¹

is required before guaiacol is detected (Pettipher *et al.*, 1997; Bahçeci *et al.*, 2005). The present results correspond to these, as guaiacol was detected only after 2 d, once cell concentrations of 10^4 - 10^5 cfu.mL⁻¹, depending on the strain, had been reached (Figs. 8-10, as discussed in Chapter 3). The sub-optimal growth temperature increases the lag phase of growth, resulting in a longer period elapsing before the critical cell concentration is reached and detectable amounts of guaiacol is produced.

However, maximum guaiacol concentrations are not necessarily lower at a lower growth temperature. At 25°C the maximum guaiacol concentrations were 0.33, 0.19 and 0.35 mM for *A. acidoterrestris* DSM 3922^T, FB2 and FB38, respectively. It is interesting that the maximum guaiacol concentrations detected in *A. acidoterrestris* DSM 3922^T and FB38 at 25°C were greater than the maximum concentrations detected at 45°C (Figs. 3 and 5). One would expect it to be less, as observed in *A. acidoterrestris* FB2 (Fig. 4), as cell concentrations of all three *A. acidoterrestris* strains are lower at 25°C (Figs. 8-10, as discussed in Chapter 3). Metabolic processes are also usually less rapid at lower temperatures. However, Pettipher *et al.* (1997) also detected higher guaiacol concentrations after 6 d at 25°C compared to 44°C in orange juice. The fact that *A. acidoterrestris* cells can produce equal and even higher concentrations of guaiacol at a sub-optimum compared to an optimum growth temperature shows that its ability to spoil products stored at ambient temperatures (usually 20°-25°C) should not be underestimated. Where spoilage incidents were previously thought to be more severe at higher temperatures, these results show that equal and even worse degrees of spoilage can also occur at lower temperatures.

Guaiacol was again detected in the two *A. acidocaldarius* samples at 45°C (Figs. 6 and 7), but in significantly ($p \leq 0.05$) lower concentrations than observed previously in Fig. 1 and in the *A. acidoterrestris* strains. Maximum guaiacol concentrations detected in the *A. acidocaldarius* DSM 446^T and FB19 samples at 45°C were 0.045 and 0.052 mM, respectively, while no guaiacol was detected in either sample at 25°C. This is because a decrease in cell concentrations rather than an increase was observed for these two strains at this temperature (Figs. 11 and 12, as discussed in Chapter 3), as 25°C is below the temperature growth range of 45°-70°C of this highly thermophilic species (Uchino & Doi, 1967; Darland & Brock, 1971; Wisotzkey *et al.*, 1992). Since no growth occurred, the cell concentration remained

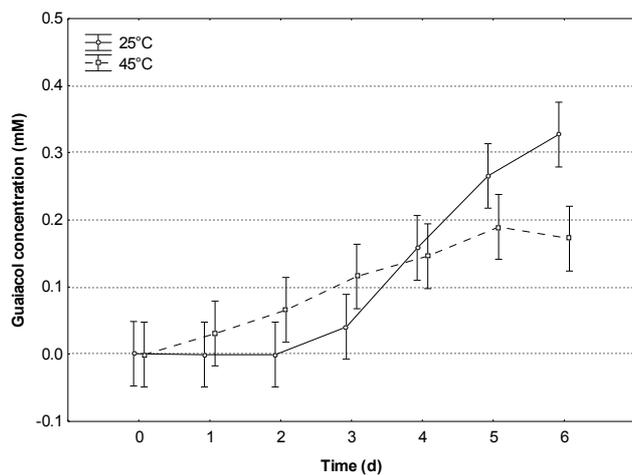


Figure 3 Guaiacol production by *A. acidoterrestris* DSM3922^T at 25°C and 45°C over 6 d as determined by the PECA. Vertical bars denote 0.95 confidence intervals.

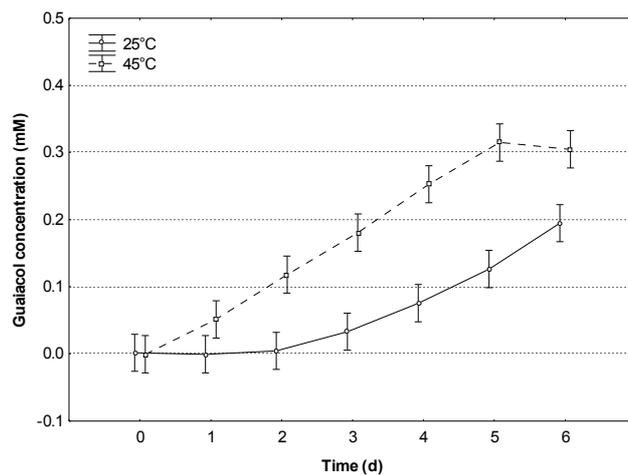


Figure 4 Guaiacol production by *A. acidoterrestris* FB2 at 25°C and 45°C over 6 d as determined by the PECA. Vertical bars denote 0.95 confidence intervals.

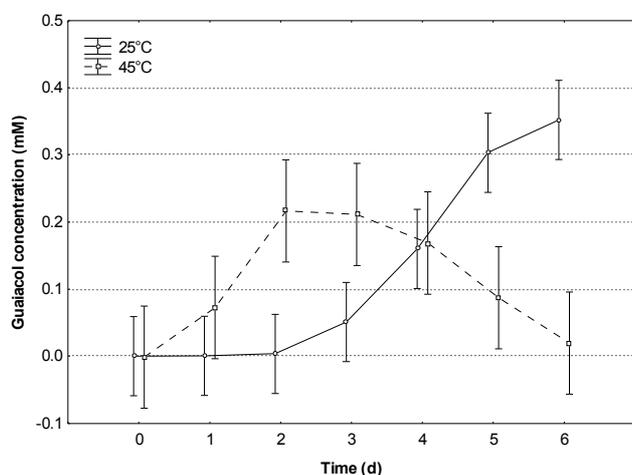


Figure 5 Guaiacol production by *A. acidoterrestris* FB38 at 25°C and 45°C over 6 d as determined by the PECA. Vertical bars denote 0.95 confidence intervals.

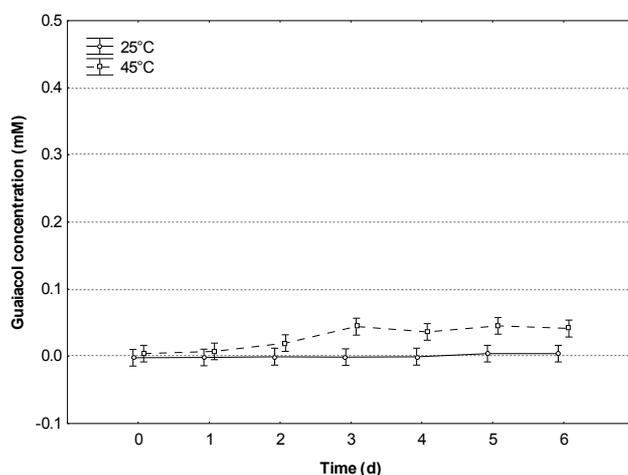


Figure 6 Guaiacol production by *A. acidocaldarius* DSM446^T at 25°C and 45°C over 6 d as determined by the PECA. Vertical bars denote 0.95 confidence intervals.

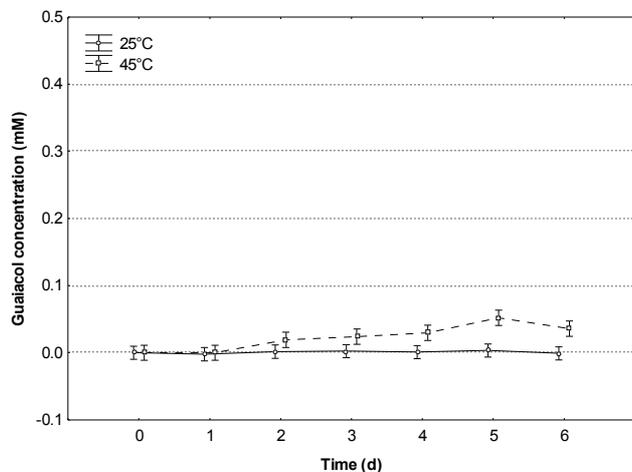


Figure 7 Guaiacol production by *A. acidocaldarius* FB19 at 25°C and 45°C over 6 d as determined by the PECA. Vertical bars denote 0.95 confidence intervals.

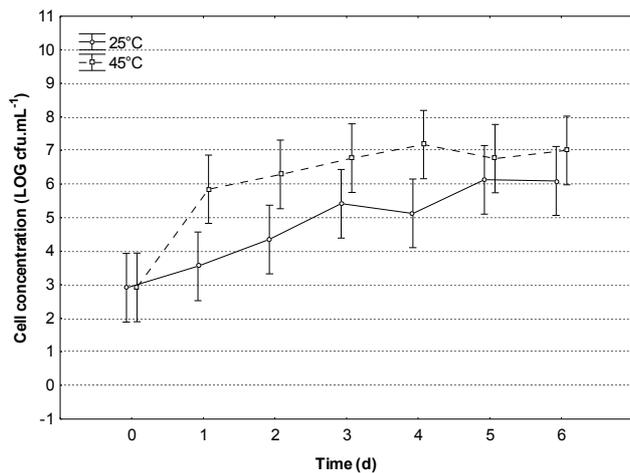


Figure 8 Change in cell concentration of *A. acidoterrestris* DSM 3922^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

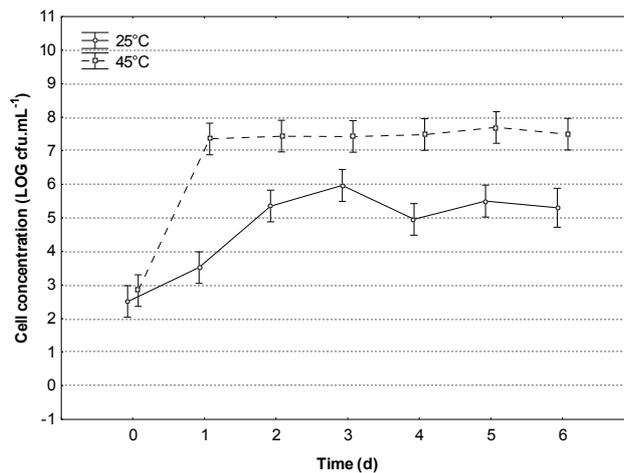


Figure 9 Change in cell concentration of *A. acidoterrestris* FB2 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

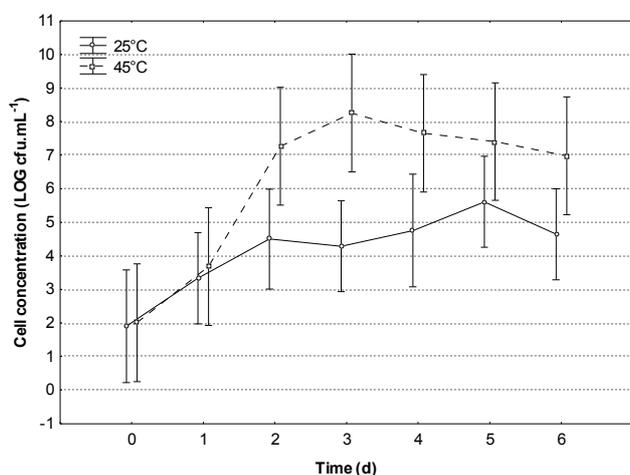


Figure 10 Change in cell concentration of *A. acidoterrestris* FB38 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

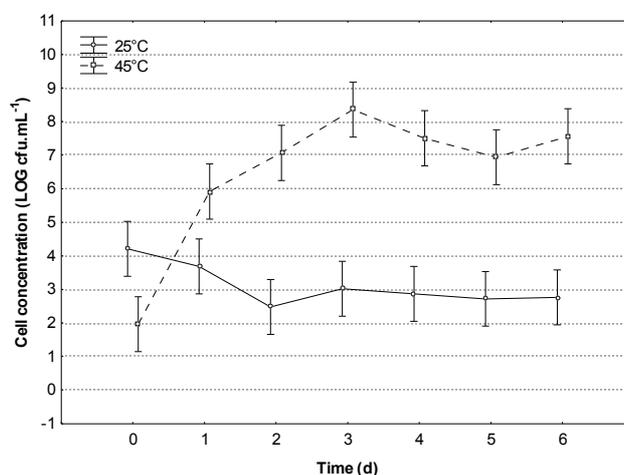


Figure 11 Change in cell concentration of *A. acidocaldarius* DSM 446^T at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

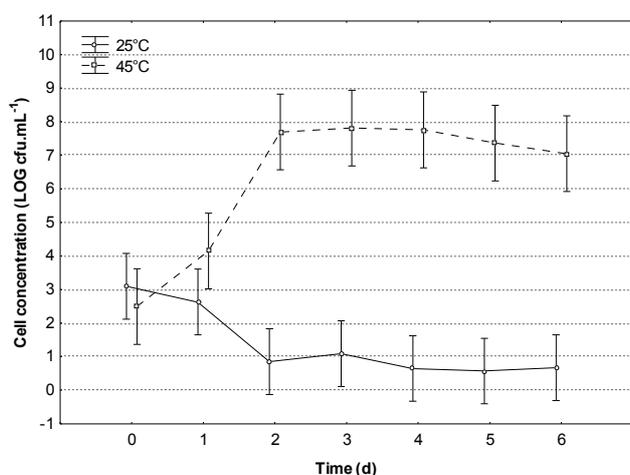


Figure 12 Change in cell concentration of *A. acidocaldarius* FB19 at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

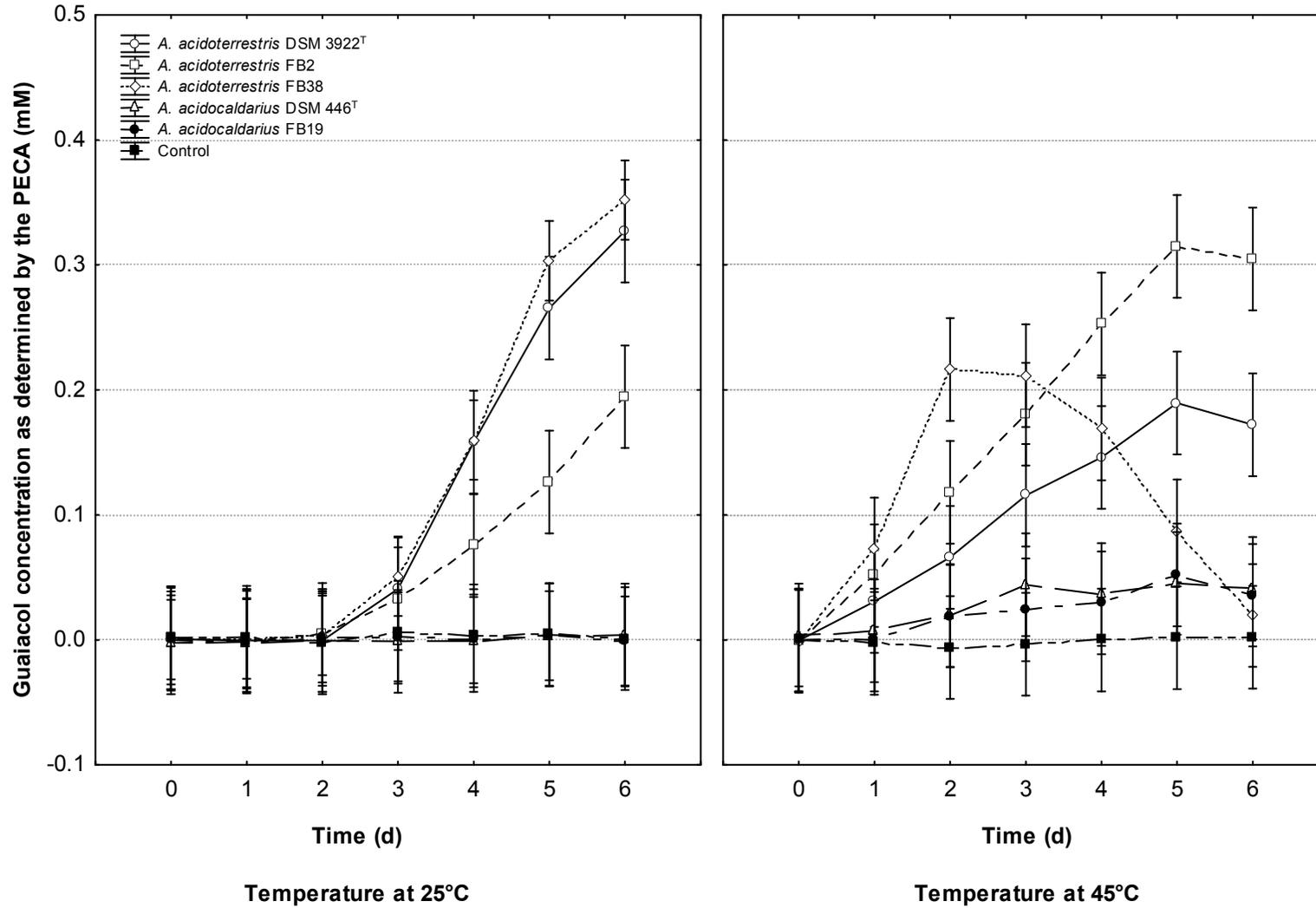


Figure 13 Guaiacol concentrations detected with the PECA in the *A. acidoterrestris* DSM 3922^T, FB2 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 samples at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

lower than the critical cell concentration of 10^4 - 10^5 cfu.mL⁻¹ necessary for guaiacol to be produced in detectable amounts by these strains, resulting in no guaiacol being detected.

Fig. 13 shows the change in guaiacol concentrations as measured by the PECA at 25°C and 45°C in all five strains of *Alicyclobacillus*. Control values, from uninoculated BAT media supplemented with 100 mg.L⁻¹ vanillin, remained constant over the 6 d at both temperatures.

Guaiacol production at different temperatures as measured with HS GC-MS

The guaiacol concentrations detected in the *Alicyclobacillus* strains at different temperatures were also measured with HS GC-MS. A typical chromatogram of guaiacol as determined by HS GC-MS is presented in Fig. 14. Although, like most analytical methods, it is a fairly expensive and time consuming method that requires specialised equipment, HS GC-MS has the advantage of providing a very accurate and sensitive detection system while requiring minimal sample preparation.

The guaiacol production patterns determined by HS GC-MS were similar to those determined by the PECA in all five *Alicyclobacillus* strains (Figs. 15-19). The guaiacol concentrations detected in the *A. acidoterrestris* DSM 3922^T and FB2 samples increased steadily, although guaiacol concentrations fluctuated more than the concentrations detected by the PECA, especially at 45°C (Figs. 15 and 16). At 45°C *A. acidoterrestris* FB38 also had a faster rate of guaiacol production followed by a decrease in the concentration after reaching a maximum, while at 25°C guaiacol was also only detected after 2 d in the *A. acidoterrestris* strains (Figs. 15-17). Significantly less guaiacol was also detected in the *A. acidocaldarius* samples at 45°C than in the *A. acidoterrestris* samples, while no guaiacol was detected at 25°C (Figs. 18 and 19).

The data produced with HS GC-MS did, however, also differ from that of the PECA. Guaiacol concentrations detected with HS GC-MS were significantly ($p \leq 0.05$) higher than those detected by the PECA. HS GC-MS also found that guaiacol concentrations were higher at 45°C in all the strains, although in *A. acidoterrestris* DSM 3922^T and FB38 the differences in guaiacol concentrations detected at 45°C and 25°C were not always significantly different ($p \leq 0.05$) (Figs. 15 and 17).

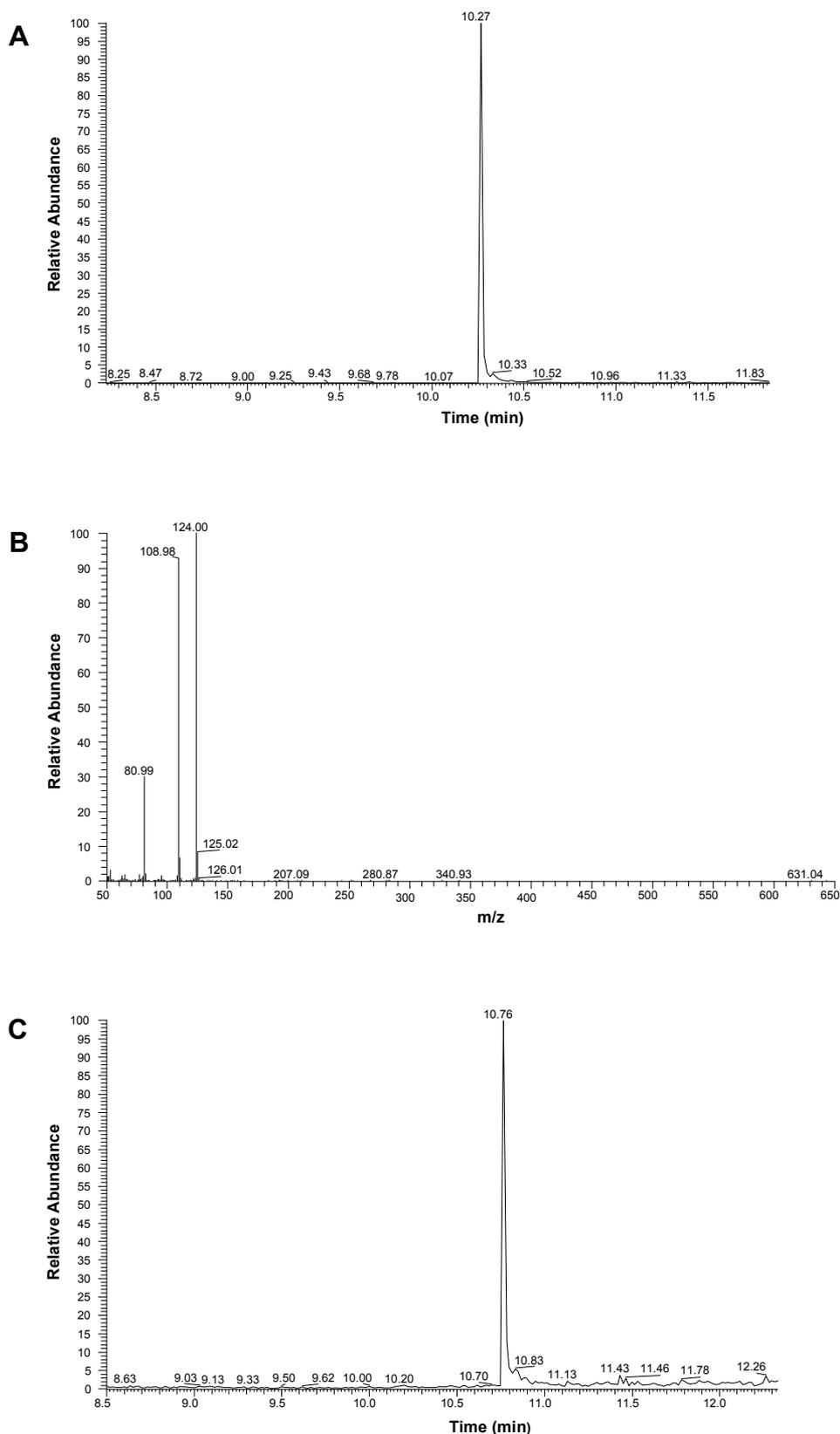


Figure 14 A typical HS GC-MS chromatogram (TIC) of (A) a standard dilution of guaiacol (Sigma-Aldrich, USA) (B) EI fragmentations of guaiacol (C) guaiacol detected in a sample containing a strain of *A. acidoterrestris*.

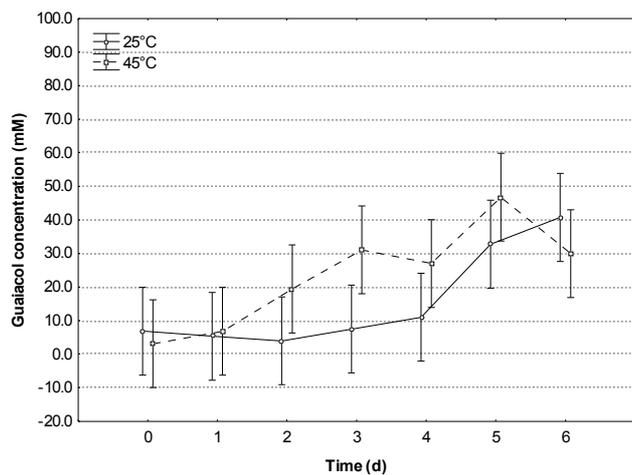


Figure 15 Guaiacol production by *A. acidoterrestris* DSM 3922^T at 25°C and 45°C over 6 d as determined by HS GC-MS. Vertical bars denote 0.95 confidence intervals.

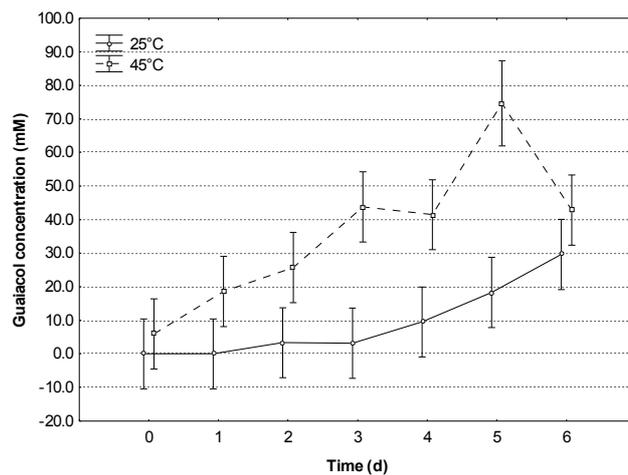


Figure 16 Guaiacol production by *A. acidoterrestris* FB2 at 25°C and 45°C over 6 d as determined by HS GC-MS. Vertical bars denote 0.95 confidence intervals.

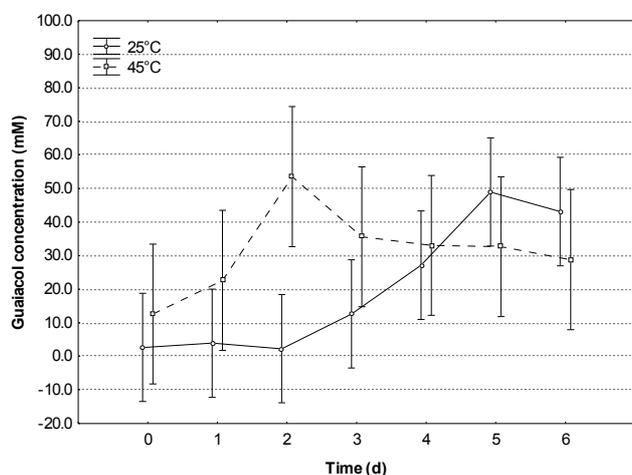


Figure 17 Guaiacol production by *A. acidoterrestris* FB38 at 25°C and 45°C over 6 d as determined by HS GC-MS. Vertical bars denote 0.95 confidence intervals.

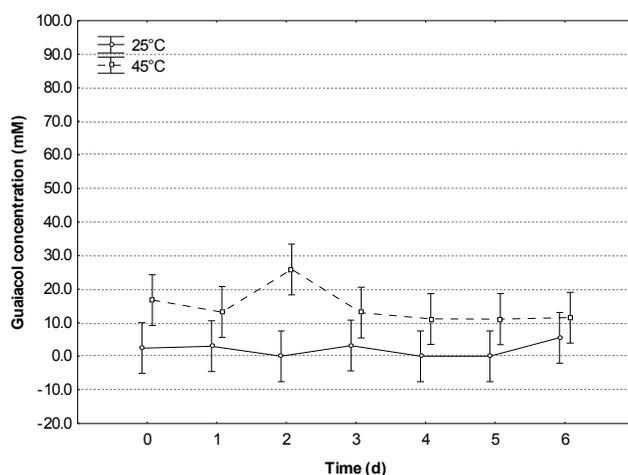


Figure 18 Guaiacol production by *A. acidocaldarius* DSM 446^T at 25°C and 45°C over 6 d as determined by HS GC-MS. Vertical bars denote 0.95 confidence intervals.

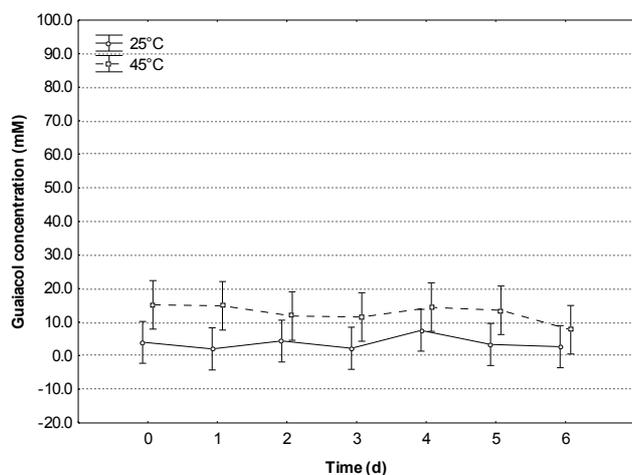


Figure 19 Guaiacol production by *A. acidocaldarius* FB19 at 25°C and 45°C over 6 d as determined by HS GC-MS. Vertical bars denote 0.95 confidence intervals.

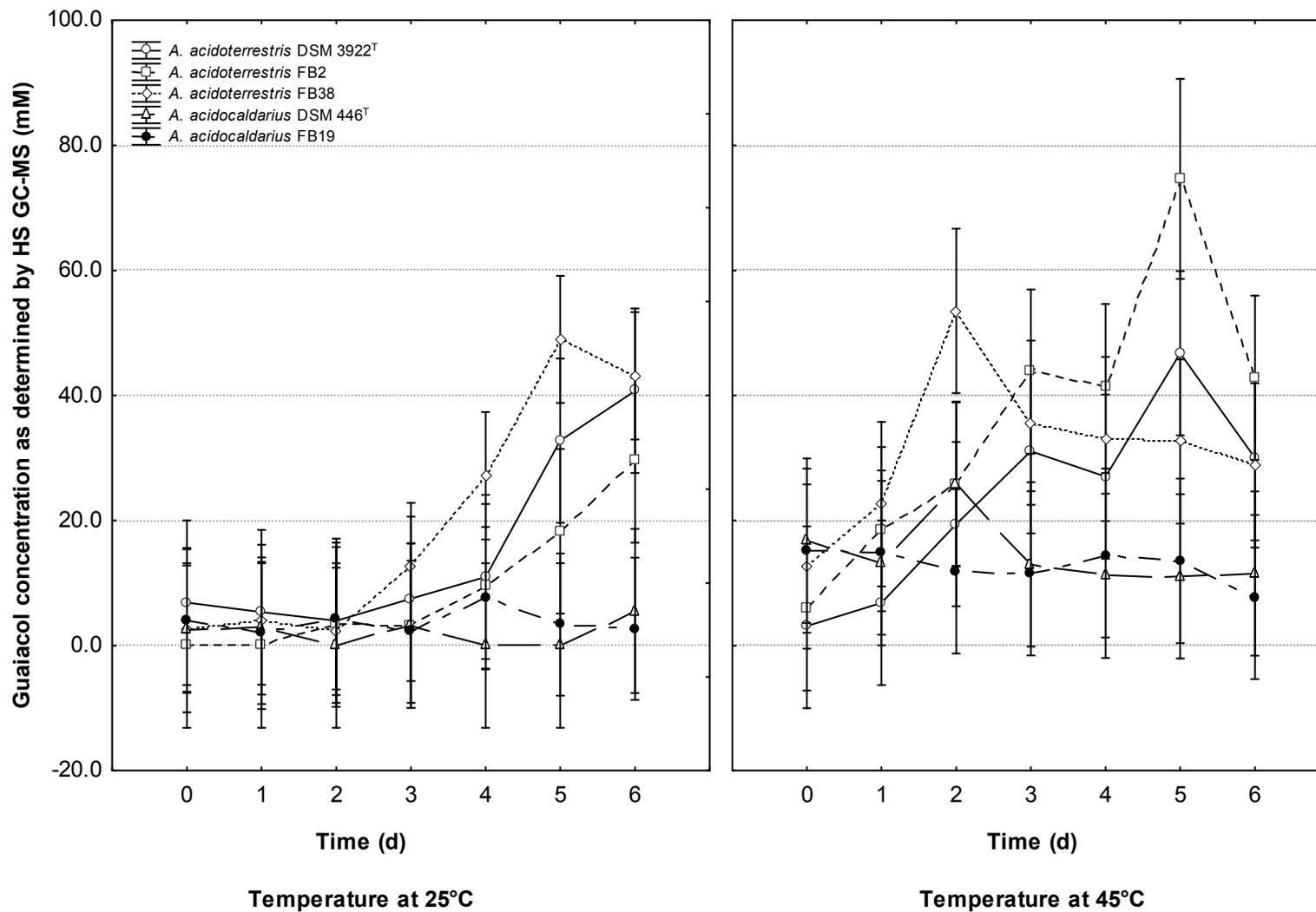


Figure 20 Guaiacol concentrations detected with HS GC-MS^T in the *A. acidoterrestris* DSM 3922^T, FB2 and FB38 and *A. acidocaldarius* DSM 446^T and FB19 samples at 25°C and 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

Maximum guaiacol concentrations detected by HS GC-MS for *A. acidoterrestris* DSM 3922^T, FB2, FB38 and *A. acidocaldarius* DSM 446^T and FB19 were 46.80, 74.60, 53.50, 25.90 and 15.20 mM at 45°C and at 25°C were 40.70, 29.60 and 49.00 mM for *A. acidoterrestris* DSM 3922^T, FB2 and FB38.

Fig. 20 shows the change in guaiacol concentrations as measured by HS GC-MS at 25°C and 45°C in all five strains of *Alicyclobacillus*. When compared to Fig. 13 the similarity between the guaiacol detection trends is clear. The higher guaiacol concentrations detected at 45°C compared to 25°C is also apparent in this figure.

Comparison of the PECA and HS GC-MS for the detection of guaiacol

Peroxidase enzyme colourimetric assay (PECA)

This assay is a commonly used method for quantifying peroxidase enzyme activity based on the change in absorbance (Maehly & Chance, 1954; Chance & Maehly, 1964). A standard curve of known guaiacol concentrations versus absorbance values is presented in Fig. 21. The equation of the regression line is indicated and a highly significant correlation ($r^2=0.9979$) was observed. The detection limit of the PECA in this study was determined to be between 0.0125 and 0.025 mM guaiacol (Fig. 21). This assay has been reported to be susceptible to interference by other phenols, such as pyrogallol and hydroquinone (Doerge *et al.*, 1997). Although guaiacol does not appear to be the only compound that can produce a colour change in the reaction, the brown colour change observed seems to be specific for guaiacol as it is due to the production of the compound 3,3'-dimethoxy-4,4'-biphenylquinone, an oxidation product of guaiacol (Doerge *et al.*, 1997). Vanillic acid has been observed to cause a pale yellow colour change, while the presence of catechol causes a blue colour change (Doerge *et al.*, 1997).

Headspace gas-chromatography mass-spectrometry (HS GC-MS)

This analytical method is widely used to detect and quantify specific compounds from complex matrices. A standard curve for the detection of guaiacol using HS GC-MS is presented in Fig. 22. The equation of the regression line is indicated and a significant correlation ($r^2=0.8822$) was observed. This method is very accurate, sensitive and highly specific for the compound being analysed.

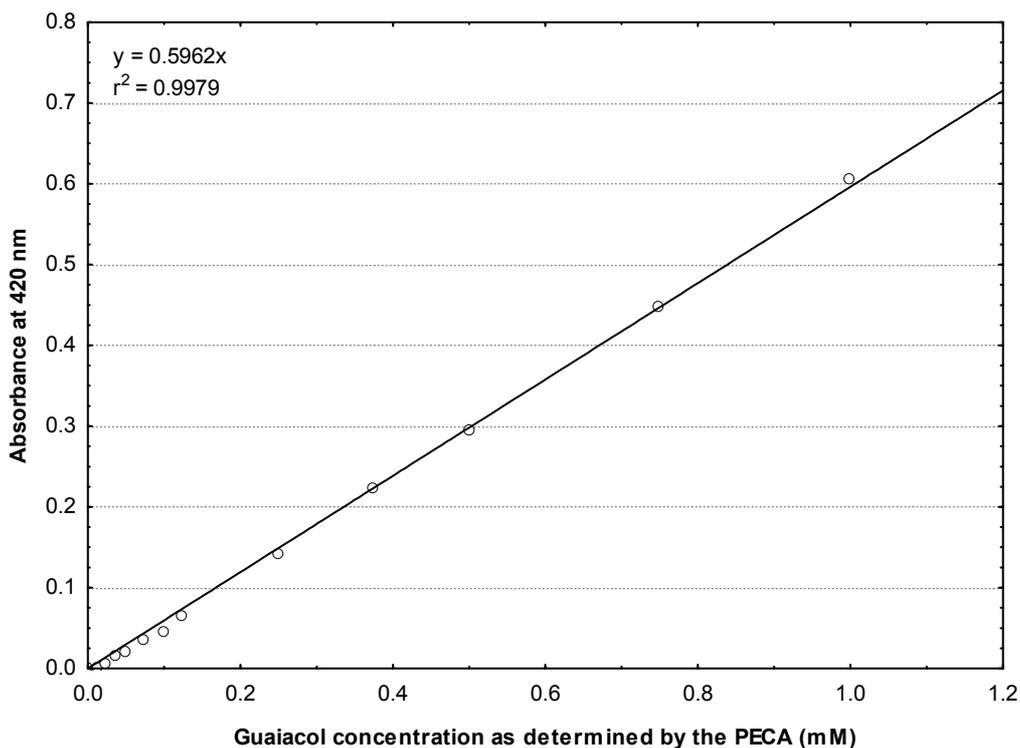


Figure 21 Calibration curve of known guaiacol concentrations versus absorbance. The equation of the regression line as well as the r^2 value is indicated on the graph.

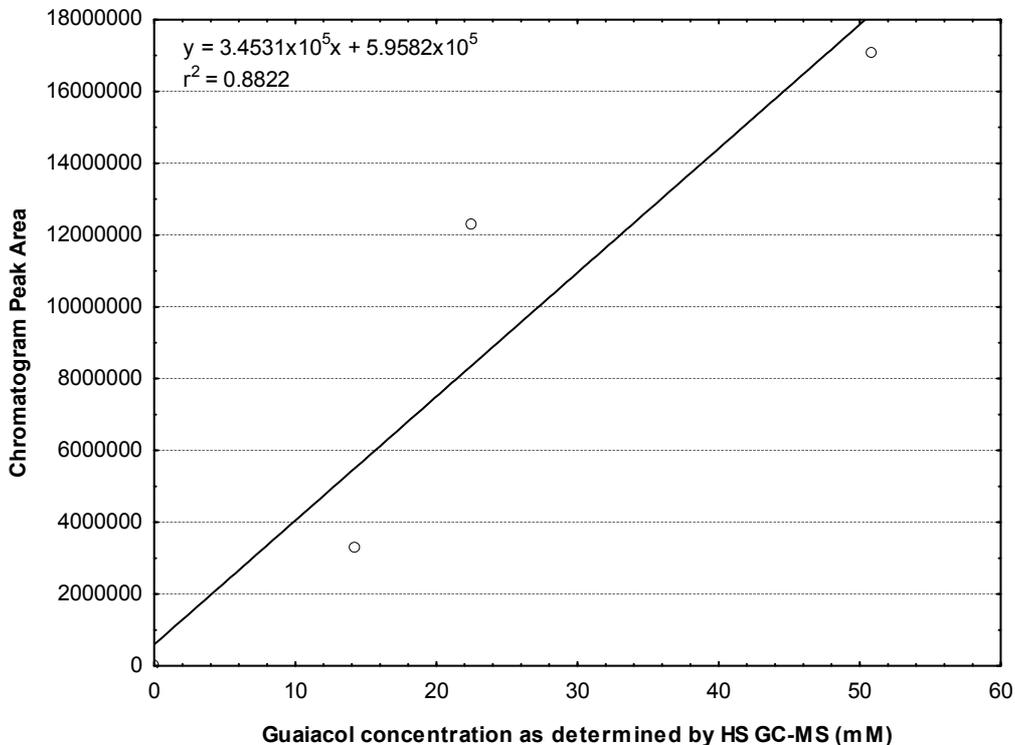


Figure 22 Calibration curve of known guaiacol concentrations versus chromatogram peak area. The equation of the regression line as well as the r^2 value is indicated on the graph.

Comparison of the PECA with HS GC-MS

The mean and range of guaiacol concentrations determined by the two different methods, as well as their correlation and agreement is presented in Table 1. Although a moderate positive correlation of 0.76 was observed between the two methods (Fig. 23), indicating that the trends of the data tend to be the same between the methods, the corresponding sensitivity differed. The HS GC-MS detected significantly higher guaiacol concentrations than the PECA. A number of factors could have influenced this observation. Firstly, mass spectrometry is a more sensitive detection method than spectrophotometry. It also detects all the configurations of a compound (D- and L-configuration), as well as guaiacol present both in- and outside the cell. Mass spectrometry is also a closed system, whereas the PECA is an open system and some guaiacol could, therefore, have evaporated while the assay was performed, leading to lower concentrations being detected with the PECA. Although mass spectrometry is a more sensitive detection method and therefore, should show saturation faster than the PECA, this was not observed, most probably because guaiacol is such a small and volatile compound.

CONCLUSION

Research on the *Alicyclobacillus* genus has mainly been focused on *A. acidoterrestris*, as this species has been the only one consistently associated with spoilage incidents due to its ability to produce the spoilage compound guaiacol. However, in this study the type strain of *A. acidocaldarius* and a strain of *A. acidocaldarius* isolated from vinegar flies in a South African fruit processing plant were able to produce guaiacol at an incubation temperature of 45°C, although in significantly smaller concentrations than the *A. acidoterrestris* strains. Therefore, studies should not only focus on *A. acidoterrestris* and guaiacol production, but should investigate the abilities of other *Alicyclobacillus* species to produce guaiacol and also other spoilage compounds.

Two of the *A. acidoterrestris* strains used in this study were able to produce higher concentrations of guaiacol at 25°C than at 45°C (as determined by the PECA). The ability of *A. acidoterrestris* to cause spoilage of acidic products at sub-optimal growth temperatures should, therefore, not be underestimated, as the degree of

Table 1 Mean and range of guaiacol concentrations measured by the PECA and HS GC-MS in 273 and 230 samples, respectively

Detection technique	Guaiacol range (mM)	Guaiacol concentration (mM) [mean (lower 95% CI - upper 95% CI)]	Correlation and agreement			
			Methods compared	Regression equation	r ²	p
PECA	-0.010-0.443	0.063 (0.051-0.076)	PECA vs. HS GC-MS	y = 134.59x + 6.47	0.61	≤0.0000
HS GC-MS	0.000-104.55	16.41 (14.03-18.79)				

PECA, peroxidase enzyme colourimetric assay; HS GC-MS, headspace gas-chromatography mass-spectrometry; CI, confidence interval

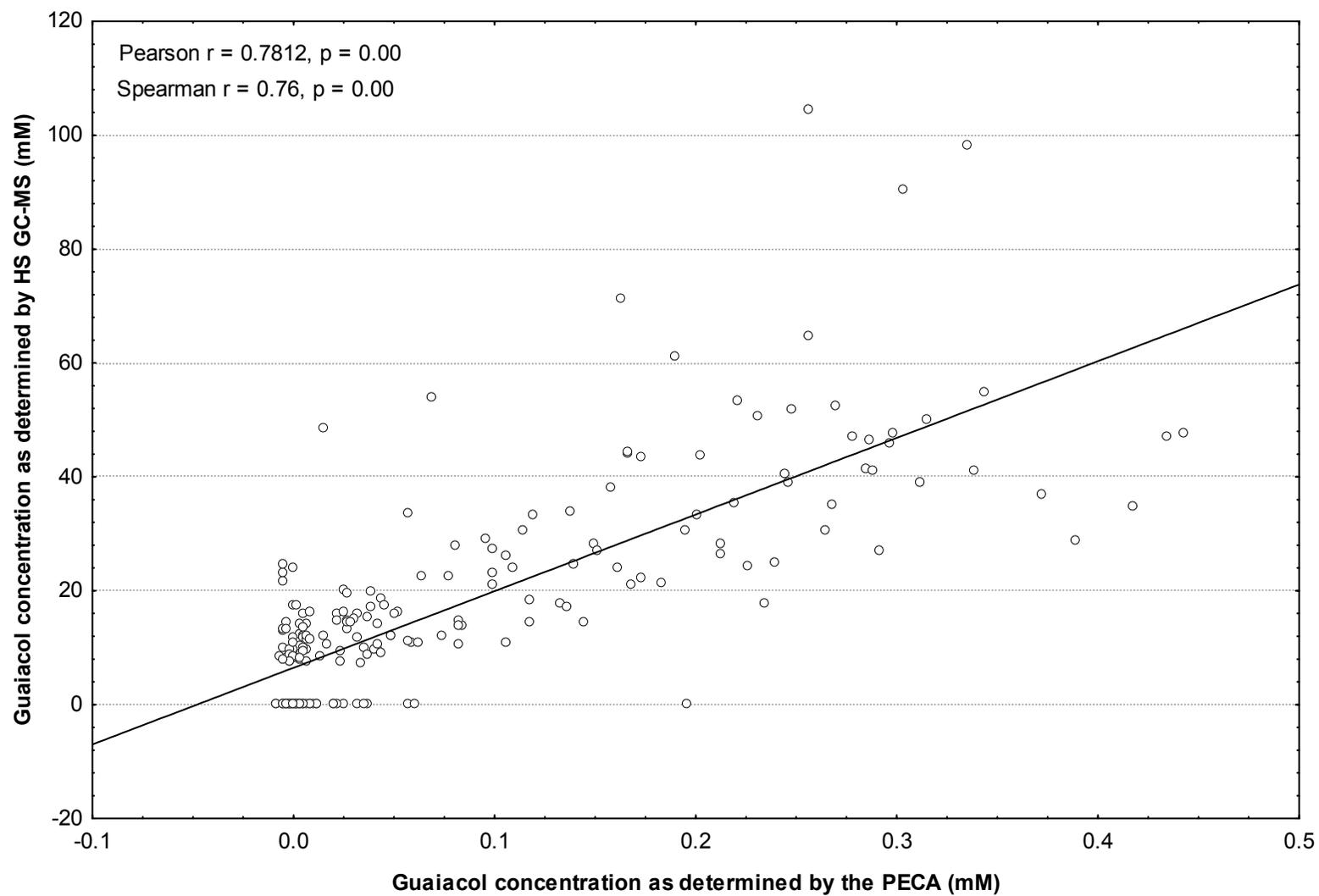


Figure 23 Comparison of concentrations of guaiacol obtained by HS GC-MS and the PECA. The Pearson and Spearman correlation (r) values, as well as their p -values, are indicated.

spoilage can be as, or even more, severe than at higher temperatures.

The isolation of guaiacol producing *Alicyclobacillus* strains from soil outside the factory, fruit wash water, factory flume water, evaporator water and vinegar flies confirm, as suggested by Groenewald *et al.* (2009), that these areas in the processing environment can all act as possible sources of *Alicyclobacillus* strains capable of causing product spoilage. Because the sources of contaminating organisms are so widespread, total elimination of *Alicyclobacillus* spp. from the processing environment is not feasible. The best approach to control the spread and growth of contaminating organisms in the processing environment is to adopt good manufacturing practices based on hazard analysis critical control point (HACCP) principles as suggested by the *Alicyclobacillus* Best Practice Guideline compiled by the European Fruit Juice Association (AIJN) (AIJN, 2007).

Although the PECA has the advantage of being a fast, simple and inexpensive guaiacol detection method, uncertainty exists regarding its accuracy. It is suggested that this method be primarily used as a presence/absence test for guaiacol producing *Alicyclobacillus* strains or to determine guaiacol production patterns of *Alicyclobacillus* strains, but that HS GC-MS should be used when accurate quantification of compounds is required.

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CHAPTER 5

INFLUENCE OF DIFFERENT VANILLIN CONCENTRATIONS IN DILUTED WHITE GRAPE JUICE CONCENTRATE ON THE GROWTH AND GUAIACOL PRODUCTION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS*

ABSTRACT

The metabolic pathway of guaiacol production by species of *Alicyclobacillus* is most commonly accepted as being one of non-oxidative decarboxylation of vanillic acid. Vanillic acid can be present in fruit juice products as an oxidation product of vanillin, which is a derivative of ferulic acid, a component of the plant polymer, lignin. The aim of this study was to establish the influence of different vanillin concentrations on the growth and guaiacol production of a strain of *A. acidoterrestris* isolated from the South African fruit processing environment. *Alicyclobacillus acidoterrestris* FB2 was incubated at 45°C for 6 d in white grape juice concentrate diluted 1:10 to 8.50°Brix and supplemented with vanillin at concentrations of 0, 10, 50, 100, 250 and 500 mg.L⁻¹. Samples were analysed every 24 h and the absorbance at 540 nm, cell concentration and pH were measured, as well as the guaiacol concentration using the peroxidase enzyme colourimetric assay (PECA). In the samples supplemented with 500 mg.L⁻¹ vanillin, growth was inhibited initially, as a longer lag phase of growth of 2 d compared to 1 d for the other samples was observed. Maximum cell concentrations in the sample containing 500 mg.L⁻¹ vanillin were also reached only after 4 d versus 3 d for the samples containing 0-250 mg.L⁻¹ vanillin. Guaiacol production increased as vanillin concentration increased, except in the sample containing 500 mg.L⁻¹ vanillin, where less guaiacol was produced than in the sample containing 250 mg.L⁻¹ vanillin. This was due to the initial growth inhibition caused by the higher vanillin concentration, which lengthened the time it took to reach the critical cell concentration of 10⁴-10⁵ cfu.mL⁻¹ necessary for detectable guaiacol production. Although there was a slight increase in the guaiacol concentration in the sample containing 10 mg.L⁻¹ vanillin, it did not differ significantly (p≤0.05) from the changes observed in the sample containing 0 mg.L⁻¹ vanillin or the control, where no significant change in guaiacol concentration was observed. The pH increased

slightly during growth in all the samples. *Alicyclobacillus acidoterrestris* FB2 is able to grow and produce guaiacol from vanillin in diluted white grape juice concentrate, with maximum guaiacol production at 250 mg.L⁻¹ vanillin. An increased concentration of vanillin in the juice will lead to production of guaiacol in increased concentrations, provided that the vanillin concentration is below the critical threshold that causes growth inhibition.

INTRODUCTION

The spores of the thermo-acidophilic bacteria from the genus *Alicyclobacillus* are able to survive pasteurisation processes normally applied to acidic products such as fruit juice (Splittstoesser *et al.*, 1998; Eiroa *et al.*, 1999; Vieira *et al.*, 2002). Because of their acidophilic nature the spores can germinate and grow in these products to cell concentrations high enough to produce the chemical compound guaiacol, which causes a medicinal/disinfectant-like off-flavour and/or -odour in the products (Pettipher *et al.*, 1997; Orr *et al.*, 2000; Gocmen *et al.*, 2005). *Alicyclobacillus* species have been known to be fastidious regarding their growth requirements and growth is only observed on specific growth media and only in certain fruit products. Fruit juices that support growth of *Alicyclobacillus* spp. and are, therefore, highly susceptible to spoilage include apple, orange, tomato, pineapple, grapefruit, pear and white grape juice (Pettipher *et al.*, 1997; Splittstoesser *et al.*, 1998; Walls & Chuyate, 2000).

Guaiacol is produced in food products either as a result of thermal decomposition, as in roasted products (Mayer *et al.*, 1999), or as a product of microbial metabolism. A number of micro-organisms are able to produce guaiacol. *Paecilomyces variotti* (Rahouti *et al.*, 1989), *Rhodotorula rubra* (Huang *et al.*, 1993) and *Sporotrichum thermophile* (Topakas *et al.*, 2003) produce guaiacol through the metabolism of ferulic acid. During its metabolism, ferulic acid is decarboxylated to 4-vinylguaiacol (Rahouti *et al.*, 1989; Karmakar *et al.*, 2000; Topakas *et al.*, 2003), which is in turn transformed into vanillic acid, with (Rahouti *et al.*, 1989) or without (Topakas *et al.*, 2003) production of vanillin as a precursor to vanillic acid. Ferulic acid can also be directly transformed into either vanillin (Peleg *et al.*, 1992) or vanillic acid (Huang *et al.*, 1993) without 4-vinylguaiacol acting as an intermediate. Where vanillin is produced it is rapidly converted to other products, which is attributed to its

toxicity (Bahçeci & Acar, 2007; Ferrante *et al.*, 2007; Char *et al.*, 2009). The vanillic acid that is formed by *P. variotti*, *R. rubra* and *S. thermophile* during ferulic acid metabolism undergoes a non-oxidative decarboxylation to form guaiacol.

Vanillic acid has in the metabolic pathway been identified as the immediate precursor to guaiacol (Rahouti *et al.*, 1989; Huang *et al.*, 1993; Topakas *et al.*, 2003). Several strains of *Bacillus megaterium* (Crawford & Olson, 1978), *Bacillus subtilis* (Álvarez-Rodríguez *et al.*, 2003), a number of unknown *Streptomyces* strains (Crawford & Olson, 1978; Álvarez-Rodríguez *et al.*, 2003) and *Streptomyces setonii* (Pometto *et al.*, 1981) are also able to produce guaiacol from vanillic acid.

Alicyclobacillus acidoterrestris is able to produce guaiacol from vanillin (Bahçeci *et al.*, 2005; Bahçeci & Acar, 2007) and vanillic acid (Niwa & Kuriyama, 2003). It has been reported that the conversion of vanillic acid to guaiacol is more rapid than that of vanillin (Niwa & Kuriyama, 2003), which is in agreement with the finding that vanillic acid is the immediate precursor to guaiacol in the metabolic production pathway. Although the precise metabolic pathway of guaiacol production by species of *Alicyclobacillus* has not been fully investigated, it is generally hypothesised that guaiacol is produced by *Alicyclobacillus* spp. as a product during ferulic acid metabolism (Chang & Kang, 2004). Guaiacol is most probably produced by *Alicyclobacillus* spp. through the non-oxidative decarboxylation of vanillic acid, which is present in products as an oxidation product of vanillin, a derivative of ferulic acid. Ferulic acid is abundantly found in nature in fruits, vegetables, grains, beans, leaves, seeds, nuts, grasses and flowers (Rosazza *et al.*, 1995) and is also a component of the structural plant cell wall polymer, lignin. Ferulic acid cross-links lignin to plant cell wall polysaccharides (Provan *et al.*, 1994; Mathew & Abraham, 2004). The proposed metabolic pathway of guaiacol production from ferulic acid and further transformation of guaiacol by various micro-organisms is presented in Fig. 1.

The concentration of guaiacol precursors such as vanillin, vanillic acid, ferulic acid or even lignin in fruit juices could, therefore, influence guaiacol production by *Alicyclobacillus* spp. It is also important to characterise newly discovered strains of *Alicyclobacillus* with regards to their ability to grow in fruit juice and their guaiacol producing potential, to establish whether they pose a spoilage threat to fruit juice manufacturers. The aim of this study was to establish the influence of different vanillin concentrations in diluted white grape juice concentrate on the growth and

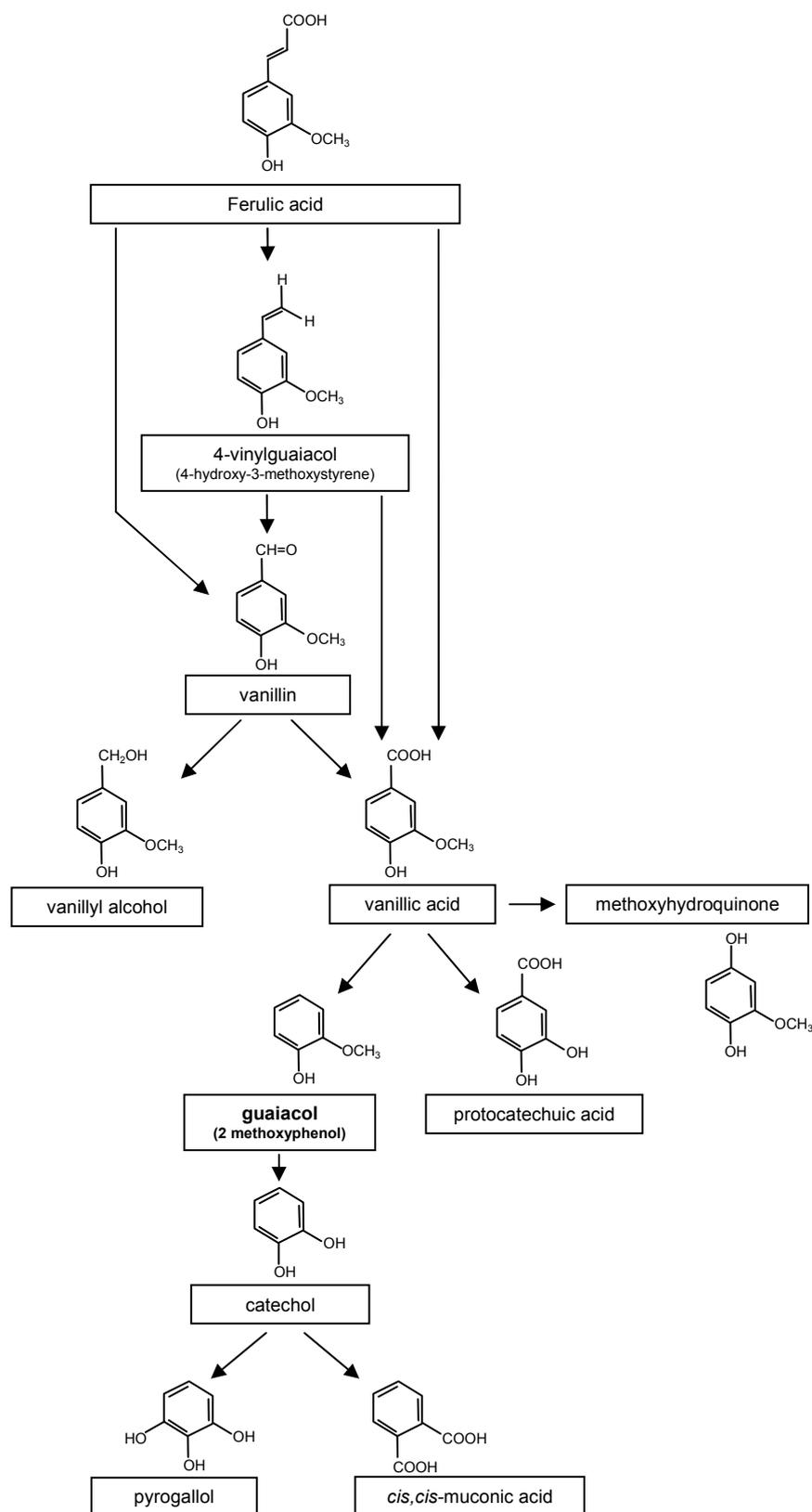


Figure 1 Microbial production pathways of guaiacol and other products through the metabolism of ferulic acid (Crawford & Olson, 1978; Pometto *et al.*, 1981; Rahouti *et al.*, 1989; Huang *et al.*, 1993; Rosazza *et al.*, 1995; Karmakar *et al.*, 2000; Topakas *et al.*, 2003).

guaiacol production of *A. acidoterrestris* FB2 isolated from the South African fruit processing environment.

MATERIALS AND METHODS

Bacterial strains

Alicyclobacillus acidoterrestris FB2 was isolated from fruit concentrate at a fruit processing plant in the Western Cape Province of South Africa (Groenewald *et al.*, 2009).

Bacterial inoculation cultures

An isolate of *A. acidoterrestris* FB2 was inoculated into yeast starch glucose (YSG) broth (Goto *et al.*, 2002) [2.00 g.L⁻¹ yeast extract (Biolab, supplied by Merck, Cape Town, South Africa), 1.00 g.L⁻¹ glucose (AnalAR, supplied by Merck) and 2.00 g.L⁻¹ soluble starch (Pro Analyti, supplied by Merck)] acidified to pH 4.00 with 1 M H₂SO₄ (AnalAR, supplied by Merck), and incubated at 45°C for 5 d.

A volume of 100 µL of this culture was spread onto *Bacillus acidoterrestris* (BAT) agar plates (IFU, 2007) and incubated at 45°C for 4 d. BAT agar was prepared by mixing equal volumes of BAT broth and a 3-4% (m/v) agar (Biolab, supplied by Merck) solution after autoclaving. BAT broth consists of 0.25 g.L⁻¹ CaCl₂·H₂O (Saarchem, supplied by Merck), 0.50 g.L⁻¹ MgSO₄·7H₂O (Saarchem, supplied by Merck), 0.20 g.L⁻¹ (NH₄)₂SO₄ (Pro Analyti, supplied by Merck), 3.00 g.L⁻¹ KH₂PO₄ (AnalAR, supplied by Merck), 2.00 g.L⁻¹ yeast extract (Biolab, supplied by Merck), 5.00 g.L⁻¹ glucose (AnalAR, supplied by Merck) and 1.00 mL trace element solution, consisting of 0.66 g.L⁻¹ CaCl₂·H₂O (Saarchem, supplied by Merck), 0.18 g.L⁻¹ ZnSO₄·7H₂O (Merck), 0.16 g.L⁻¹ CuSO₄·5H₂O (Merck), 0.15 g.L⁻¹ MnSO₄·H₂O (Merck), 0.18 g.L⁻¹ CoCl₂·5H₂O (Merck), 0.10 g.L⁻¹ H₃BO₃ (Merck) and 0.30 g.L⁻¹ Na₂MoO₄·2H₂O (Merck). The trace element solution was prepared separately and sterilised by autoclaving. The broth was adjusted to pH 4.00 using 1 M H₂SO₄ (AnalAR, supplied by Merck) and sterilised by autoclaving.

Colonies from the BAT agar plates were aseptically removed and streaked onto fresh BAT agar plates, followed by incubation at 45°C for 4 d, to ensure a pure culture. A colony was aseptically removed from one of the BAT agar plates and

inoculated into YSG broth acidified to pH 4.00 with 1 M H₂SO₄ (AnalAR, supplied by Merck). After incubation at 45°C for 4-5 d the approximate cell concentration was determined by measuring the optical density (OD) at 540 nm. The cell concentration of the inoculum was also confirmed by preparing serial dilutions (10⁰-10⁻⁶) of the culture in sterile saline solution (SSS) [0.85% (m/v) NaCl (Merck)] and plating out in duplicate using the pour plate technique on PDA (Biolab, supplied by Merck) acidified to pH 4.00 after autoclaving with 1 M H₂SO₄ (AnalAR, supplied by Merck), followed by incubation at 45°C for 4 d.

Influence of different vanillin concentrations on growth and guaiacol production

White grape juice concentrate obtained from a fruit processing plant in the Western Cape province of South Africa was diluted 1:10 with distilled water containing either 0, 10, 50, 100, 250 or 500 mg.L⁻¹ vanillin (Merck) to 8.50°Brix. The pH of all the juice samples was adjusted to pH 4.00 using either 1 M H₂SO₄ or 1 M NaOH, followed by autoclaving to sterilise the fruit juice. Three hundred ml of each of the media containing the different vanillin concentrations was inoculated with *A. acidoterrestris* FB2 to a final cell concentration of approximately 10³ cfu.ml⁻¹. Cultures were incubated at 45°C for 6 d and samples were analysed every 24 h. The OD, pH, cell concentration and guaiacol concentration of the samples was measured. The guaiacol concentration was measured using the peroxidase enzyme colourimetric assay (PECA) and the experiment was performed in triplicate.

OD

One ml of the sample was used to measure the OD at 540 nm using a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA).

pH

Four ml of the sample was used to determine the pH using a Mettler Toledo 320 pH meter (Mettler-Toledo Ltd., Leicester, England).

Cell concentration

The cell concentration was measured by serially diluting the sample (10^0 - 10^{-6}) in SSS and using the pour plate technique with PDA (Biolab, supplied by Merck) adjusted to pH 4.00 after autoclaving with 1 M H_2SO_4 (AnalAR, supplied by Merck). Plates were poured in duplicate and incubated at 45°C for 4 d.

Peroxidase enzyme colourimetric assay (PECA)

The guaiacol concentration was determined using the PECA as described by Sheu and Chen (1991) and Bahçeci and Acar (2007). One ml sample was centrifuged for 10 min at 2300 g using an Eppendorf 5415 D centrifuge (Hamburg, Germany). A volume of 300 μ L of the supernatant of the centrifuged sample was vortexed with 2.00 ml 0.20 M potassium phosphate buffer [$4.18 \text{ g.L}^{-1} K_2HPO_4$ (Merck), $23.95 \text{ g.L}^{-1} KH_2PO_4$ (Merck), calculated using the Henderson-Hasselbalch equation (Segel, 1976) adjusted to pH 6.00 with 1 M H_2SO_4 (AnalAR, supplied by Merck) or 1 M NaOH (AnalAR, supplied by Merck)], 300 μ L (5 U) peroxidase enzyme (Merck) and 300 μ L 0.5% (m/v) H_2O_2 (AnalAR, supplied by Merck). A blank was prepared in the same way using 300 μ L potassium phosphate buffer instead of the sample. Colour changes were detected by measuring the OD at 420 nm using a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA). The guaiacol concentrations were quantified using a standard curve of OD versus guaiacol (Sigma-Aldrich, USA) concentrations.

Statistical analysis

The effect of different vanillin concentrations over time on the various measurements was analysed using repeated measures ANOVA. The Vepac (mixed) module of Statistica 8 was used to do the analyses. A 5% significance level was used as guideline for indicating significant results.

RESULTS AND DISCUSSION

Influence of different vanillin concentrations on growth and guaiacol production

The change in absorbance followed a similar pattern for all the inoculated juice

samples containing different vanillin concentrations with the exception of the sample containing 500 mg.L⁻¹ vanillin, which had a lag phase of 3 d compared to the absorbances of the other samples that showed a lag phase of only 2 d (Fig. 2). A maximum absorbance of 0.40 was reached after 6 d by the sample containing 0 mg.L⁻¹ vanillin, while the samples containing 10, 50, 100, 250 and 500 mg.L⁻¹ vanillin reached maximum absorbances of 0.33, 0.25, 0.23, 0.29 and 0.32, respectively, after 6 d. The OD of the control samples (containing uninoculated white grape juice concentrate diluted 1:10 with water containing 100 mg.L⁻¹ vanillin) increased slightly over the 6 d period due to slight darkening of the juice in the incubator at 45°C. From these results no clear conclusion can be drawn regarding the correlation between the absorbance and vanillin concentration, except that the presence of vanillin is able to inhibit growth, as the sample containing no vanillin was able to multiply to a higher OD measurement than the samples containing vanillin. This conclusion is also strengthened by the longer lag phase of growth and, therefore, greater growth inhibition observed in the sample containing the highest (500 mg.L⁻¹) vanillin concentration.

All the samples showed similar cell growth patterns (Fig. 3). The samples containing 0-250 mg.L⁻¹ vanillin have a lag phase of growth of 1 d, followed by an exponential growth phase and a stabilisation around maximum cell concentrations ranging from 10⁷-10⁸ cfu.ml⁻¹ after 3 d. A longer lag phase of growth (2 d) is also observed in the cell concentration of the sample containing 500 mg.L⁻¹ vanillin and a maximum cell concentration of 10⁷ cfu.ml⁻¹ was only reached after 5 d. Therefore, there may be a concentration where vanillin becomes inhibitory to cell growth. Bahçeci and Acar (2007) reported that a vanillin concentration of 1000 mg.L⁻¹ had an inhibitory effect on cell growth and guaiacol formation. In agreement with the observations in this study, in the study by Bahçeci and Acar (2007) the inhibitory effect of vanillin was also observed at a vanillin concentration of 500 mg.L⁻¹, as the guaiacol concentration was two times higher after 24 h in a sample containing 100 mg.L⁻¹ compared to 500 mg.L⁻¹ vanillin (Bahçeci & Acar, 2007). The inhibitory effect of vanillin has also been observed in other bacteria (Cerrutti *et al.*, 1997; Ferrante *et al.*, 2007; Char *et al.*, 2009), yeasts (Cerrutti *et al.*, 1997; Fitzgerald *et al.*, 2003) and fungi (López-Malo *et al.*, 1998). The rapid metabolism of vanillin formed as an intermediate in the metabolic pathway was also attributed to its toxicity (Ander *et al.*, 1980; Rahouti *et al.*, 1986).

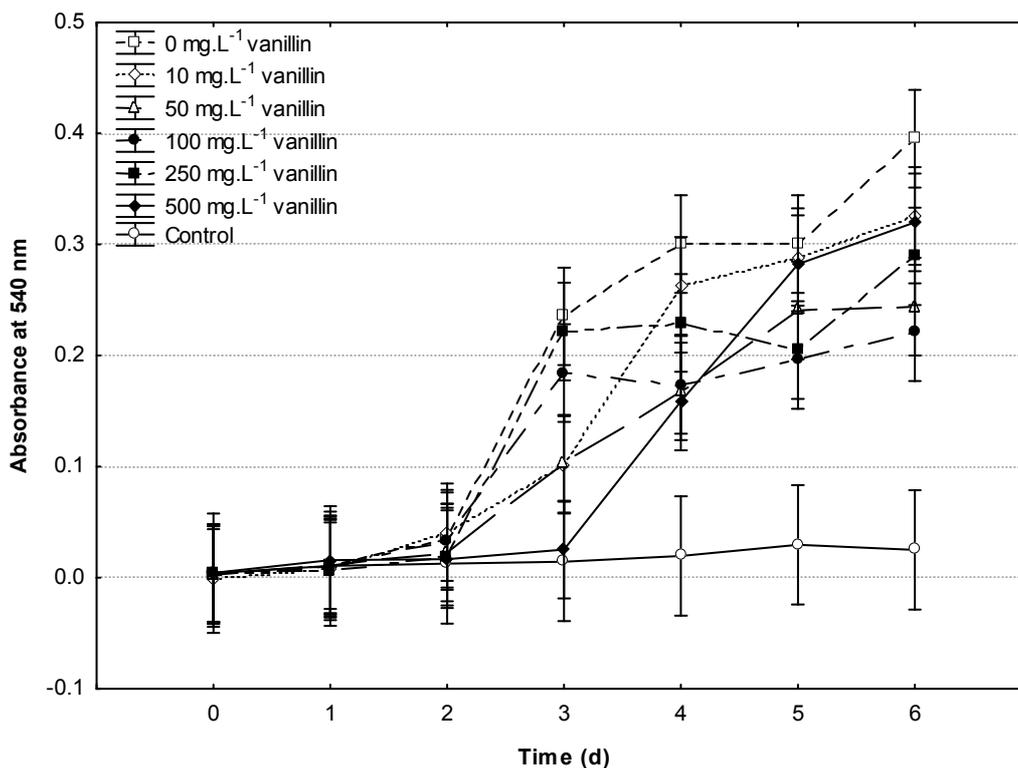


Figure 2 Change in absorbance of *A. acidoterrestris* FB2 at different vanillin concentrations at an incubation temperature of 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

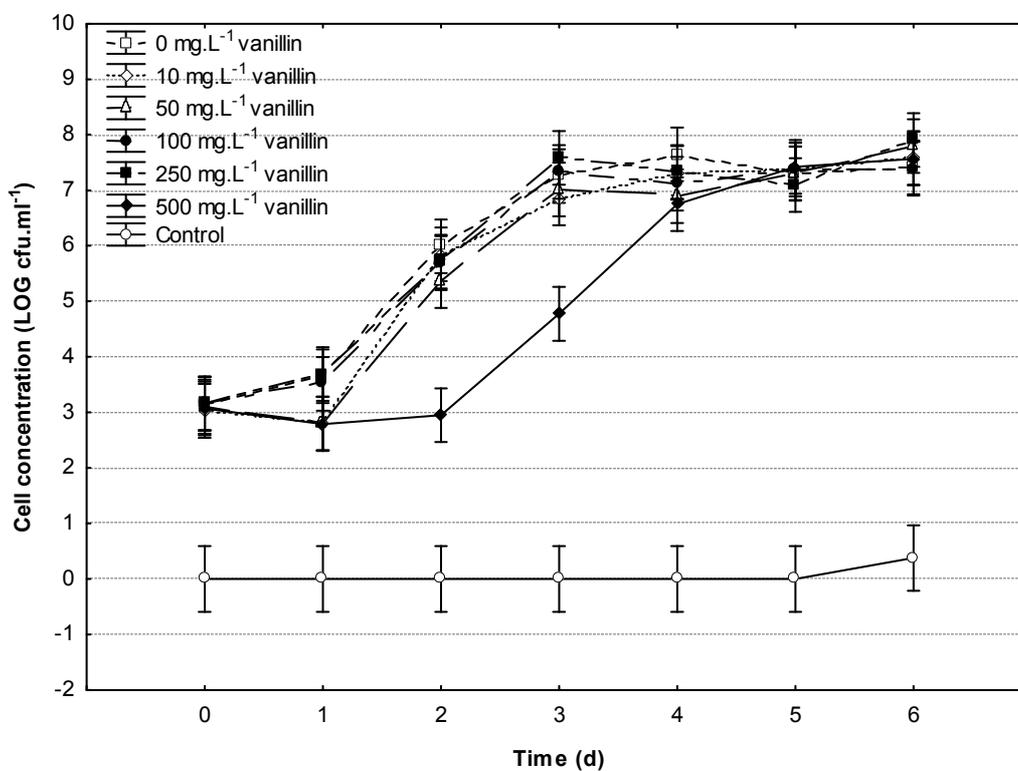


Figure 3 Change in cell concentrations of *A. acidoterrestris* FB2 at different vanillin concentrations at an incubation temperature of 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

Guaiacol concentrations, after an initial lag phase of 2 d for the samples containing 50, 100 and 250 mg.L⁻¹ vanillin and 3 d for the sample containing 500 mg.L⁻¹ vanillin, increased steadily to maximums of 0.14, 0.22, 0.36 and 0.27 mM after 6 d in the samples containing 50, 100, 250 and 500 mg.L⁻¹ vanillin, respectively (Fig. 4). No guaiacol was detected in the control (from uninoculated white grape juice concentrate diluted 1:10 with water containing 100 mg.L⁻¹ vanillin) or the sample containing 0 mg.L⁻¹ vanillin. Although guaiacol was detected and the concentration increased slightly from day 0 to day 4 in the sample containing 10 mg.L⁻¹ vanillin, the changes in guaiacol concentration did not differ significantly ($p \leq 0.05$) from the sample containing 0 mg.L⁻¹ vanillin or the control, where no significant change in guaiacol concentration was observed over the 6 d. Bahçeci *et al.* (2005) observed that guaiacol was produced in amounts higher than their control in samples containing 10 mg.L⁻¹ vanillin and incubated at 46°C. The maximum guaiacol concentration produced in these samples was 0.064 mM, where in the present study the maximum guaiacol concentration reached in the sample containing 10 mg.L⁻¹ vanillin was determined at 0.040 mM, which is in the same range as the value observed in the study by Bahçeci *et al.* (2005).

There was a clear correlation between the vanillin concentration and the guaiacol concentrations produced by *A. acidoterrestris* FB2 (Fig. 4). Guaiacol production was dependent on the vanillin concentration, as there was a clear increase in guaiacol concentration as the vanillin concentration increased from 0-500 mg.L⁻¹. However, the sample containing 500 mg.L⁻¹ vanillin was an exception, as less guaiacol was detected in this sample than in the sample containing 250 mg.L⁻¹ vanillin. Previous studies have found that a critical cell concentration ranging from 10⁴-10⁵ cfu.mL⁻¹ has to be present before guaiacol is detected (Pettipher *et al.*, 1997; Bahçeci *et al.*, 2005). Since the higher vanillin concentration in the sample containing 500 mg.L⁻¹ vanillin initially inhibited cell growth and, therefore, increased the lag phase of growth (Fig. 3), it took longer for the critical cell concentration to be reached, causing a lag in guaiacol production and resulting in less guaiacol being produced compared to the sample containing 250 mg.L⁻¹ vanillin. A higher vanillin concentration, therefore, may not always lead to higher guaiacol concentrations. Above a certain threshold concentration the vanillin starts to inhibit cell growth, leading to lower detectable guaiacol production.

The change in pH varied between the different samples (Fig. 5). The pH

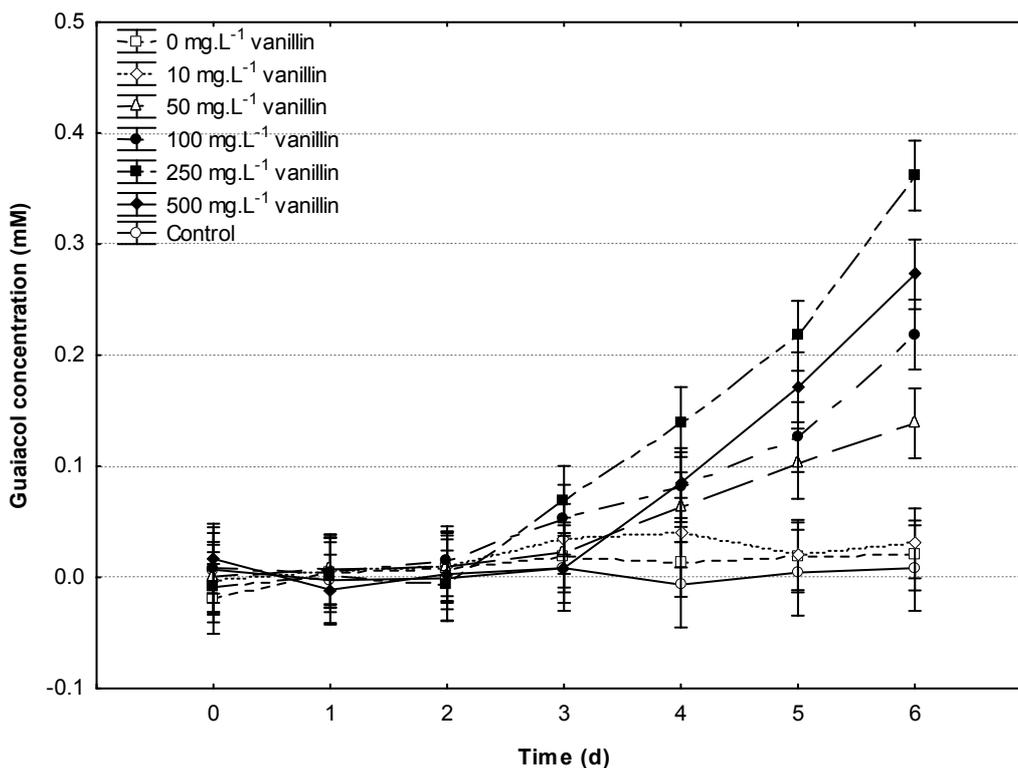


Figure 4 Guaiacol concentrations detected with the PECA at different vanillin concentrations in *A. acidoterrestris* FB2 at an incubation temperature of 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

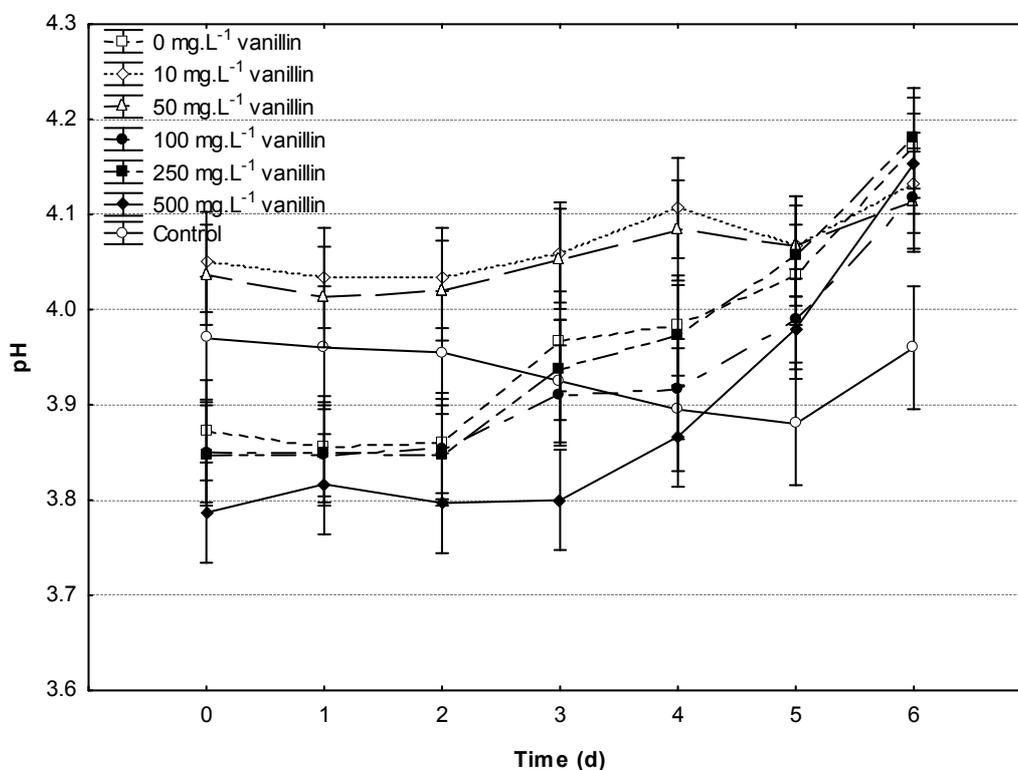


Figure 5 Change in pH of *A. acidoterrestris* FB2 at different vanillin concentrations at an incubation temperature of 45°C over 6 d. Vertical bars denote 0.95 confidence intervals.

values of all the samples were adjusted to pH 4.00 before autoclaving. Autoclaving seemed to have a slight influence on the pH of the growth medium, as pH values were slightly below or above pH 4.00 after autoclaving. However, the change was not more than 0.20 pH units either way. The pH of the samples containing 0, 100 and 250 mg.L⁻¹ vanillin remained constant from day 0 to day 2 at pH values ranging between 3.80 and 3.90, followed by an increase from day 2 to day 6 to end pH values ranging between 4.10 and 4.20. The sample containing 500 mg.L⁻¹ vanillin followed the same pattern, except that the initial pH remained constant until day 3 before an increase was observed. The samples containing 10 and 50 mg.L⁻¹ vanillin showed a slight increase from the initial pH values between 4.00 and 4.10 to end pH values of just above 4.10. The pH of the control sample (from uninoculated white grape juice concentrate diluted 1:10 with water containing 100 mg.L⁻¹ vanillin) decreased from pH 4.00 to pH 3.90 from day 0 to day 5, followed by an increase to approximately pH 3.95 at day 6. The confidence intervals are large, indicating much variation in the measurements. However, overall the pH tends to increase with cell growth and guaiacol production. This observation is supported by Jensen (2000) who also observed an increase in medium pH during the growth of *A. acidoterrestris*.

CONCLUSION

Species of *Alicyclobacillus* have been reported to be fastidious with regards to their growth medium and conditions, as *Alicyclobacillus* species do not necessarily grow in all products and growth will not always lead to guaiacol production (Splittstoesser *et al.*, 1994; Pettipher *et al.*, 1997; Pinhatti *et al.*, 1997; Splittstoesser *et al.*, 1998; Walls & Chuyate, 2000). The composition of the growth medium and the concentration of guaiacol precursors do influence the production of guaiacol by *A. acidoterrestris*.

This study has confirmed the potential of a strain of *A. acidoterrestris* isolated from fruit juice concentrate to act as a spoilage organism in fruit juice, as this strain was able to grow to high cell densities in diluted white grape juice concentrate and produced detectable guaiacol concentrations from vanillin concentrations as low as 10 mg.L⁻¹. Although vanillin was used as the guaiacol precursor in this study, it is unlikely that vanillin or vanillic acid, the immediate guaiacol precursors in the proposed pathway of guaiacol production, would be naturally present in fruit juice products in concentrations high enough to result in detectable guaiacol production.

The toxicity of vanillin above a certain critical concentration makes this even more unlikely. Rather, it would be the metabolism of ferulic acid by *Alicyclobacillus* spp. which would lead to guaiacol being formed. Therefore, when attempting to determine whether a product would be susceptible to spoilage through guaiacol production by *Alicyclobacillus* spp., one should investigate not only the concentration of immediate precursors, such as vanillin and vanillic acid, but also other substrates such as lignin or ferulic acid.

Other factors may also play a role in whether or not guaiacol is produced in amounts high enough to cause spoilage. The composition of juices plays a significant role, as some juices such as red grape juice have been shown not to support the growth of *Alicyclobacillus* spp. (Splittstoesser *et al.*, 1998). Another approach to prevent guaiacol production and product spoilage could be to control the pH of juices so as to render conditions unfavourable for growth and guaiacol production.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Spoilage of fruit juice and other acidic products by species of *Alicyclobacillus* has become a growing concern in the fruit processing industry worldwide. Although spoilage incidents seem to be coupled to specific circumstances, for example high environmental temperatures or products left to cool for long time periods at ambient temperatures (Cerny *et al.*, 1984; Walls & Chuyate, 1998), spoilage and product recalls can lead to massive financial losses, as well as loss of consumer confidence.

An effective and accurate method for the isolation of *Alicyclobacillus* spp. from products is essential for efficient quality control. The International Federation of Fruit Juice Producers (IFU) Method No. 12 was identified in this study as the most effective method for the isolation of *Alicyclobacillus* spp. when compared to other frequently used methods. These results support the suggestion of the IFU for the use of the IFU Method No. 12 as the standard international method for the isolation and detection of *Alicyclobacillus* spp. from acidic products.

The research results presented in this thesis have shown that a variety of areas in the fruit processing environment can act as possible sources of taint-producing *Alicyclobacillus* spp. *Alicyclobacillus acidoterrestris* strains, isolated from fruit concentrate, wash water, flume water and evaporator water, and an *A. acidocaldarius* strain, isolated from vinegar flies, were all able to produce guaiacol in concentrations high enough to cause product spoilage. Good manufacturing practices should, therefore, be employed in order to minimise contamination of products through these sources.

The presence of *Alicyclobacillus* spp. in fruit products will, however, not always lead to spoilage (Pettipher *et al.*, 1997; Pinhatti *et al.*, 1997), which is why a demand for complete elimination of these bacteria from the processing environment is impractical. Guaiacol production and spoilage is subject to a number of conditions. This study found that detectable guaiacol concentrations are only produced once a cell concentration of 10^4 - 10^6 cfu.mL⁻¹ is present, an observation that has also been made by other researchers (Pettipher *et al.*, 1997; Bahçeci *et al.*, 2005). The presence or even growth of *Alicyclobacillus* spores in food products will, therefore,

not necessarily result in detection of spoilage compounds. This is in agreement with a study where a large number of commercial fruit juices and concentrates were analysed for the presence of *Alicyclobacillus* spp. Species of *Alicyclobacillus* were present in nearly all of the products, in some products in concentrations as high as 10^3 cfu.mL⁻¹, but none of the products were spoiled (Pinhatti *et al.*, 1997). Therefore, if populations of *Alicyclobacillus* can be kept below 10^4 cfu.mL⁻¹, either through good manufacturing practices, storage at low temperatures or other control measures, it may be possible to prevent the production of taint compounds and product spoilage.

Certain guaiacol precursors, such as vanillin, vanillic acid or ferulic acid, needs to be present for guaiacol production to occur. In this study it was determined that vanillin must be present in concentrations higher than 10 mg.L⁻¹ for detectable guaiacol production to occur in white grape juice. No guaiacol was produced in white grape juice containing 0 mg.L⁻¹ vanillin that was inoculated with *A. acidoterrestris*, confirming that the presence and even growth of *Alicyclobacillus* spp. to cell concentrations high enough to produce guaiacol will not always lead to spoilage. The presence of these precursors in sufficient amounts may be a contributing factor to the higher susceptibility of certain juices to spoilage by *Alicyclobacillus* spp. If these compounds can be removed from or minimised in products it could also contribute to preventing product spoilage.

Alicyclobacillus species are fastidious with regards to their growth requirements, as they will only grow on certain agars and in certain juices, even if the pH is favourable for growth (Splittstoesser *et al.*, 1994; Pettipher *et al.*, 1997; Splittstoesser *et al.*, 1998; Walls & Chuyate, 2000). Growth of these bacteria will not even necessarily always occur in the same type of juice. Even though product spoilage incidents have been the most frequent in apple juice, (Pettipher *et al.*, 1997; Splittstoesser *et al.*, 1998; Cerny *et al.*, 2000, Jensen, 2000; Bahçeci *et al.*, 2005) a study reported the inability of *A. acidoterrestris* to grow in 100% apple juice, although it was able to grow when the apple juice was diluted to 30% (Oita, 2002). Even in some preliminary studies conducted in our laboratory it was found that out of five commercial, unpreserved, shelf-stable apple juices, *A. acidoterrestris* only grew in one type of juice, and only occasionally (data not shown). Therefore, there seems to be a combination of factors which must all be favourable before *Alicyclobacillus* spp. growth occurs. Some of these factors include storage temperature, pH of the juice and specific juice constituents which can influence growth.

The specific strain of *Alicyclobacillus* that is present also plays a role in spoilage. Most of the strains of *A. acidoterrestris* used in this study accumulated guaiacol in the medium, but one strain was also identified that produced guaiacol more rapidly. Furthermore, not all the species of the genus *Alicyclobacillus* are able to produce spoilage compounds and, therefore, the identification of *Alicyclobacillus* spores found in products during quality control procedures need to be done to species level or their guaiacol producing ability needs to be determined in order to establish whether they pose a spoilage threat. Species other than *A. acidoterrestris* that have been found to be able to produce guaiacol and/or halophenols include *A. acidiphilus* (Matsubara *et al.*, 2002; Goto *et al.*, 2008), *A. hesperidum* (Goto *et al.*, 2008), *A. herbarius* (Goto *et al.*, 2008) and *A. cycloheptanicus* (Gocmen *et al.*, 2005). Species that have been implicated as possible spoilage species due to their isolation from spoiled products include *A. acidocaldarius* (Gouws *et al.*, 2005) and *A. pomorum* (Goto *et al.*, 2003). *Alicyclobacillus acidocaldarius*, previously thought to be a non-guaiacol producing species, was shown to be able to produce guaiacol in this study. This observation warrants further investigation as this species has also been shown to be able to produce other spoilage compounds (Lottici *et al.*, 2006).

Alicyclobacillus acidoterrestris, the species most often associated with spoilage incidents, is able to grow and produce guaiacol in concentrations high enough to cause spoilage even at 25°C. It is possible for certain strains of *A. acidoterrestris* to produce equal or even higher concentrations of guaiacol at 25°C than at 45°C, the optimum growth temperature for this species. Therefore, as other studies have also found (Pettipher *et al.*, 1997; Jensen & Whitfield, 2003; Siegmund & Pöllinger-Zierler, 2007), storing products at room temperature (normally 20°-25°C) will not prevent growth of and guaiacol production by *A. acidoterrestris*. It is, therefore, recommended that products be stored below 20°C, preferably at 4°C. Cooling of shelf-stable products traditionally stored at ambient temperatures will, however, present a major cost factor that will have to be considered by manufacturers.

Concluding remarks

Spoilage incidents caused by *Alicyclobacillus* species have challenged the quality and safety of acidic products, because of the ability of these thermo-acidophilic

spore-formers to survive the traditional pasteurisation processes applied to fruit products and subsequently cause spoilage. This study has identified the IFU Method No. 12 as the most effective method for the isolation and detection of *Alicyclobacillus* spp. South African strains of *A. acidoterrestris* and *A. acidocaldarius* have also been characterised with regards to their growth characteristics and spoilage potential. These strains were all able to produce guaiacol, even at 25°C in the case of the *A. acidoterrestris* strains. The significant difference observed between the sensitivity of the peroxidase enzyme colourimetric assay (PECA) and headspace gas-chromatography mass-spectrometry (HS GC-MS) based on the detection of guaiacol suggests that a distinction should be made between the specific uses of the respective guaiacol detection methods. It is suggested that the PECA be used for presence/absence determination of guaiacol, while HS GC-MS be used where quantification of guaiacol is necessary.

Since it would be difficult to totally eliminate *Alicyclobacillus* spp. from the fruit processing environment the focus should rather, as suggested by the *Alicyclobacillus* Best Practice Guideline compiled by the European Fruit Juice Association (AIJN) (AIJN, 2008), be placed on the effective management of a fruit processing facility according to hazard analysis critical control point (HACCP) principles in order to either prevent these bacteria from entering the process or minimising their opportunities to grow. A proactive approach such as this will ensure that spoilage incidents by *Alicyclobacillus* spp. are minimised or possibly even eliminated.

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