IDENTIFICATION OF PRECURSORS PRESENT IN FRUIT JUICE THAT LEAD TO THE PRODUCTION OF GUAIACOL BY *ALICYCLOBACILLUS ACIDOTERRESTRIS*

ENETTE VAN DER MERWE

Thesis presented in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN FOOD SCIENCE

at the Stellenbosch University

Department of Food Science
Faculty of AgriSciences

Study leader: Prof. R.C. Witthuhn
Co-study leader: Prof. P. Venter
Dr. M. Cameron

March 2011

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

_____ _____

Enette van der Merwe Date

ABSTRACT

Alicyclobacilli are endospore-forming, thermophilic, acidophilic bacteria (TAB) that survive the pasteurisation process and spoil acidic fruit juices through the production of the taint compound guaiacol. Guaiacol causes an undesirable odour with an unpleasant smoky, medicinal or phenolic-like taste. This thesis reports on the precursors, vanillin and vanillic acid metabolised to guaiacol by *Alicyclobacillus* spp. in fruit juice, the pathway of guaiacol production and the spoilage potential of contaminated fruit juices supplemented with these precursors.

A high performance liquid chromatography method with UV-diode array detection (HPLC-DAD) was developed for the simultaneous detection and quantification of guaiacol and its precursors. *Alicyclobacillus acidoterrestris* FB2 was incubated at 45 °C for 7 d in *Bacillus acidoterrestris* (BAT) broth supplemented with ferulic acid, vanillin or vanillic acid. The cell concentrations were determined every 24 h and the concentration of the precursors and the production of guaiacol was determined using HPLC-DAD. The guaiacol production was also determined using the peroxidase enzyme colourimetric assay (PECA). *Alicyclobacillus acidoterrestris* produced guaiacol from vanillin and vanillic acid, confirming both vanillin and vanillic acid as precursors for guaiacol production by *A. acidoterrestris* FB2. Furthermore, a metabolic pathway directly from vanillin to guaiacol was identified in this study. However, guaiacol was not produced by *A. acidoterrestris* FB2 in the samples supplemented with ferulic acid and it is, therefore, not considered a direct precursor for guaiacol production by *A. acidoterrestris*.

The spoilage potential of apple juice supplemented with either 10 mg L^{-1} or 100 mg L^{-1} vanillin or vanillic acid by *A. acidoterrestris* FB2 (10^6 cfu mL^{-1}) was also evaluated. The production of guaiacol increased with the increase in vanillin or vanillic acid concentrations (in BAT broth and apple juice) indicating that the concentration of vanillin and vanillic acid present in fruit juice will influence the spoilage potential of the juice. Guaiacol concentrations in apple juice well above the best estimated threshold value of guaiacol for taste (0.24 – 2.00 µg L^{-1}) and odour (0.50 - 2.32 µg L^{-1}) was produced by *A. acidoterrestris* FB2 in the apple juice supplemented with 10 mg L^{-1} vanillin or vanillic acid. This indicates that fruit juice with a vanillin or vanillic acid concentration as low as 10 mg L^{-1} has the potential to spoil if the juice is contaminated with *A. acidoterrestris.*

The concentrations of vanillin and vanillic acid in different fruit juices can be used to indicate if a specific fruit juice is susceptible to guaiacol spoilage by *Alicyclobacillus* spp. In the development of juice products and different blends of fruit juices, special care must be taken not to concentrate the amount of vanillin and vanillic acid present in the fruit juices.

UITTREKSEL

Alicyclobacilli is endospoor-vormende, termofiliese, asidofiliese bacterie (TAB) wat die pasteurisasie proses oorleef en suur vrugtesappe bederf met die produksie van ‘n taint komponent guaiakol. Guaiakol veroorsaak ‘n ongewensde reuk en onaangename rookagtige, medisinale of fenoliese smaak. Hierdie tesis doen verslag oor die voorloper komponente, vanillien en vanilliensuur in vrugtesappe wat gemetaboliseer word na guaiakol deur *Alicyclobacillus* spesies, die padweg van guaiakol produksie en die bederfbaarheid van gekontamineerde vrugtesap aangevul met hierdie voorloper komponente.

‘n Hoëprestasie vloeistof chromatografie metode met UV-deteksie (skanderend) (HPVC) is ontwikkel vir die gesamentlike deteksie en kwantifisering van guaiakol en die voorloper komponente. *Alicyclobacillus acidoterrestris* FB2 is geïnkubeer by 45 °C vir 7 d in *Bacillus acidoterrestris* (BAT) medium aangevul met feruliensuur, vanillien of vanilliensuur. Die sel konsentrasies is elke 24 h bepaal en die aangevulde komponente en die geproduseerde guaiakol is bepaal deur van HPVC gebruik te maak. Die guaiakol konsentrasies is ook bepaal deur van die peroksidase ensiem kolorimetriese bepaling (PEKB) gebruik te maak. *Alicyclobacillus acidoterrestris* het guaiakol geproduseer vanaf vanillien en vanilliensuur, dus is beide vanillien en vanilliensuur bevestig as voorlopers van guaiakol produksie deur *A. acidoterrestris* FB2. A padweg direk van vanillien na guaiakol is in hierdie studie geïdentifiseer. Guaiakol is nie geproduseer deur *A. acidoterrestris* FB2 in the monsters wat met feruliensuur aangevul is nie en feruliensuur is dus nie ‘n direkte voorloper van guaiakol produksie deur *A. acidoterrestris*.

Die bederf potensiaal van appelsap aangevul met 10 mg L^{-1} of 100 mg L^{-1} vanillien of vanilliensuur deur *A. acidoterrestris* (10^6 kve mL^{-1}) is ook geëvalueer. Die produksie van guaiakol het toegeneem met die toename in vanillien of vanilliensuur konsentrasies (in beide BAT en appelsap) wat aandui dat die konsentrasie vanillien en vanilliensuur teenwoordig in vrugtesap die bederfbaarheid van die sap sal beïvloed. Guaiakol konsentrasies in appelsap hoog bo die drumpel waardes van guaiacol vir smaak (0.24 – 2.00 µg L^{-1}) en reuk (0.50 - 2.32 µg L^{-1}) is geproduseer deur *A. acidoterrestris* FB2 in die appelsap monsters aangevul met 10 mg L^{-1} vanillien of vanilliensuur. Hierdie verskynsel dui aan dat vrugtesap met ‘n vanillien of vanilliensuur konsentrasies van so laag as 10 mg L^{-1} die potensiaal het om te bederf indien die sap gekontamineer is met *A. acidoterrestris.*

Die konsentrasies van vanillien en vanilliensuur in verskillende vrugtesappe kan gebruik word om aan te dui of ‘n spesifieke vrugtesap ‘n hoë risiko het vir guaiakol bederf deur *Alicyclobacillus* spesies. Tydens die ontwikkeling van vrugtesap produkte en verskillende mengsels van vrugtesappe moet seker gemaak word dat die hoeveelhede vanillien en vanilliensuur in die sappe nie gekonsentreer word nie.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people and institutions for their valuable contributions to the successful completion of this research:

Prof. Corli Witthuhn, study leader, for her help, guidance, expert advice and support during the course of my studies;

Prof. Pierre Venter, co-study leader, for his guidance and expert advice and for always making me feel welcome during my visits to the Central University of Technology (CUT) in Bloemfontein;

Dr. Michelle Cameron, co-study leader, for her advice and guidance throughout the completion of this study and thesis;

National Research Foundation (NRF) for financial support of this research and a Scare skills bursary (2009-2010) (*Any opinion, findings and conclusions or recommendations expressed in this material are those of the author and therefore the NRF does not accept any liability in regard thereto*), the Consumer Goods Council South Africa (CGCSA) (FoodBev SETA Bursary 2009) and the South African Association for Food Science and Technology (SAAFoST) (FoodBev SETA Bursary 2010) for financial support;

Professor Martin Kidd, for statistical analyses and help with regards to statistical interpretations;

The staff and my fellow post-graduate students at the Department of Food Science for their help, support and friendship;

Yvette Smit for her help and advice, as well as Amy Strydom, Ingrid Bester, Cato' Steyn and Donna Cawthorn from the Molecular Food Microbiology Laboratory at the Department of Food Science for their support and friendship;

Mareanna van der Merwe, my mother, for her unconditional love, support, encouragement, inspiration and friendship;

Christiaan Adlem for his love, support, patience and encouragement;

My brothers, sister-in-law, family and friends for their love and support; and

My Lord and Saviour, Jesus Christ who made this all possible, for giving me the strength and guidance to successfully complete my studies.

Dedicated to my mother
for giving me the opportunity
and all the love in the world

CONTENTS

Chapter		Page
	Declaration	ii
	Abstract	iii
	Uittreksel	v
	Acknowledgements	vii
Chapter 1	Introduction	1
Chapter 2	Literature review	6
Chapter 3	Separation and quantification of guaiacol, vanillic acid, vanillin and ferulic acid with HPLC	46
Chapter 4	Guaiacol production from ferulic acid, vanillin and vanillic acid by *Alicyclobacillus acidoterrestris*	61
Chapter 5	Production of guaiacol by *Alicyclobacillus acidoterrestris* in apple juice from the precursors vanillin and vanillic acid	90
Chapter 6	General discussion and conclusions	110

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

Fruit juice and related raw material stored in shelf-stable packaging at room temperature is generally preserved by a combination of heat treatment and low pH. The low pH of fruit juice do not support the germination of most bacterial endospores (Blocher & Busta, 1983) and hot-fill and hold pasteurisation is deemed sufficient to destroy non-endospore-forming microbes yeasts, mycelial fungi and lactic acid bacteria (Eiora *et al*., 1999; Vieira *t al.,* 2002) to render the product commercially sterile (Pontius *et al.,* 1998; Silva *et al.,* 2000; Vieira *et al.,* 2002; Walker & Phillips, 2008). However, since the isolation of *Alicyclobacillus* spp. in fruit juice products, the natural low pH of fruit juices, as well as the pasteurisation process has been reported to be inadequate for the prevention of spoilage caused by this thermophilic, acidophilic bacterium (Splittstoesser *et al.,* 1994; Pettipher *et al.,* 1997; Walls & Chuyate, 1998). *Alicyclobacillus* endospores survive the pasteurisation process and germinate, grow and spoil these products at a pH formerly considered to be below the range that supported the growth of endospore-forming bacteria (Walls & Chuyate, 1998).

Alicyclobacillus was first identified in aseptically packed apple juice in Germany in 1982 (Cerny *et al.,* 1984). Since then and with more frequent isolation of *Alicyclobacillus* as the spoilage bacterium from fruit juices, it has become a major concern to the beverage industry (Duong & Jensen, 2000; Durak *et al.,* 2010). Spoilage by species of *Alicyclobacillus* are difficult to detect and can occur without visible changes (Walls & Chuyate, 1998; Walker & Phillips, 2005). The formation of a medicinal or phenolic off-flavour is the only obvious indication of spoilage (Walls & Chuyate, 1998; Silva *et al.,* 1999; Pettipher & Osmundson, 2000). The off-flavours that are associated with the spoilage of food products caused by *Alicyclobacillus* spp. can either be due to the production of guaiacol (Pettipher *et al.,* 1997; Splittstoesser *et al.,* 1998) or the halophenols, 2,6-dibromophenol (2,6-DBP) and 2,6-dichlorophenol (2,6-DCP) (Borlinghaus & Engel, 1997). However, guaiacol is accepted as the predominant metabolite associated with taint production in fruit juices (Pettiper *et al.,* 1997; Jensen, 2000; Jensen & Whitfield, 2003). Guaiacol causes an undesirable odour in fruit juice resulting in an unpleasant smoky, medicinal or phenolic-like taint (Pettipher *et al.,* 1997; Jensen, 2000).

A number of factors affect *Alicyclobacillus* spoilage of fruit juice. These include the species of *Alicyclobacillus* present, cell concentration of *Alicyclobacillus*, storage

temperature, the pH and juice composition (Splittstoesser *et al*., 1994; Lusardi *et al.,* 2000; Chang & Kang, 2004). *Alicyclobacillus acidoterrestris* is primarily responsible for the production of guaiacol (Yamazaki *et al.*, 1996; Pettipher *et al.,* 1997), although other species including *A. acidiphilus* (Matsubara *et al.,* 2002; Goto *et al.,* 2008), *A. acidocaldarius* (Smit, 2009), *A. cycloheptanicus* (Gocmen *et al.,* 2005), *A. herbarius* (Goto *et al.,* 2008) and *A. hesperidum* (Gocmen *et al.,* 2005; Goto *et al.,* 2008) also have the ability to produce guaiacol. The availability of precursors for guaiacol production is also an important factor for spoilage by *Alicyclobacillus* spp. to occur. The exact metabolic pathway of guaiacol production by *Alicyclobacillus* has not been fully investigated, however, it is currently believed that guaiacol is produced as a product of ferulic acid metabolism (Jensen, 2000; Niwa & Kawamoto, 2003; Chang & Kang, 2004; Bahçeci *et al*., 2005).

There are currently no preservation techniques available to ensure the complete elimination of *Alicyclobacillus* spp. from the fruit possessing environment or fruit juice products. However, numerous factors need to be favourable for *Alicyclobacillus* spp. to produce guaiacol and the elimination of one of the factors can possibly prevent or limit the spoilage of fruit juice. Little information exists on the metabolic pathway of *Alicyclobacillus* and the substrates, specifically in fruit juice, that are required for guaiacol production. The aim of this study was to confirm the anabolic pathway for guaiacol production by *Alicyclobacillus acidoterrestris*. A high performance liquid chromatography method was developed to separate and quantify guaiacol and the possible precursor compounds, ferulic acid, vanillin and vanillic acid. The precursor compounds required for guaiacol production by *A. acidoterrestris* was identified and the spoilage potential of apple juice supplemented with the precursor compounds was evaluated.

References

Bahçeci, K.S., Gökmen, V. & Acar, J. (2005). Formation of guaiacol from vanillin by *Alicyclobacillus acidoterrestris* in apple juice: a model study. *European Food Research and Technology*, **220**, 196-199.

Borlinghaus, A. & Engel, R. (1997). *Alicyclobacillus* incidence in commercial apple juice concentrate (AJC) supplies - method development and validation. *Fruit Processing*, **7**, 262-266.

Cerny, G., Hennlich, W. & Poralla, K. (1984). Spoilage of fruit juice by bacilli: isolation and characterisation of the spoiling microorganism. *Zeitschrift für Lebensmitteluntersuchung und -Forschung,* **179**, 224-227.

Chang, S.S. & Kang, D.H. (2004). *Alicyclobacillus* spp. in the fruit juice industry: history, characteristics, and current isolation/detection procedures. *Critical Reviews in Microbiology*, **30**, 55-74.

Duong, H.A. & Jensen, N. (2000). Spoilage of iced tea by *Alicyclobacillus*. *Food Australia*, **52**, 292.

Durak, M.Z., Churey, J.J, Danyluk, M.D. & Worobo, R.W. (2010). Identification and haplotype distribution of *Alicyclobacillus* spp. from different juices and beverages. *International Journal of Food Microbiology,* **142**, 286-291.

Eiora, M.N.U., Junqueira, V.C.A. & Schmidt, F.L. (1999). *Alicyclobacillus* in orange juice: occurrence and heat resistance of spores. *Journal of Food Protection*, **62**, 883-886.

Gocmen, D., Elston, A., Williams, T., Parish, M. & Rouseff, R.L. (2005). Identification of medicinal off-flavours generated by *Alicyclobacillus* species in orange juice using GC-olfactometry and GC-MS. *Letters in Applied Microbiology*, **40**, 172-177.

Goto, K., Nishibori, A., Wasada, Y., Furuhata, K., Fukuyama, M. & Hara, M. (2008). Identification of thermo-acidophilic bacteria isolated from the soil of several Japanese fruit orchards. *Letters in Applied Microbiology*, **46**, 289-294.

Jensen, N. (2000). *Alicyclobacillus* in Australia. *Food Australia*, **52**, 282-285.

Jensen, N. & Whitfield, F.B. (2003). Role of *Alicyclobacillus acidoterrestris* in the development of a disinfectant taint in shelf-stable fruit juice. *Letters in Applied Microbiology*, **36**, 9-14.

Lusardi, C., Previdi, M.P., Colla, F. Barbieri, G. & Bolzoni, L. (2000). Ability of *Alicyclobacillus* strains to spoil fruit juices and nectars. *Industria Conserve,* **75**, 151-161.

Matsubara, H., Goto, K., Matsumura, T., Mochida, K., Iwaki, M., Niwa, M. & Yamasato, K. (2002). *Alicyclobacillus acidiphilus* sp. nov., a novel thermo-acidophilic, ω-alicyclic fatty acid-containing bacterium isolated from acidic beverages. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 1681-1685.

Niwa, M. & Kuriyama, A. (2003*). A. acidoterrestris* rapid detection kit. *Fruit Processing*, **13**, 328-331.

Pettipher, G.L. & Osmundson, M.E. (2000). Methods for the detection, enumeration and identification of *Alicyclobacillus acidoterrestris*. *Food Australia*, **52**, 293-295.

Pettipher, G.L., Osmundson, M.E. & Murphy, J.M. (1997). Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit juice-containing drinks. *Letters in Applied Microbiology*, **24**, 185-189.

Pontius, A.J., Rushing, J.E. & Foegeding, P.M. (1998). Heat resistance of Alicyclobacillus acidoterrestris spores as affected by various pH values and organic acids. Journal of Food Protection, 61, 41-46.

Silva, F.M.S., Gibbs, P., Vieira, M.C. & Silva, C.L.M. (1999). Thermal inactivation of *Alicyclobacillus acidoterrestris* spores under different temperature, soluble solids and pH conditions for the design of fruit processes. *International Journal of Food Microbiology,* **51**, 95-103.

Silva, F.V.M., Gibbs, P. & Silva, C.L.M. (2000). Establishing a new pasteurisation criterion based on *Alicyclobacillus acidoterrestris* spores for shelf-stable high-acidic fruit products. *Fruit Processing*, **4**, 138-141.

Smit, Y. (2009). Growth and guaiacol production of species of *Alicyclobacillus* isolated from the South African fruit processing environment. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Splittstoesser, D.F., Churey, J.J. & Lee, C.Y. (1994). Growth characteristics of aciduric sporeforming bacilli isolated from fruit juices. *Journal of Food Protection*, **57**, 1080-1083.

Splittstoesser, D.F., Lee, C.Y. & Churey, J.J. (1998). Control of *Alicyclobacillus* in the juice industry. *Dairy, Food and Environmental Sanitation*, **18**, 585-587.

Vieira, M.C., Teixeira, A.A., Silva, F.M., Gaspar, N. & Silva, C.L.M. (2002). *Alicyclobacillus acidoterrestris* spores as a target for Cupuaçu (*Theobroma grandiflorum*) nectar thermal processing: kinetic parameters and experimental methods. *International Journal of Food Microbiology*, **77**, 71-81.

Walker, M. & Phillips, C.A. (2005). The effect of intermittent shaking, headspace and temperature on the growth of *Alicyclobacillus acidoterrestris* in stored apple juice. *International Journal of Food Science and Technology*, **40**, 557-562.

Walker, M. & Phillips, C.A. (2008). *Alicyclobacillus acidoterrestris:* an increasing threat to the fruit juice industry? *International Journal of Food Science and Technology,* **43**, 250-260.

Walls, I. & Chuyate, R. (1998). *Alicyclobacillus* - historical perspective and preliminary characterization study. *Dairy, Food and Environmental Sanitation*, **18**, 499-503.

Yamazaki, K., Teduka, H. & Shinano, H. (1996). Isolation and identification of *Alicyclobacillus acidoterrestris* from acidic beverages. *Bioscience, Biotechnology, and Biochemistry*, **60**, 543-545.

CHAPTER 2

LITERATURE REVIEW

A. Background

Over the past few years the international fruit juice market has grown substantially. This is due to trends towards healthier eating, as well as an increase in the demand for natural food products and functional foods. There is also an increased demand for fresh or minimally processed fruits (Walker & Phillips, 2008). Consumers are replacing carbonated soft drinks with healthier alternatives and juice provides a convenient and tasty option (Anon, 2008). However, concomitant to general foods, consumers have high expectations of juice quality and safety and a spoilage incidence could possibly lead to loss of brand or product loyalty. The focus in the juice market has been on product differentiation, sensory quality and functionality (Anon, 2008).

A combination of heat treatment and low pH are used to preserve fruit juice products stored in shelf-stable packaging at room temperature. The natural acidity of the fruit juice prevents the growth of many types of bacteria and supports the growth of microbes of low heat resistance, such as yeasts, mycelial fungi and acid-tolerant non-endospore-forming bacteria (Murdock, 1976; Splittstoesser & Mundt, 1976; Eiora *et al*., 1999). Pasteurisation is used to inhibit the growth of heat-labile lactic acid bacteria and fungi (Splittstoesser & Mundt, 1976) and to destroy pathogens such as *Escherichia coli* O157:H7 (Walls & Chuyate, 1998). Hot-fill and hold pasteurisation at 88 ° - 96 °C for 2 min is usually sufficient to destroy most non-endospore-forming microbes and to render the product commercially sterile (Murdock, 1976; Pontius *et al.,* 1998).

Fruit juice and other acidic foods that have a pH below 4 do not support the germination of most bacterial endospores such as *Bacillus stearothermophilus* that is non-viable at pH<5.3 (Blocher & Busta, 1983; Brown, 2000). Therefore, a heat treatment is not needed to eliminate these (Walls & Chuyate, 1998). It is assumed that if fruit juices are properly processed and handled, it will remain commercially sterile during the specified shelf-life until the packaging is opened (Blocher & Busta, 1983; McIntyre *et al.,* 1995). However, due to the identification and frequent isolation of *Alicyclobacillus* spp. from aseptically packed apple juice and other juices (Cerny *et al.,* 1984; Duong & Jensen, 2000; Durak *et al.,* 2010), it is evident that the natural low pH of fruit juice and pasteurisation is not adequate to prevent spoilage caused by these thermophilic, acidophilic bacteria (Splittstoesser *et al.,* 1994; Pettipher *et al.,* 1997;

Walls & Chuyate, 1998). *Alicyclobacillus* endospores can germinate, grow and spoil these products at a pH formerly considered to be below the range that supported the growth of endospore-forming bacteria (Walls & Chuyate, 1998).

The growth of *Alicyclobacillus* can be inhibited by refrigeration, but fruit juice is pasteurised to be marketed at ambient temperatures and chilling of these products would increase the costs. Amongst other treatments, increased pasteurisation temperatures could be a control measurement for spoilage, however, sterilisation temperatures (above 100 °C) negatively affect the quality of the fruit juice by producing unacceptable organoleptic changes (Walls & Chuyate, 1998; Palop *et al.,* 2000; Bahçeci *et al.,* 2003; Chang & Kang, 2004). Furthermore, endospores can penetrate ultra-filtration membranes and this may also not be an effective method to inhibit the spoilage caused by *Alicyclobacillus* spp. (Cerny *et al.,* 2000; Bahçeci *et al*., 2003).

B. Taxonomy of *Alicyclobacillus* spp.

Alicyclobacillus was discovered in the late 1960 and the bacteria were presumed to belong to the genus *Bacillus*. In 1967, the first acidophilic endospore-forming bacteria were isolated from thermal waters in Tokohu, Japan (Uchino & Doi, 1967). The pH range of growth was between 2.3 and 5.0 with a temperature range of 45 °- 71 °C. These bacteria were named *Bacillus acidocaldarius*, but were originally classified as *Bacillus coagulans* based on their morphology (Uchino & Doi, 1967). Bacteria with similar characteristics were isolated from a variety of thermal acidic environments in 1971 in the United States of America (USA), including the hot springs in Yellowstone National Park (Darland & Brock, 1971). The pH range of growth was reported to be between 2 and 6, with a temperature range of 45 °- 70 °C.

In 1981, Hippchen *et al.* isolated an unknown heat-acid-tolerant *Bacillus* from soil with a lower optimum growth temperature, different biochemical characterisation and DNA base composition compared to *Bacillus acidocaldarius*. In addition to this, in 1982, the first spoilage incident caused by acidophilic endospore-formers was reported in Germany in aseptically packed apple juice with a pH of 3.15 (Cerny *et al.,* 1984). The spoilage was characterised by a light cloudiness and an unpleasant taste and odour, similar to that of disinfectant (Eiora *et al*., 1999). In 1987 Deinhard *et al.* showed that the bacteria previously isolated by Hippchen *et al.* (1981) and Cerny *et al.* (1984) were different from the *B. acidocaldarius* described by Darland & Brock (1971), therefore, the new species was named *B. acidoterrestris* (Deinhard *et al.,* 1987a).

A new genus, *Alicyclobacillus* was proposed in 1992 due to the three stains *B. acidocaldarius*, *B. acidoterrestris* and *B. cycloheptanicus* being sufficiently different from other *Bacillus* spp. based on comparative ribosomal DNA (rDNA) sequencing (Wisotzkey *et al.,* 1992). Several new species belonging to the genus *Alicyclobacillus* have been isolated from a variety of environments and species first classified in the genus *Sulfobacillus* have also been reclassified as members of the genus *Alicyclobacillus* (Karavaiko *et al*., 2005). To date, 20 species and two subspecies belong to this genus *Alicyclobacillus* (Anon., 2010a).

C. Characteristics of *Alicyclobacillus* spp.

Alicyclobacilli are thermophilic, acidophilic bacteria (TAB) with a growth temperature of 20 ° -70 °C and a growth pH range of between 2.5 and 6 (Pettipher *et al.,* 1997; Walls & Chuyate, 1998; Orr *et al.,* 2000; Yamazaki *et al.,* 2000). The species of *Alicyclobacillus* are aerobic with the exception of *A. pohliae* that is sometimes facultative anaerobic (Imperio *et al*., 2008). *Alicyclobacillus* spp. are rod-shaped, non-pathogenic, endospore-formers and usually Gram-positive. In many of the species the 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) and *Alicyclobacillus sendaiensis* is the only Gram-negative species that is a member of the genus (Tsuruoka *et al*., 2003).

Species of *Alicyclobacillus* have a unique and characteristic fatty acid profile that contains ω-alicyclic fatty acid as the major membrane component (Wisotzkey *et al.,* 1992; Walls & Chuyate, 1998; Albuquerque *et al.,* 2000). The exception to this is *Alicyclobacillus pomorum* that has no ω-alicyclic fatty acids in its membrane, but straight- and branched-chain saturated fatty acids. This bacterium is classified as belonging to the genus *Alicyclobacillus* based on phylogenetic analyses of the 16S ribosomal RNA (rRNA) and DNA gyrase B subunit (gyrB) gene sequences (Goto *et al.,* 2003). The following bacteria were also classified as belonging to the genus *Alicyclobacillus* with a similar fatty acid profile than *A. pomorum,* namely *A. contaminans, A. macrosporangiidus* (Goto *et al.,* 2007)*, A. pohliae* (Imperio *et al.,* 2008) and *A. ferrooxydans* (Jiang *et al.,* 2008). The membrane of *A. acidocaldarius, A. acidoterrestris, A. hesperidium*, *A. acidophilus*, *A. disulfdooxidans, A. sendaiensis, A. vulcanalis, A. sacchari* and *A. fastidiosus* consist predominately of ω-cyclohexane fatty acids (Darland & Brock, 1971; De Rosa *et al.,* 1971; Oshima & Ariga, 1975; Deinhard *et al.,* 1987a; Dufresne *et al.,* 1996; Albuquerque *et al.,* 2000; Matsubara *et al.,* 2002;

Tsuruoka *et al.,* 2003; Simbahan *et al.,* 2004; Karavaiko *et al.,* 2005; Goto *et al.,* 2007) while *A. cycloheptanicus, A. herbarius, A. kakegawensis* and *A. shizuokensis* are mainly compose of ω-cycloheptane fatty acids (Poralla & König, 1983; Allgaier *et al.,* 1985; Deinhard *et al.,* 1987b; Goto *et al.,* 2002; Goto *et al.,* 2007).

Alicyclobacillus spp. has a resistance to high temperatures and low pH due to the ω-alicyclic fatty acids and hopanoids. The ω-alicyclic fatty acids-containing lipids in the membrane of these bacteria are packed densely in a cyclohexane ring structure. This structure stabilise the membrane and influences the permeability of the membrane, resulting in a protective coating enabling the cells to survive extreme conditions of acidity and high temperatures (Kannenberg *et al.,* 1984; Wisotzkey *et al.,* 1992; Chang & Kang, 2004). This is an important mechanism of thermoacidophilic bacteria for membrane adaptation under various conditions (Kannenberg *et al.,* 1984). Hopanoids are also present in the membrane of a number of species (Poralla *et al.,* 1980; Hippchen *et al.,* 1981) and also have a stabilising effect by decreasing the mobility of the acyl chain of the lipids in the cytoplasmic membrane. This mechanism provides an advantage in a low pH environment by hindering the passive diffusion of protons and solutes through the membrane. Therefore, the organism can easily establish approximately neutral cytopalsmic pH (Poralla *et al.,* 1980). A high concentration of hapanoids counterbalances the low viscosity of the branched-chain fatty acids, providing a more stable membrane (Krischke & Poralla, 1990).

D. Spoilage by *Alicyclobacillus*

The spoilage caused by *Alicyclobacillus* spp. was previously regarded as sporadic, however, a survey done in 1998 by the National Food Processors Association (NFPA) of the USA showed that a large amount of fruit juice spoilage are directly associated with members of this genus. The survey indicated that 35% of respondents experienced spoilage consistent with the growth of acidophilic endospore-formers (Walls & Chuyate, 1998). The spoilage of products occurred seasonally, in spring or summer, and most commonly occurred in apple juice (Walls & Chuyate, 1998; Pettipher & Osmundson, 2000). A similar survey was conducted by the European Fruit Juice Association (AIJN) in 2005. Of the 68 participants involved, 31 (45 %) experienced *Alicyclobacillus* spp. related problems in the three years preceding the survey. Sixteen of the incidences was intermediately to majorly severe. Eighteen of the incidences involved apple raw materials and in 13 incidences *A. acidoterrestris* was reported to be the cause of the spoilage (Howard, 2006).

The source of contamination of fruit juices has been identified as *Alicyclobacillus* contaminated soil present on the fruit and vegetables that enter the processing plant (Deinhard *et al.,* 1987a; Brown, 1995; Groenewald *et al.,* 2008). *Alicyclobacillus acidoterrestris* was isolated from orchard soil, wash water, water from evaporator inlet, soil outside the fruit concentrate factory and flume water. While strains of *A. acidocaldarius* were isolated from orchard soil, pre-pasteurised pear purees and from vinegar flies (Groenewald *et al.,* 2008; 2009). Water is a possible vector of contamination in the processing environment, aspecially when recycled water is used in the production of fruit juices and concentrates (McIntyre *et al.,* 1995; Walls & Chuyate, 1998; Jensen, 1999).

Spoilage by species of *Alicyclobacillus* are difficult to detect and can occur without visible changes. Only a light sediment and a cloudiness or haze may be present in contaminated fruit juices (Walker & Phillips, 2005), with no gas production (Walls & Chuyate, 1998). The growth of *Alicyclobacillus* spp. does not affect the pH of the juice (Brown, 2000) and the only obvious indication of spoilage is the formation of a medicinal or phenolic off-flavour (Walls & Chuyate, 1998; Pettipher & Osmundson, 2000; Walker & Phillips, 2005). The spoilage was described as being a flat-sour type spoilage. In many incidences spoilage was only reported as customer complaints (Walls & Chuyate, 1998; Walker & Phillips, 2005).

According to studies done by Splittstoesser *et al.* in 1994 and 1998, apple, white grape and tomato juices are predominantly vulnerable to spoilage by *A. acidoterrestris.* Although spoilage of more diverse products such as self-stable iced tea containing berry juice, the ingredients of rose hip and hibiscus teas (Duong & Jensen, 2000), a carbonated fruit drink (Pettipher & Osmundson, 2000), diced canned tomatoes (Chang & Kang, 2004) and Aloe vera juice (Duvenagen, 2006) have been reported, spoilage of orange (Pinhatti *et al.,* 1997; Eiora *et al.,* 1999; Komitopoulou *et al.,* 1999) and apple juice (Walls & Chuyate, 1998; Pettipher & Osmundson, 2000; Durak *et al.,* 2010) are most frequently reported. *Alicyclobacillus acidoterrestris* was also isolated from an apple-cranberry juice blend (Splittstoesser *et al.,* 1994), blending water used in fruit juice, berry juice, citrus juices (McIntyre *et al.,* 1995; Durak *et al.,* 2010) and pear juice (Duvenage *et al.*, 2007). Four strains isolated by Yamazaki *et al.* (1996) were all identified as *A. acidoterrestris* and was responsible for the production of guaiacol in various acidic juices, isotonic water, lemonade, fruit juice blends and fruit-carrot juice blends. *Alicyclobacillus acidoterrestris* was also responsible for guaiacol formation at levels of 1 to 100 ppb in orange juice, apple juice and non-carbonated fruit juice

containing drinks (Pettipher *et al.,* 1997). Although *A. acidoterrestris* is primarily responsible for spoilage and the production of the taint compound, guaiacol other species including *A. acidiphilus* (Matsubara *et al.,* 2002; Anon., 2006; Goto *et al.,* 2008), *A. hesperidum* (Gocmen *et al.,* 2005; Goto *et al.,* 2008), *A. cycloheptanicus* (Gocmen *et al.,* 2005), *A. herbarius* (Anon., 2006; Goto *et al.,* 2008) and *A. acidocaldarius* (Smit, 2009) also have the ability to produce guaiacol.

Certain neutral phenolic compounds in red grape juice prevent spoilage by *Alicyclobacillus* spp. Catechingallate, the phenolic compound in red grape juice was added to apple juice and inhibited the germination of *Alicyclobacillus* endospore (Splittstoesser *et al.,* 1994; 1998). The acidific phenolics in red grape juice, such as trans-caffeoyl tartaric acid and cis-coumaroyl tartaric acid did not have an inhibitory effect on the growth of *Alicyclobacillus* (Splittstoesser *et al.,* 1994). Table wines also do not support the spoilage by *Alicyclobacillus* spp., possibly due to the high ethanol concentration that exceeds 6 % (v/v) (Splittstoesser & Churey, 1996).

The growth of *Alicyclobacillus* spp. is affected by the soluble solids (SS) content of the juices (Splittstoesser *et al*., 1994). Tomato juice, with a higher pH and lower SS permitted the most growth of *Alicyclobacillus*, followed by apple juice and growth did not occur in the presence of the high sugar levels common in grape juice. However, a reduction in the sugar concentration of the white grape juice to 16 °Brix resulted in maximum growth (Splittstoesser *et al*., 1994), while a sugar content exceeding 18 °Brix inhibits the growth of *Alicyclobacillus* (Splittstoesser *et al.,* 1994). Thus, the growth of *Alicyclobacillus* spp. is inhibited in juice concentrates, but after dilution to form single strength juice, the endospores present in the concentrate can germinate and cause spoilage (Pettipher & Osmundson, 2000).

Not all the strains belonging to the genus *Alicyclobacillus* can grow in fruit juices. Endospores of *Alicyclobacillus acidoterrestris* (DSM 2498) and *Alicyclobacillus* spp. (SSICA 278/B, SSICA Ar23) germinated and grew in apple juice at 30 °, 37 ° and 50 °C (pH 3.4), the strains SSICA B and SSICA 3998A (with similar characteristics than *A. acidocaldarius*) grew at 50 °C, while SSICA 3998A also grew at 37 °C. *Alicyclobacillus acidocaldarius* (DSM 446) was the only strain that did not grow in apple juice. None of these strains germinated in apricot nectar (pH 3.6), while DSM 2498, SSICA 278/B and SSICA Ar23 grew at 30 °, 37 ° and 50 °C in orange (pH 3.65), pear (pH 3.6) and tropical fruit (pH 3.51) nectars. Strains DSM 2498 and SSICA Ar23 were able to grow at 37 ° and 50 °C in peach nectar and at 30 °, 37 ° and 50 °C in pineapple nectar.

Alicyclobacillus acidocaldarius strain DSM 446 was unable to germinate in any of the products (Lusardi *et al.,* 2000).

The growth of *A. acidoterrestris* is also influenced by the amount of headspace in the packaging of fruit juice. The growth rates are higher in partially filled containers than in full containers. The partially filled containers provide optimum aerobic conditions for the growth of *A. acidoterrestris.* It has also been shown that intermittent shaking of the contaminated juice before sampling increases the growth of *A. acidoterrestris* on isolation media and increase the probable detection of the bacteria in fruit juice (Walker & Philips, 2005).

E. Isolation and enumeration of species of *Alicyclobacillus*

A selection of media used for the isolation and enumeration of *Alicyclobacillus* spp. have been summarised in Table 1. Studies have shown a great contrast of which media to use for the enumeration of *Alicyclobacillus* spp. According to Pettipher *et al.* (1997) *Bacillus acidocaldarius* (BAM), potato dextrose agar (PDA) and orange serum agar (OSA) all performed well, with OSA having the highest recovery. The medium for the most effective recovery of *Alicyclobacillus* endospores is K agar (Orr & Beuchat, 2000; Marray *et al.,* 2007), *Alicyclobacillus* (ALI) medium and *Bacillus acidoterrestris* (BAT) agar (Marray *et al.,* 2007). K agar and BAT agar are also effective for the recovery of *A. acidoterrestris* and *A. acidocaldarius,* respectively (Jensen, 2005). BAT agar (pH 4), using the spread plate technique, gave the highest recovery of cells and is used as a standard international method for the detection of *Alicyclobacillus* spp. (IFU, 2007). According to Witthuhn *et al.* (2007), PDA (pH 3.7) and OSA (pH 5.5) plates, incubated at 50 °C for 3 to 5 days, gave the highest recovery of *Alicyclobacillus* vegetative cells and endospores compared to BAM (pH 4), K agar (pH 3.7) and YSG (pH 3.7). The pH of the media (either 3.7 or 5.5) did not have a significant influence on the recovery, however, higher recovery was reported at 50 °C than at 43 °C. An incubation time longer than 3 days did not give a significantly different recovery of *Alicyclobacilllus* endospores (Witthuhn *et al.,* 2007). Marray *et al.* (2007) reported that spread plating recovered higher numbers than pour plating and that viable endospores were detected after 3 days of incubation at 43 °C (Marray *et al.,* 2007). In contrast with this and a previous study (Pettipher *et al.,* 1997), Jensen (2000) found that spread and pour plating had similar recoveries of *Alicyclobacillus* spp. from a high oxygen environment and spread plates gave higher recoveries from reduced oxygen environments.

Table 1 Media used for the isolation of *Alicyclobacillus* species

Name	**Reference**
Bacillus acidocaldarius medium (BAM)	Deinhard *et al*., 1987a; IFU, 2007
Bacillus acidoterrestris (BAT) medium	Deinhard *et al*., 1987a; IFU, 2007
Alicyclobacillus (ALI) medium	Wisse & Parish, 1998
Potato dextrose agar (PDA)	Bevilacqua *et al*., 2008
K agar	Walls & Chuyate, 1998
SK agar	Chang & Kang, 2005
Orange serum agar (OSA)	Bevilacqua *et al*., 2008b
Yeast starch glucose (YSG) medium	Goto *et al*., 2002

F. Taint compounds

The food industry often receives customer complaints concerning off-flavours or taints in food products (Whitfield, 1998; Chang & Kang, 2004). The term taint is frequently used to describe the compounds that are responsible for off-flavour and odours in spoiled food products (Whitfield, 1998). The off-flavours that are associated with the spoilage of food products caused by *Alicyclobacillus* spp. can either be due to the production of guaiacol (Pettipher *et al.,* 1997; Splittstoesser *et al.,* 1998) or the halophenols, 2,6-dibromophenol (2,6-DBP) and 2,6-dichlorophenol (2,6-DCP) (Borlinghaus & Engel, 1997). However, guaiacol is accepted as the predominant metabolite associated with taint production in fruit juices (Pettiper *et al.,* 1997; Jensen, 2000; Jensen & Whitfield, 2003). The lower concentration of halophenols that occur in fruit juice and the high volatility of guaiacol is probably the reason for the predominance of guaiacol as taint compound (Jensen, 2000). In pear and orange juice inoculated with *A. acidoterrestris* strains (SSICA Ar23 and SSICA 278/B) and incubated at 37 °C, 2,6-DBP was not detected (detection limit of 0.05 µg kg^{-1}), while guaiacol was detected in all the samples ranging from 87 – 294 µg kg^{-1} (Lusardi *et al.,* 2000). In the United Kingdom and Germany, guaiacol was identified as the off-flavour in orange juice (Pettipher *et al.,* 1997) and 2,6-DBP was identified in apple juice (Borlinghaus & Engel, 1997). Guaiacol, 2,6-DBP and 2,6-DCP were identified in mixed juices contaminated with *A. acidoterrestris* (Jensen, 1999).

Guaiacol (2-methoxyphenol)

Guaiacol (Fig. 1) produces an undesirable odour in fruit juice resulting in an unpleasant smoky, medicinal or phenolic-like taint (Pettipher *et al.,* 1997; Jensen, 2000). Guaiacol can also be produced in acidic beverages of non-fruit juice content when vanilla flavour is added for aromatic essence (Niwa & Kawamoto, 2003). Incidences of guaiacol production have been reported in different substrates, including wine (Simpson *et al.,* 1986), chocolate ice cream (Saxby, 1993), chocolate milk (Jensen *et al.,* 2001), vanilla yoghurt (Whitfield, 1998), fruit juice and fruit juice-containing drinks (Pettipher *et al.,* 1997; Orr *et al.,* 2000). Bacterial strains present in cork stoppers, such as *Bacillus subtilus* and *Streptomyces* strains can be responsible for the presence of guaiacol in wines, referred to as cork taint (Simpson *et al.,* 1986; Álvarez-Rodríquez *et al.,* 2003).

Guaiacol may not only be present in food products due to microbial activity, but may also be present as a product of the heat degradation of phenolic compounds

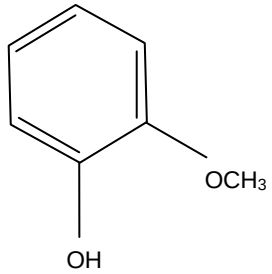

Figure 1 The structure of guaiacol (Chang & Kang, 2004).

(Mayer *et al.,* 1999; Chang & Kang, 2004). In the case of roasted products, wine oak barrels and natural cork guaiacol is formed by the thermal decomposition of phenolic compounds (Simpson *et al.,* 1986; Chang & Kang, 2004). Although guaiacol is better known for its undesirable smell or off-flavour (Whitfield, 1998), it gives the characteristic smell to roasted Arabica coffee (De Maria *et al.,* 1994; Mayer *et al.,* 1999) and barley malt (Fickert & Schieberle, 1998). The flavour is recorded as a sweet, burnt aroma with a smoky taste (Wasserman, 1966). In fruit based products, guaiacol has been identified as the chemical responsible for off-odour described as medical or antiseptic (Brown, 1995; Walls & Chuyate, 1998). The aroma of guaiacol was also described by Pettipher & Osmundson (2000) as a hammy off-odour similar to smoky bacon.

The best estimated threshold (BET) value for taste in apple juice is 2 µg L^{-1} (2 ppb) and for odour it is 2.32 ppb (Pettipher *et al.,* 1997; Orr *et al.,* 2000). Eisele & Semon (2005) indicated the BET values for taste in water and apple juice as 0.17 ppb and 0.24 ppb, respectively with the odour threshold in water and apple juice being 0.48 ppb and 0.91 ppb, respectively. However, Siegmund & Pöllinger-Zierler (2006) reported an odour threshold of 0.5 ppb in apple juice. The variation in the different threshold values might be due to differences in the sensitivity and training of the panel members used to conduct the sensory studies (Eisele & Semon, 2005).

Guaiacol is formed from ferulic acid via vanillin and vanillic acid by *A. acidoterrestris* (Fig. 2) (Niwa & Kawamoto, 2003). The formation of guaiacol in apple juice is dependent on the concentration of vanillin present in the juice. When the vanillin concentration is no longer available, the increase in the guaiacol concentration in the apple juice stabilises (Bahçeci *et al*., 2005a). Furthermore, the guaiacol production by *Alicyclobacillus* spp. is affected by the concentration of *Alicyclobacillus* spp. (Chang & Kang, 2004). A concentration of 10^5 cfu mL^{-1} of *A. acidoterrestris* was needed in apple and orange juice for the production of guaiacol (Pettipher *et al.,* 1997). According to Bahçeci *et al*. (2005a) guaiacol formation from vanillin starts when the cell concentration in the apple juice reaches 10^4 cfu mL^{-1}.

Storage temperature influences the growth of *Alicyclobacillus* spp. and as a result the production of guaiacol (Pettipher *et al.,* 1997; Bahçeci *et al*., 2005a). The growth of *A. acidoterrestris* is not favoured at 25 °C (Bahçeci *et al*., 2005a) and the rate of guaiacol production increases with the increase in growth (Chang & Kang, 2004). Although guaiacol can be produced at 25 °C, a longer lag phase in guaiacol production is observed (Smit, 2009). Higher concentrations of guaiacol were produced in orange

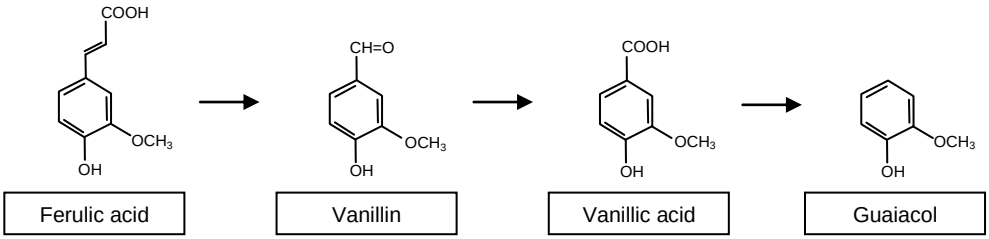

Figure 2 The proposed pathway of guaiacol production by *A. acidoterrestris* from ferulic acid via vanillin and vanillic acid (Niwa & Kawamoto, 2003).

and apple juice at 46 °C than at 37 °C (Jensen, 2000) and guaiacol was not detected in apple and orange juice stored at 4 °C (Pettipher *et al.,* 1997).

Vegetative cells of *Alicyclobacillus* spp. have to be present for guaiacol production to occur. Therefore, a heat shock treatment is needed to activate the dormant endospores. At a low cell concentration of *Alicyclobacillus* a heat shock treatment of 80 °C for 10 min gave significantly higher cell concentrations than 60 °C for 10 min or 100 °C for 5 min. However, no significant differences were observed between the heat shock methods when endospores were inoculated at higher concentrations (Walls & Chuyate, 2000). According to Pettipher *et al.* (1997) a heat shock treatment is not required to induce germination, but the rate of germination is slower when heat shock is not applied.

Halophenols

Although guaiacol is the predominant off-flavour compound associated with fruit juice spoilage by *Alicyclobacillus* species, several halophenols, particularly 2,6-DBP (Fig. 3) and 2,6-DCP (Fig. 3) are produced by *A. acidoterrestris* strains (Jensen & Whitfield, 2003). The halophenols are also described as having a medicinal, antiseptic or disinfectant-like odour and flavour (Jensen, 2000; Gocmen *et al*., 2005). Halophenols occur in lower concentrations than guaiacol (Jensen, 2000) and in most cases, the halophenols have been detected in concomitant to guaiacol (Gocmen *et al*., 2005). The production of the halophenols also seems to be strain or species specific (Gocmen *et al*., 2005). In a study done by Gocmen *et al.* (2005) *Alicyclobacillus hesperidum* and a presumptive *Alicyclobacillus* strain (UFL-CA8) produced 2,6-DCP in orange juice incubated at 45 °C, while *A. acidoterrestris* (ATCC 49025), *Alicyclobacillus cycloheptanicus* (ATCC 49029) and a presumptive *Alicyclobacillus* strain (UFL-CA11) produced 2,6-DBP. In this study *Alicyclobacillus cycloheptanicus* was the only species tested that produced both 2,6-DCP and 2,6-DBP.

G. Pathway of guaiacol production

Several microbes can produce guaiacol, including *Bacillus megaterium* (Crawford & Olson, 1978)*, Bacillus subtilis* (Álvarez-Rodríguez *et al*., 2003), *Pseudomonas acidovorans* (Vicuña *et al.,* 1987)*, Paecilomyces variotii* (Rahouti *et al*., 1989), *Rhodotorula rubra* (Huang *et al*., 1993), *Sporotrichum thermophile* (Topakas *et al*., 2003), *Streptomyces setonni* and *Streptomyces* strains (Pometto *et al*., 1981;

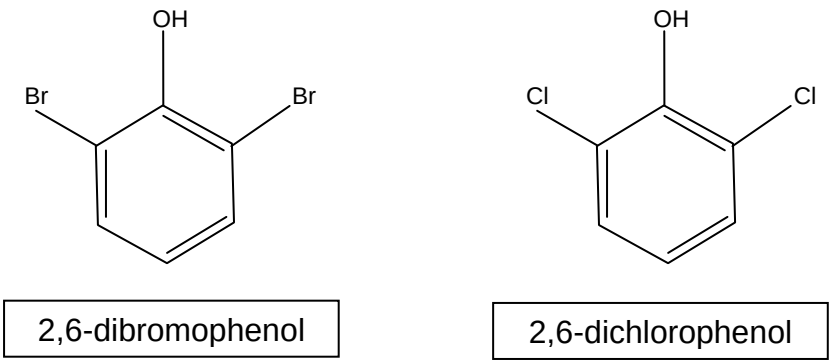

Figure 3 The structure of 2,6-dibromophenol (Anon., 2010b) and 2,6-dichlorophenol (Anon., 2010c).

Crawford & Olson, 1978; Álvarez-Rodríguez *et al*., 2003), as well as *Alicyclobacillus acidoterrestris* (Niwa & Kawamoto, 2003; Bahçeci *et al*., 2005a). Guaiacol is formed from vanillic acid by *B. megaterium* (Crawford & Olson, 1978), *B. subtilis* (Álvarez-Rodríguez *et al*., 2003) and *Streptomyces* strains (Crawford & Olson, 1978) and during the metabolism of ferulic acid by *P. variotii* (Rahouti *et al*., 1989), *R. rubra* (Huang *et al*., 1993) and *Sporotrichum thermophile* (Topakas *et al*., 2003). In fruit juice guaiacol is formed by *Alicyclobacillus* from ferulic acid via vanillin (Jensen, 2000; Niwa & Kawamoto, 2003; Bahçeci *et al*., 2005a). In these pathways vanillic acid is the immediate precursor of guaiacol (Jensen *et al.*, 2001).

The microbial production pathway of guaiacol and other products from ferulic acid is illustrated in Fig. 4. Ferulic acid, the precursor compound in lignin biosynthesis or the product of lignin degradation (Ishikawa *et al.,* 1963), is metabolised to vanillic acid and vanillin by different bacteria (Kawakami, 1980; Sutherland *et al.,* 1983) and fungi (Henderson, 1961; Ishikawa *et al.,* 1963; Toms & Wood, 1970; Tadasa, 1977). *Fusarium solani* (Nazareth & Mavinkurve, 1986), *P. variotii, Pestalotia palmarum* (Rahouti *et al.,* 1989), *Pseudomonas cepacia* (Andreoni *et al.,* 1984), *Bacillus coagulans* (Karmakar *et al.,* 2000), *S. thermophile* (Topakas *et al.,* 2003) and *Debaryomyces hansenii* (Mathew *et al.,* 2007) can also convert ferulic acid to 4-vinylguaiacol. Vanillin is mostly formed directly from ferulic acid by oxidative two-carbon fragmentation (Sutherland *et al.,* 1983; Peleg *et al.,* 1992). Vanillin mayalso be formed via 4-vinylguaiacol by the cleavage of the vinyl bond (Nazareth & Mavinkurve, 1986; Rahouti *et al.,* 1989). Vanillin is then oxidised to vanillic acid, which can form methoxyhydroquinone by oxidative decarboxylation or guaiacol is formed from vanillic acid by non-oxidative decarboxylation (Rahouti *et al.,* 1989). Vanillyl alcohol can also be formed from vanillin (Ishikawa *et al.,* 1963; Ander, 1983; Hatakka, 1985). This pathway is possibly followed to eliminate toxic levels of vanillin (Ander *et al.,* 1980; Rahouti *et al.,* 1989).

The pathway for the formation of guaiacol by different fungi and bacteria is the non-oxidative decarboxylation of vanillic acid (Crawford & Olsen, 1978; Pometto *et al.*, 1981; Sutherland *et al.,* 1983; Rahouti *et al.,* 1989). *Streptomyces setonii* and *Streptomyces* strains produce guaiacol from vanillic acid by decarboxylation and guaiacol can be demethylated to catechol (Pometto *et al*., 1981, Rahouti *et al.,* 1989). Vanillic acid can also be demethylated to protocatechuic acid by the fungi, *Polyporus dichrous* and *F. solani* (Nazareth & Mavinkurve, 1986) and by the bacteria, *P. cepacia* (Andreoni *et al.,* 1984) and *S. setonii* (Sutherland *et al.,* 1983).

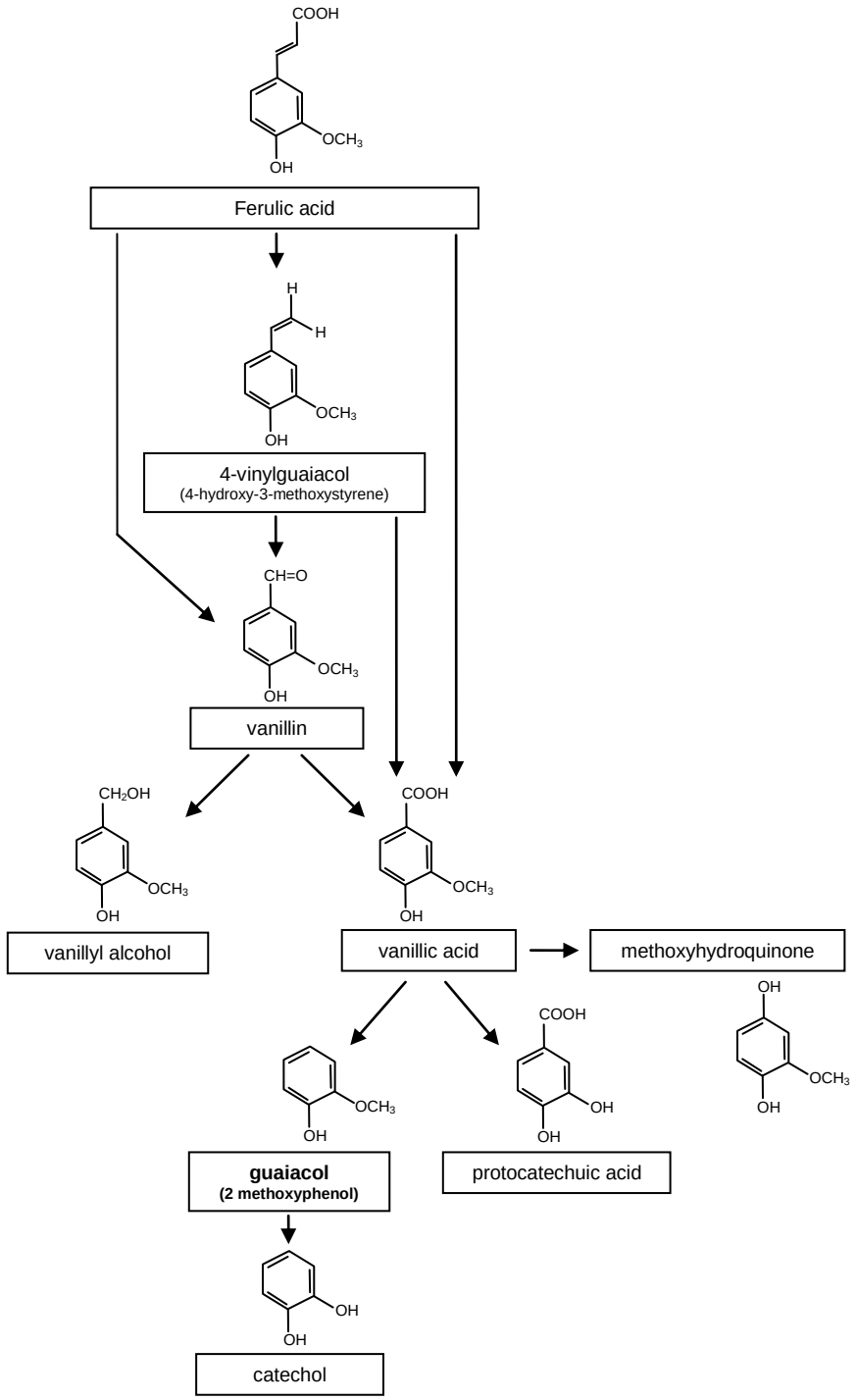

Figure 4 Metabolic pathway for guaiacol and other product production from ferulic acid (Ishikawa *et al.,* 1963; Crawford & Olson, 1978; Pometto *et al*., 1981; Nazareth & Mavinkurwe, 1986; Rahouti *et al*., 1989; Huang *et al*., 1993).

The metabolic pathway for guaiacol formation by members of the genus *Alicyclobacillus* is also one of non-oxidative decarboxylation of vanillic acid, the oxidation product of vanillin (Chang & Kang, 2004). In this pathway, ferulic acid is converted to vanillin or 4-vinylguaiacol by decarboxylation and 4-vinylguaiacol can then be converted back to vanillin by oxidation. Vanillin is oxidised to vanillic acid, which then transform to guaiacol by non-oxidative decarboxylation (Crawford & Olsen, 1978; Pometto *et al.,* 1981; Huang *et al.,* 1993; Rosazza *et al.,* 1995). Vanillic acid is rapidly converted to guaiacol by vanillic acid decarboxylase (Niwa & Kawamoto, 2003). This vanillic acid decarboxylase gene is present in *A. acidoterrestris,* in the actinomycetes, *Streptomyces* strain D7 and *Streptomyces lividans* and in *Bacillus subtilus* (Chow *et al.,* 1999).

H. Possible precursors of guaiacol production

According to the proposed pathway for guaiacol production, the phenolic acids, vanillic acid, vanillin and ferulic acid as well as the amino acid tyrosine are precursors for guaiacol (Crawford & Olsen, 1978; Pometto *et al.,* 1981; Huang *et al.,* 1993; Rosazza *et al.,* 1995; Jensen, 2000; Niwa & Kawamoto, 2003). Orange juice contains 3.4 - 13.5 µg mL^{-1} tyrosine and apple juice contains approximately 4.1 µg mL^{-1} tyrosine (Jensen, 2000). However, the widely accepted guaiacol synthetic pathway is that of lignin or ferulic acid degradation (Chang & Kang, 2004).

Ferulic acid is an abundant natural aromatic phenolic compound (Rosazza *et al.,* 1995), commonly found in fruits, vegetables, grains, beans, leaves, seeds, nuts, grasses, flowers and other types of vegetation (Herrmann, 1989). This compound is responsible for the structural rigidity of plants, strengthens the cell wall by cross-linking pentosan chains, arabinoxylans and hemicelluloses (Graf, 1992). The phenolic acids, vanillic acid and vanillin that form part of the proposed pathway for guaiacol production are also found in nature. Vanillic acid is present in the fruit juice as a derivative of lignin or product of several microbes (Chang & Kang, 2004). The vanillic acid content of apple juice tested by Piacquadio *et al.* (2002) was 10.07 (± 1.13) mg L^{-1}. Vanillin is present in several fruit and fruit products (Goodner *et al.,* 2000), including mango (Sakho *et al.,* 1997), orange juice (Martin *et al.,* 1992), strawberries (Pyysalo *et al.,* 1979), wines (Spillman *et al.,* 1997) and appel cider brandy (Mangas *et al*., 1997). In orange, tangerine, lemon, lime and grape fruit juice vanillin concentrations were present from 0.20 to 0.60 ppm and the pasteurisation treatment of grape fruit juice increased the vanillin concentration with 15% (Goodner *et al.,* 2000). Peleg *et al.* (1992) showed that

vanillin is formed in model solutions of orange juice stored at 35 ° and 45 °C as a possible product of ferulic acid degradation.

Ferulic acid is metabolically derived in plants from cinnamic acid (Gross, 1985) and exist naturally as *trans* or *cis* isomers (Agarwal & Atalla, 1990). Figure 5 illustrates the biosynthetic pathway of CO_2 to lignin, showing this compound as a phenolic monomer of lignin (Higuchi *et al.,* 1977). Ferulic acid can be present in its free state as the primary product of thermal decomposition of lignin (Fiddler, 1967) or linked to carbohydrates as glycosidic conjugates and forms various esters and amides with a wide variety of natural products, including polysaccharides, flavonoids, tryptamine and amino acids (Friend, 1981; Martin-Tanguy, 1985; Herrmann, 1989; Graf, 1992). The ability of bound and conjugated ferulic acid to serve as a source of ferulic acid depends on the type of each linkage with ferulic acid (Peleg *et al.,* 1988). The potential sources of free ferulic acid in orange and grapefruit are feruloylputrescine, feruloylglucose, feruloylglucaric, diferuloylglucaric and feruloylgalactaric acids (Wheaton & Stewart, 1965; Reschke & Herrmann, 1981; Risch *et al.,* 1987; Risch *et al.,* 1988). In apple juice, apple pomace and peach juice, ferulic acid, caffeic acid and *p*-coumaric acid occur as free cinnamic acids derived from the corresponding naturally-occuring esters (Van Buren, 1976; Treutter, 2001), feruloyl glucose, chlorogenic acid and p-coumaroyl quinic acid (Pérez-Ilzarbe *et al.*, 1991; Hernandez *et al.,* 1997). Fruit and fruit juices contain different concentrations of ferulic acid (Karmakar *et al.,* 2000) and commercial pectic enzymes or the enzymatic activity of microbes in the juice can be responsible for the release of ferulic acid from the plant derived tissues. The apple juice tested by Piacquadio *et al.* (2002) consisted of 14.46 (± 1.31) mg L^{-1} ferulic acid. Therefore, the substrates, ferulic acid, vanillin and vanillic acid required for the production of guaiacol can be naturally present in fruit juices and if microbes with the appropriate enzyme systems are present at the ideal environmental condition, ataint is formed (Chang & Kang, 2004).

I. Influence of processing technologies on phenolic compounds

Each fruit species and variety can be characterised by the composition of their phenolic compounds (Peréz-Ilzarbe *et al.*, 1991). The composition of the chemicals in the fruit product varies according to the cultivar, growing region, climate, maturity, cultural practices (Van Buren, 1976; Hernandez *et al.,* 1997), manufacturing treatments, including thermal and enzymatic treatments (Schols *et al.,* 1991; Treutter, 2001), and

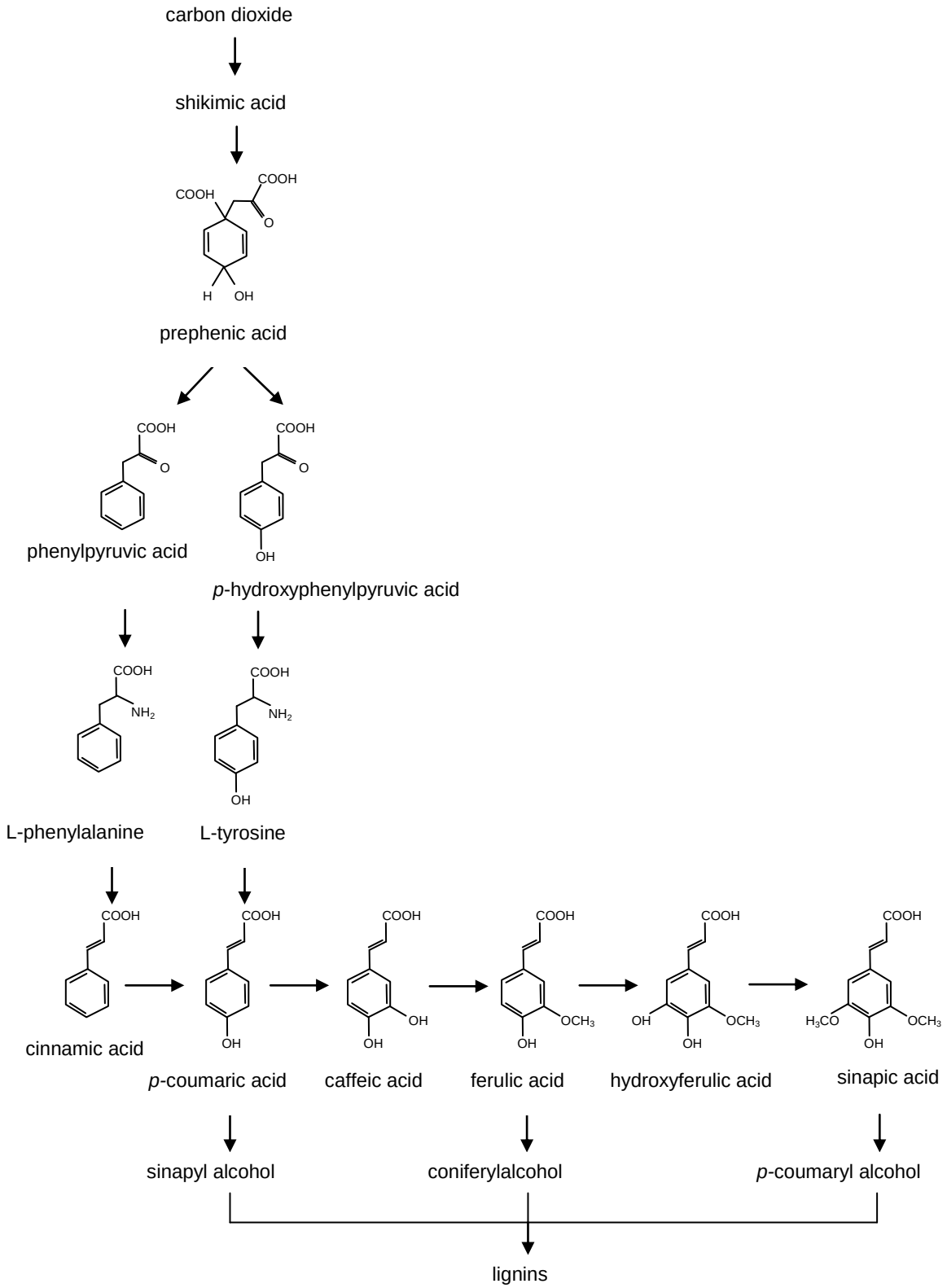

Figure 5 The biosynthetic pathway of CO_2 to lignin (Higuchi *et al.,* 1977).

the storage conditions of the fruit or final fruit products (Spanos & Wrolstad, 1992). These treatments may result in an increased degradation and oxidation of the phenolic compounds (Treutter, 2001).

The phenolic compounds in fruits are also not uniformly distributed at subcellular level or in the tissue. Simple soluble phenols are located mainly in the cell vacuoles and lignins are located in the cell walls (Higuchi, 1990). This is important in relation to the chemical composition of industrial manufactured food products (Macheix *et al*., 1990). The particular distribution of phenolic compounds and the technological processes employed influence the release of compounds through the rupture of vacuoles and cell walls (Hernandez *et al.,* 1997).

Pectic enzymes can also be used to facilitate juice extraction or pressing. During manufacturing of concentrates, pectinase and cellulase are used to break down pectins and improve the yield during crushing and pressing (Joshi *et al.,* 1991; Di Cesare *et al.,* 1993). Pectinase and amylase are used to remove suspended particulate matter and clarify the concentrates (Bengoechea *et al.,* 1997). Addition of pectinase before thermal treatment causes specific changes in the phenolic composition of juice. The esters of caffeic, *p*-coumaric and ferulic acid are hydrolysed to their corresponding free cinnamic acids (Bengoechea *et al.,* 1997). However, the final effect of hydrolysis depends on the nature of the fruit juice (Spanos *et al*., 1990; Kermasha *et al.,* 1995; Bengoechea *et al.,* 1997; Hernandez *et al.,* 1997). Donaghy *et al.* (1999) reported that yeasts in unpasteurised juice, present as pre-processing contaminants, can also release free phenolic acids into the apple juice by feruloyl esterase activity.

The initial heat treatment after pressing protects the cinnamic acids from enzymatic oxidation and inactive polyphenol oxidase. Polyphenol oxidase activity continues before and during processing of pulp until the high temperature short time treatment commences (Van Buren, 1976; Spanos *et al.,* 1990). At the bottling stage clarification enzymes are precipitated by pasteurisation (Montgomery & Petropakis, 1980; Spanos *et al.,* 1990; Spanos & Wrolstad, 1992).

J. Detection and quantification of guaiacol and phenolic acids

The taint compound, guaiacol can be detected by using sensory (Orr *et al.,* 2000; Eisele & Semon, 2005), chemical (Bahçeci & Acar, 2007) or analytical (Zierler *et al*., 2004; Bahçeci *et al*., 2005a) methods. Analytical methods, such as gas chromatography (GC) and high performance liquid chromatography (HPLC) are used for the identification of guaiacol (Chang & Kang, 2004) and can be used to quantify the amount of guaiacol that

is present in a sample (Gökmen *et al*., 2001; Chang & Kang, 2004; Bahçeci & Acar, 2007). These methods are also used in quantitative analysis of phenolic acids in media and beverages (Kermasha *et al.,* 1995; Proestos *et al.,* 2006; Bahçeci & Acar, 2007). A chemical method, also described as the peroxidase method, can also be applied to identify and quantify guaiacol and is based on a chemical reaction between guaiacol, peroxidase and hydrogen peroxide and the presence of guaiacol is measured as a colour or absorbance change (Niwa, 2004; Bahçeci & Acar, 2007). Sensory methods are mostly used to indicate the presence or absence of off-flavours (Chang & Kang, 2004).

Sensory methods

Sensory methods make use of olfactory senses to detect the presence of guaiacol (Chang & Kang, 2004). Experienced panels are used to determine the recognition threshold of guaiacol odour (Orr *et al.,* 2000) and taste by using a forced-choice ascending concentration method of limits (Eisele & Semon, 2005). The sensory threshold of guaiacol is low and consumers without formal sensory training will not be able to detect the presence of guaiacol in fruit juices (Chang & Kang, 2004). Orr *et al.* (2000) conducted a study to correlate levels of *A. acidoterrestris* cells in apple juice with guaiacol levels measured by chromatographic analysis and sensory perception of spoilage. Guaiacol was detected by the panel in several samples in which guaiacol was not detected by the chromatographic analysis. This indicated that the detection limit of BET is lower than that of chromatographic analysis (Orr *et al.,* 2000). However, Siegmund and Pöllinger-Zierler (2006) indicated that headspace solid phase microextraction (HS-SPME) is more sensitive than sensory methods.

Chemical methods

Chemical detection methods of guaiacol in products employs a colourimetric assay based on the oxidation of guaiacol in the presence of hydrogen peroxide by peroxidase enzymes (Doerge *et al*., 1997). In this reaction, guaiacol radicals are produced formed guaiacol and the free guaiacol radicals react with each other to form a red-brown tetraguaiacol (Bahçeci & Acar, 2007). This brown component, which has been identified as 3,3'-dimethoxy-4,4'-biphenoquinone (Doerge *et al*., 1997) cause a change in absorbance and is measured with a spectrophotometer at 420 nm (Bahçeci *et al*., 2005a; b) or 470 nm (Doerge *et al*., 1997; Niwa & Kawamoto, 2003; Niwa & Kuriyama, 2003). Guaiacol can be quantified by comparing the absorbance of the sample to a

standard curve of the absorbance of known guaiacol concentrations. The amount of peroxidase and hydrogen peroxidase is constant and there is stoichiometry between the colour formed and the amount of guaiacol present in the sample (Bahçeci & Acar, 2007).

Spectrophotometric methods and HPLC method for guaiacol determination is not significantly different ($p > 0.05$) regarding sensitivity (Bahçeci & Acar, 2007). The colourimetric method is a fast and simple method to determine guaiacol production if advanced instruments like a HPLC is not available. However, there are some limitations with the use of spectrophotometric measurements, as the Ultraviolet-Visable (UV-Vis) spectrophotometric method that is based on peroxidise activity (Bahçeci *et al*., 2005b) do not give satisfactory results at concentrations below 10 mg L^{-1} (Bahçeci & Acar, 2007).

The colourimetric assay is widely used (Doerge *et al*., 1997) and also forms the basis of the guaiacol detection kit (Niwa & Kawamoto, 2003; Niwa & Kuriyama, 2003) developed by the Kyokuto Pharmaceutical Industrial Company Ltd, Japan and distributed by Cosmo Bio Company Ltd. (Anon., 2005). The kit is used to determine the presence of guaiacol or the presence of species of *Alicyclobacillus* that can produce guaiacol (Niwa & Kawamoto, 2003; Niwa & Kuriyama, 2003). A kit was also developed by the Döhler Group in Germany (Anon., 2006). The detection kit do not give a clear prediction on the extent and risk of beverage spoilage, but can only be used for the detection of species of *Alicyclobacillus* that can produce guaiacol (Anon., 2006).

Analytical methods

Physico-chemical analyses usually include sample preparation, separation and detection. The most common separation procedures for guaiacol detection are chromatography performed by HPLC (Bahçeci *et al*., 2005a; Bahçeci & Acar, 2007) and GC (Pettipher *et al*., 1997; Jensen *et al*., 2001; Pollnitz *et al*., 2004; Zierler *et al*., 2004; Gocmen *et al*., 2005). Sample preparation or extraction procedure can be performed using liquid-liquid extraction (Pollnitz *et al.,* 2004; Gocmen *et al.,* 2005), solid phase extraction (Bieniek, 2003), and solid phase microextraction (SPME) (Wardencki *et al.,* 2004). Mass spectrometry (MS) is a very specific, accurate and sensitive detection method that is often used with GC analysis (Pettipher *et al*., 1997; Orr *et al*., 2000; Zierler *et al*., 2004; Gocmen *et al*., 2005). The mass spectrometer enables the simultaneous detection of the analyte and the determination of its structure (Wilson & Walker, 2005). Headspace (HS) SPME GC-MS was used by Zierler *et al*. (2004) and

HS GC-MS used by Smit (2009) for the detection of guaiacol in juices. Headspace-SPME is a variation on the SPME extraction procedure, where volatile compounds are collected in the headspace of the container before the absorption by the SPME fiber and then the fiber is transferred directly to the injection port of the GC–MS system for analysis (Zierler *et al*., 2004).

Reports of cinnamic acids in apple juice were previously based on semi-quantitative methods, including thin-layer chromatography or colorimetric procedures, however, with advances in analytical techniques, including diode array detection (DAD), characterisation of the phenolic profiles and the changes that occur during pasteurisation, enzymatic clarification, fining, bottling, concentration and storage can be identified more accurately (Spanos *et al.,* 1990). Diode array detectors are used with HPLC methods to record the complete absorption spectrum of each analyte and aid in the identification by scanning the complete wavelength spectrum of the eluent within 0.01 s and display a real time three-dimensional plot (Wilson & Walker, 2005). Reverse-phase (RP) HPLC with DAD is generally used to separate and quantify the phenolic compounds in grape juice (Spanos & Wrolstad, 1990), apple juice (Spanos *et al.,* 1990; Kermasha *et al.,* 1995; Hernandez *et al.,* 1997), pear juice (Schieber *et al*., 2001), peach juice (Hernandez *et al.,* 1997) and the purée and concentrates of peach and apple fruits (Bengoechea *et al*., 1997). RP HPLC applied a column consisting of a non-polar stationary phase, usually octadecyl silane (C_{18}) groups attached to silica. The mobile phase is relatively polar and water, aqueous buffers, methanol, acetonitrile, tetrahydrofuran or mixtures thereof are generally used (Wilson & Walker, 2005; Proestos *et al.,* 2008).

Although the analyses of the major phenolic compounds in apple juice are well documented (Wilson, 1981; Lea, 1982; Spanos *et al.,* 1990), only a few studies reported on the analyses procedure applied for free hydroxycinnamic and hydroxybenzoic acids, the minor phenolic compounds in apple juice (Kermasha *et al.,* 1995). Kermasha *et al*. (1995) quantified caffeic, chlorogenic, *p*-coumaric, coumaroylquinic and ferulic acid in appel juice with RP-HPLC. The detection limit of caffeic acid, chlorogenic acid and *p*-coumaric acid was 0.1 mg L^{-1} (at 320 nm) and of ferulic acid was 0.05 mg L^{-1} (at 320 nm). The major phenolic compounds, chlorogenic acid and coumaroylquinic acid were present at 31.12 - 52.04 mg L^{-1} and 18.49 - 29.21 mg L^{-1} (Kermasha *et al.,* 1995). These results are in agreement with the concentrations reported by Spanos *et al.* (1990). Of the minor phenolic compounds in apple juice, caffeic acid was present in a range of 2.06 - 3.00 mg L^{-1}, *p*-coumaric acid was present at concentrations lower than

2.00 mg L^{-1} and ferulic acid was present from 0.41 - 2.74 mg L^{-1} (Kermasha *et al.,* 1995). Since a considerable fraction of phenolic acids is in bound form, hydrolysis of glycosides or esters is needed before HPLC analysis to determine the concentration of the phenolic compounds in free and bound form (Lee & Widmer, 1996). Hernandez *et al.* (1997), Bengoechea *et al*. (1997) and Spanos *et al.* (1990) incorporated a sample extraction procedure in the analyses of phenolic compounds before HPLC analyses.

Gas chromatography-MS can also be used to identify phenolic acids, however, a silylation or trifluoroacetylation procedure must be incorporated for non-volatile compounds. Silylation or chemical derivatisation is the replacement of active hydrogen with a trimethylsilyl or fluoro group to reduce polarity (Proestos, *et al.,* 2008). The silylation procedure required to analyse non-volatile compounds with GC-MS is very sensitive and quantification is not easy to perform, while the RP-HPLC results are reproducible. Therefore, RP-HPLC can be use to analyse phenolic compounds without derivatisation of the compounds (Proestos *et al.,* 2008).

K. Conclusion

The widespread occurrence of *Alicyclobacillus* spp. as spoilage bacteria of fruit juice products is of great concern for the beverage industry. Since the discovery of *Alicyclobacillus* researchers have mostly focused on finding alternative methods of pasteurisation due to conventional pasteurisation methods not being adequate to prevent the growth of *Alicyclobacillus* spp. in fruit juices. Although numerous control methods have been investigated, it seems that this soil-borne bacterium is unavoidable in the fruit processing environment. These bacteria do not pose a health risk, however, the off-flavour guaiacol that may be produced by *Alicyclobacillus* species in the juice is of great importance. Therefore, the undesirable effect of guaiacol on the sensory characteristics of the fruit juice is of concern rather than the presence of the bacteria.

Numerous factors influence the production of guaiacol, which not only include the presence of *Alicyclobacillus* spp. that have the ability to produce guaiacol, but also the composition of the media. Manufactures subject fruit juice products to extreme treatments to destroy *Alicyclobacillus* endospores without confirming if the specific species have the ability to produce guaiacol. Little information exists on the metabolic pathway of *Alicyclobacillus* and the substrates specifically in fruit juice that is needed for guaiacol production. A better understanding of the precursor compounds of guaiacol production by *Alicyclobacillus* spp. and the spoilage potential of fruit juice could help control or prevent spoilage by *Alicyclobacillus.*

References

Agarwal, U.P. & Atalla, R.H. (1990). Formation and identification of *cis/trans* ferulic acid in photoyellowed white spruce mechanical pulp. *Journal of Wood Chemistry & Technology*, **10**, 169-190.

Albuquerque, L., Rainey, F.A., Chung, A.P., Sunna, A., Nobre, M.F., Grote, R., Antranikian, G. & Da Costa, M.S. (2000). *Alicyclobacillus hesperidum* sp. nov. and a related genomic species from solfataric soils of São Miguel in the Azores. *International Journal of Systematic and Evolutionary Microbiology*, **50**, 451-457.

Allgaier, H., Poralla, K. & Jung, G. (1985). ω-Cycloheptyl-α-hydroxyundecanoic acid, a new fatty acid from a thermophilic acidophilic *Bacillus* species. *Liebigs. Annalen der Chemie,* 378-382.

Álvarez-Rodríquez, M.L., Belloch, C., Villa, M., Uruburu, F., Larriba, G. & Coque, JJ. R. (2003). Degradation of vanillic acid and production of guaiacol by microorganisms isolated from cork samples. FEMS Microbiology Letters, **220**, 49-55.

Ander, P., Hatakka, A. & Eriksson, K.-E. (1980). Vanillic acid metabolism by the white-rot fungus *Sporotrichum pulverulentum*. *Archives of Microbiology*, **125**, 189-202.

Ander, P., Eriksson, K.E. & Yu, H.S. (1983). Vanillic acid metabolism by *Sporotrichum pulverulentum:* evidence for demethoxylation before ring-cleavage. *Archives of Microbiology*, **1136**, 1-6.

Andreoni, V., Galli, E. & Galliani, G. (1984). Metabolism of ferulic acid by a facultatively anaerobic strain of Pseudomonas cepacia. *Systematic and Applied Microbiology*, **5**, 299-303.

Anonymous (2005). Guaiacol detection kit. Biomedical products. Cosmo Bio Co., Ltd. [WWW document]. URL http://www.cosmobio.co.jp/export_e/products/kits/products_KYO_20050412.asp. 29 January 2007. (as cited by Smit, Y.).

Anonymous (2006). Product safety – the source for innovative microbiological detection media. DöhlerGroup Press Release. [WWW document]. URL http://www.drinksmediawire.com/upload/pdfs/cdp/DohlerGroup-ENG.pdf. 16 February 2010.

Anonymous (2008). *Emerging Opportunities in Fruit and Vegetable Juices: New markets, key innovation trends.* [WWW document]. URL http://www.marketresearch.com/product/display.asp?productid=2022490. 16 April 2010.

Anonymous (2010a). Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures) (DSMZ) Bacterial Nomenclature Up-to-Date. *Alicyclobacillus*. [WWW document]. URL http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=ALICYCLOBACILLUS&show_all_details=1. 7 December 2010.

Anonymous (2010b). Sigma-Aldrich South Africa. [WWW document]. URL. http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=252018|ALDRICH&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC. 10 December 2010.

Anonymous (2010c). Sigma-Aldrich South Africa. [WWW document]. URL. http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=D70201|ALDRICH&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC. 10 December 2010.

Bahçeci, K.S. & Acar, J. (2007). Determination of guaiacol produced by *Alicyclobacillus acidoterrestris* in apple juice by using HPLC and spectrophotometric methods, and mathematical modeling of guaiacol production. *European Food Research and Technology*, **225**, 873-878.

Bahçeci, K.S., Gökmen, V., Serpen, J. & Acar, J. (2003). The effects of different technologies on *Alicyclobacillus acidoterrestris* during apple juice production. *European Food Research and Technology*, **217**, 249-252.

Bahçeci, K.S., Gökmen, V. & Acar, J. (2005a). Formation of guaiacol from vanillin by *Alicyclobacillus acidoterrestris* in apple juice: a model study. *European Food Research and Technology*, **220**, 196-199.

Bahçeci, K.S., Serpen, A., Gökmen, V. & Acar, J. (2005b). Study of lipoxygenase and peroxidase as indicator enzymes in green beans: change of enzyme activity, ascorbic acid and chlorophylls during frozen storage. *Journal of Food Engineering*, **66**, 187-192.

Bengoechea, M.L., Sancho, A.I., Bartolom, B., Estrella, I., Gómez-Cordovés, C & Hernandez, M.T. (1997). Phenolic composition of industrially manufactured purées and concentrates from peach and apple fruits. *Journal of Agricultural and Food Chemistry,* **45**, 4071-4075.

Bevilacqua, A., Sinigaglia, M. & Corbo, M.R. (2008). *Alicyclobacillus acidoterrestris*: New methods for inhibiting spore germination. *International Journal of Food Microbiology*, **125**, 103-110.

Bieniek, G. (2003). Simultaneous determination of 2-methoxyphenol, 2-methoxy-4-methylphenol, 2,6-dimethoxyphenol and 4'-hydroxy-3'-methoxyacetophenone in urine by capillary gas chromatography. *Journal of Chromatography A*, **795**, 389-394.

Blocher, J.C. & Busta, F.F. (1983). Bacterial spore resistance to acid. *Food Technology*, **37**, 87-99.

Borlinghaus, A. & Engel, R. (1997). *Alicyclobacillus* incidence in commercial apple juice concentrate (AJC) supplies - method development and validation. *Fruit Processing*, **7**, 262-266.

Brown, K.L. (1995). New microbiological spoilage challenges in aseptic: *Alicyclobacillus acidoterrestris* spoilage in aseptically packed fruit juices. In: *Proceedings of International Symposium Advances in Aseptic Processing and Packaging Technologies,* Denmark, September 11-12. (edited by T. Ohlsson). Sweden: The Swedish Institute for Food Research.

Brown, K.L. (2000). Control of bacterial spores. *British Medical Bulletin*, **56**, 158-171.

Cerny, G., Hennlich, W. & Poralla, K. (1984). Spoilage of fruit juice by bacilli: isolation and characterisation of the spoiling microorganism. *Zeitschrift für Lebensmitteluntersuchung und -Forschung,* **179**, 224-227.

Cerny, G., Duong, H.A., Hennlich, W. & Miller, S. (2000). *Alicyclobacillus acidoterrestris*: influence of oxygen content on growth in fruit juices. *Food Australia*, **52**, 289-291.

Chang, S.S. & Kang, D.H. (2004). *Alicyclobacillus* spp. in the fruit juice industry: history, characteristics, and current isolation/detection procedures. *Critical Reviews in Microbiology*, **30**, 55-74.

Chang, S. & Kang, D.H. (2005). Development of novel *Alicyclobacillus* spp. isolation medium. *Journal of Applied Microbiology*, **99**, 1051-1060.

Chow, K.T., Pope, M.K. & Davis, J. (1999). Characterization of a vanillic acid non-oxidative decarboxylation gene cluster from *Streptomyces* sp. D7. *Microbiology,* **145**, 2393-2403.

Crawford, R.L. & Olson, P.P. (1978). Microbial catabolism of vanillate: decarboxylation to guaiacol. *Applied and Environmental Microbiology*, **36**, 539-543.

Darland, G. & Brock, T.D. (1971). *Bacillus acidocaldarius* sp. nov., and acidophilic thermophilic spore-forming bacterium. *Journal of General Microbiology*, **67**, 9-15.

De Maria, C.A.B., Trugo, L.C., Moreira, R.F.A. & Wemeck, C.C. (1994). Composition of green coffee fractions and their contribution to the volatile profile formed during roasting. *Food Chemistry,* **50,** 141-145.

De Rosa, M., Gambacorta, A., Minale, L. & Bu'Lock, J.D. (1971). Cyclohexane fatty acids from a thermophilic bacterium. *Journal of the Chemical Society, Chemical Communications*, 1334.

Deinhard, G., Blanz, P., Poralla, K. & Altan, E. (1987a). *Bacillus acidoterrestris* sp. nov., a new thermotolerant acidophile isolated from different soils. *Systematic and Applied Microbiology*, **10**, 47-53.

Deinhard, G., Saar, J., Krischke, W. & Poralla, K. (1987b). *Bacillus cycloheptanicus* sp. nov., a new thermoacidophile containing ω-cycloheptane fatty acids. *Systematic and Applied Microbiology*, **10**, 68-73.

Di Cesare, L. F.; Nani, R.; Forni, E. & Scotto, P. (1993). Influence of pectolytic enzymes on the flow rate and flavouring of peach juice. *Fruit Processing*, **7,** 254-257.

Doerge, D.R., Divi, R.L. & Churchwell, M.I. (1997). Identification of the colored guaiacol oxidation product produced by peroxidases. *Analytical Biochemistry*, **250**, 10-17.

Donaghy, J.A., Kelly, P.F. & McKay, A. (1999). Conversion of ferulic acid to 4-vinyl guaiacol by yeasts isolated from unpasteurised apple juice. *Journal of the Science of Food and Agriculture*, **79**, 453-456.

Dufresne, S., Bousquet, J., Boissinot, M. & Guay, R. (1996). *Sulfobacillus disulfdooxidans* sp. nov., a new acidophilic, disulfide-oxidizing, gram-positive spore-forming bacterium. *International Journal of Systematic Bacteriology*, **46**, 1056-1064.

Duong, H.A. & Jensen, N. (2000). Spoilage of iced tea by *Alicyclobacillus*. *Food Australia*, **52**, 292.

Durak, M.Z., Churey, J.J, Danyluk, M.D. & Worobo, R.W. (2010). Identification and haplotype distribution of *Alicyclobacillus* spp. from different juices and beverages. *International Journal of Food Microbiology,* **142**, 286-291.

Duvenagen, W. (2006). Detection and isolation of thermophilic acidophilic bacteria from fruit juice. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Duvenage, W., Gouws, P.A. & Witthuhn, R.C. (2007). PCR-based DGGE identification of bacteria present in pasteurised South African fruit juices. South African *Journal of Enology and Viticulture*, **28**, 56-60.

Eiora, M.N.U., Junqueira, V.C.A. & Schmidt, F.L. (1999). *Alicyclobacillus* in orange juice: occurrence and heat resistance of spores. *Journal of Food Protection*, **62**, 883-886.

Eisele, T.A. & Semon, M.J. (2005). Best estimated aroma and taste detection threshold for guaiacol in water and apple juice. *Journal of Food Science*, **70**, 267-269.

Fickert, B. & Schieberle, P. (1998). Identification of the key odorants in barley malt (caramalt) using GC/MS techniques and odour dilution analysis. *Nahrung,* **42**, 371.

Fiddler, W., Parker, W.E., Wasserman, A.E. & Doerr, R.C. (1967). Thermal decomposition of ferulic acid. *Journal of Agriculture & Food Chemistry*, **15**, 757-761.

Friend, J. (1981) Plant phenolics, lignifications and plant disease. In: *Progress in Phytochemistry,* Vol. 1 (edited by L. Reinhold, J.B. Harborne & T. Swain). Pp.197-261. UK: Pergamon Press.

Gocmen, D., Elston, A., Williams, T., Parish, M. & Rouseff, R.L. (2005). Identification of medicinal off-flavours generated by *Alicyclobacillus* species in orange juice using GC-olfactometry and GC-MS. *Letters in Applied Microbiology*, **40**, 172-177.

Gökmen, V., Artik, N., Acar, J., Kahraman, N. & Poyrazoğlu, E. (2001). Effect of various clarification treatments on patulin, phenolic compounds and organic acid compositions of apple juice. *European Food Research and Technology*, **213**, 194-199.

Goodner, K.L., Jella, P. & Rouseff, R. (2000). Determination of vanillin in orange, grapefruit, tangerine, lemon, and lime juices using GC-Olifactometry and GC-MS/MS. *Journal of Agricalture and Food Chemistry*, **48**, 2882-2886. Goto, K., Matsubara, H., Mochida, K., Matsumura, T., Hara, Y., Niwa, M. & Yamasato, K. (2002). *Alicyclobacillus herbarius* sp. nov., a novel bacterium containing ω-cycloheptane fatty acids, isolated from herbal tea. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 109-113.

Goto, K., Matsubara, H., Mochida, K., Matsumura, T., Hara, Y., Niwa, M. & Yamasato, K. (2002). *Alicyclobacillus herbarius* sp. nov., a novel bacterium containing ω-cycloheptane fatty acids, isolated from herbal tea. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 109-113.

Goto, K., Mochida, K., Asahara, M., Suzuki, M., Kasai, H. & Yokota, A. (2003). *Alicyclobacillus pomorum* sp. nov., a novel thermo-acidophilic, endospore-forming bacterium that does not possess ω-alicyclic fatty acids, and emended description of the genus *Alicyclobacillus*. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 1537-1544.

Goto, K., Mochida, K., Kato, Y., Asahara, M., Fujita, R., An, S.-Y., Kasai, H. & Yokota, A. (2007). Proposal of six species of moderately thermophilic, acidophilic, endospore-forming bacteria: *Alicyclobacillus contaminans* sp. nov., *Alicyclobacillus fastidiosus* sp. nov., *Alicyclobacillus kakegawensis* sp. nov., *Alicyclobacillus macrosporangiidus* sp. nov., *Alicyclobacillus sacchari* sp. nov. and *Alicyclobacillus shizuokensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, **57**, 1276-1285.

Goto, K., Nishibori, A., Wasada, Y., Furuhata, K., Fukuyama, M. & Hara, M. (2008). Identification of thermo-acidophilic bacteria isolated from the soil of several Japanese fruit orchards. *Letters in Applied Microbiology*, **46**, 289-294.

Graf, E. (1992). Antioxidant potential of ferulic acid. *Free Radical Biology & Medicine,* **13**, 435-448.

Gross, G.G. (1985). Biosynthesis and metabolism of phenolic acids. In: *Biosynthesis and Biodegradation of Wood Components* (edited by T. Higuchi). Pp. 229-271. New York: Academic Press.

Groenewald, W.H., Gouws, P.A. & Witthuhn, R.C. (2008). Isolation and identification of species of *Alicyclobacillus* from orchard soil in the Western Cape, South Africa. *Extremophiles*, **12**, 159-163.

Groenewald, W.H., Gouws, P.A., & Witthuhn, R.C. (2009). Isolation, identification and typification of *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius* strains from orchard soil and the fruit processing environment in South Africa. *Food Microbiology*, **26**, 71-76.

Hatakka, A.I. (1985). Degradation of veratric acid and other lignin-related aromatic compounds by the white-rot fungus *Pycnoporus cinnabarinus. Archives of Microbiology*, **141**, 22-28.

Henderson, M.E.K. (1961). The metabolism of aromatic compounds related to lignin by some hyphomycetes and yeastlike fungi of soil. *Journal of General. Microbiology,* **26**, 155-165.

Herrmann, K. (1989). Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *CRC Critical Review in Food Science and Nutrition,* **28**, 315-347.

Hernandez, T., Ausin, N., Bartolomé, B., Bengoechea, L., Estrella, I. & Gómez-Cordovés, C. (1997). Variations in the penolic composition of fruit juices with different treatments. *Zeitschrift für Lebensmitteluntersuchung und –Forschung A,* **204**, 151-155.

Higuchi, T. (1990). Lignin biochemistry: Biosynthesis and biodegradation. *Wood Science & Technology,* **24**, 23-63.

Higuchi, T., Shimada, M., Nakatsubo, F. & Tanahashi, M. (1977). Differences in biosynthesis of guaiacyl and syringyl lignins in woods. *Wood Science and Technology,* **11**, 153-167.

Hippchen, B., Röll, A. & Poralla, K. (1981). Occurrence in soil of thermo-acidophilic bacilli possessing ω-cyclohexane fatty acids and hopanoids. *Archives of Microbiology*, **129**, 53-55.

Howard, I. (2006). ACB Workshop October 2005 - Review. European Quality Control System (EQCS) Workshop 2006. [WWW document]. URL http://www.eqcs.org/download/Workshop2006/07_ACBworkshop2005.pdf. 1 April 2010.

Huang, Z., Dostal, L. & Rosazza, J.P.N. (1993). Mechanisms of ferulic acid conversions to vanillic acid and guaiacol by *Rhodotorula rubra*. *The Journal of Biological Chemistry*, **268**, 23954-23958.

Imperio, T., Viti, C. & Marri, L. (2008). *Alicyclobacillus pohliae* sp. nov., a thermophilic, endospore-forming bacterium isolated from geothermal soil of the north-west slope of Mount Melbourne (Antarctica). *International Journal of Systematic and Evolutionary Microbiology*, **58**, 221-225.

International Federation of Fruit Juice Producers (IFU). (2007). Method on the detection of taint producing *Alicyclobacillus* in fruit juices. *IFU Method No. 12*. Pp. 1-11. IFU, Paris.

Ishikawa, H., Schubert, W.J. & Nord, E.F. (1963). The degradation by *Polyporus versicolor* and *Fomes fomentarius* of aromatic compounds structurally related to soft wood lignin. *Archives of Biochemistry and. Biophysics,* **100**, 140-149.

Jensen, N. (1999). *Alicyclobacillus* - a new challenge for the food industry. *Food Australia*, **51**, 33-36.

Jensen, N. (2000). *Alicyclobacillus* in Australia. *Food Australia*, **52**, 282-285.

Jensen, N. (2005). Evaluation of detection methods for alicyclobacilli in fruit juice concentrates in Australia. In: *14th International Federation of Fruit Juice Producers (IFU) Congress*, Beijing, China. 14-18 August 2005.

Jensen, N. & Whitfield, F.B. (2003). Role of *Alicyclobacillus acidoterrestris* in the development of a disinfectant taint in shelf-stable fruit juice. *Letters in Applied Microbiology*, **36**, 9-14.

Jensen, N., Varelis, P. & Whitfield, F.B. (2001). Formation of guaiacol in chocolate milk by the psychrotrophic bacterium *Rahnella aquatilis*. *Letters in Applied Microbiology*, **33**, 339-343.

Jiang, C.Y., Liu, Y., Liu, Y.Y., You, X.Y., Guo, X. & Liu S.J. (2008). *Alicyclobacillus ferrooxydans* sp. nov., a ferrous-oxidizing bacterium from solfataric soil. *International Journal of Systematic and Evolutionary Microbiology*, **58**, 2898-2903.

Joshi, V.K., Chauhan, S.K. & Lal, B.B. (1991). Extraction of juices from peaches, plums and apricots by pectinolytic treatment. *Journal of Food Science & Technology*, **28**, 64-65.

Kannenberg, E., Blume, A. & Poralla, K. (1984). Properties of ω-cyclohexane fatty acids in membranes. *FEBS Letters*, **172**, 331-334.

Karavaiko, G.I., Bogdanova, T.I., Tourova, T.P., Kondrat'eva, T.F., Tsaplina, I.A., Egorova, M.A., Krasil'nikova, E.N. & Zakharchuk, L.M. (2005). Reclassification of '*Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans*' strain K1 as *Alicyclobacillus tolerans* sp. nov. and *Sulfobacillus disulfidooxidans* Dufresne *et al*. 1996 as *Alicyclobacillus disulfidooxidans* comb. nov., and emended description of the genus *Alicyclobacillus*. *International Journal of Systematic and Evolutionary Microbiology*, **55**, 941-947.

Karmakar, B., Vohra, R.M., Nandanwar, H., Sharma, P., Gupta, K.G. & Sobti, R.C. (2000). Rapid degradation of ferulic acid via 4-vinylguaiacol and vanillin by a newly isolated strain of *Bacillus coagulans*. *Journal of Biotechnology*, **80**, 195-202.

Kawakami, H. (1980). Degradation of lignin-related aromatic compounds and lignins by several *Pseudomonas.* In: *Lignin biodegradation* (edited by T.K. Kirk, T. Higuchi and H.-M. Chang). Pp. 103-125, Boca Raton: CRC Press.

Kermasha, S, Goetghebeur, M., Dumod, J. & Couturec, R. (1995). Analyses of phenolic and furfural compounds in concentrated and non-concentrated apple juices. *Food Research International,* **28**, 245-252.

Komitopoulou, E., Boziaris, I.S., Davies, E.A., Delves-Broughton, J. & Adams, M.R. (1999). *Alicyclobacillus acidoterrestris* in fruit juices and its control by nisin. *International Journal of Food Science and Technology*, **34**, 81-85.

Krischke, W. & Poralla, K. (1990). Properties of *Bacillus acidocaldarius* mutants deficient in ω-cyclohexyl fatty acid biosynthesis. *Archives of Microbiology*, **153**, 463-469.

Lea, A.G.H. (1982). Reversed-phase high performance liquid chromatography of procyanidins and other phenolics in fresh and oxidising apple juices using a pH shift technique. *Journal of Chromatography,* **238**, 253-257.

Lee, H.S. & Widmer, B.W. (1996). Phenolic compounds. In: *Handbook of Food Analysis* (edited by L.M.L. Nollet). Pp. 821-894, New York: Marcel Dekker.

Lusardi, C., Previdi, M.P., Colla, F. Barbieri, G. & Bolzoni, L. (2000). Ability of *Alicyclobacillus* strains to spoil fruit juices and nectars. *Industria Conserve,* **75**, 151-161.

Macheix, J.J., Fleuriet, A. & Billot, J. (1990). The main phenolics of fruit. In: *FruitPhenolics* (edited by J.J. Macheix, A. Fleuriet & J. Billot). Pp. 1-98. Boca Raton: CRC Press.

Mangas, J.J, Rodríguez, R., Moreno, J., Suárez, B. & Blanco, D. (1997). Furanic and phenolic composition of cider brandy. A chemometric study. *Journal of Agricaltural and Food Chemistry*, **45**, 4076-4079.

Martin, A.B., Acree, T.E., Hotchkiss, J.H. & Nagy, S. (1992). Gas chromatography-Olfactometry of orange juice to assess the effects of plastic polymers on aroma character. *Journal of Agricaltural and Food Chemistry*, **40**, 650-654.

Martin-Tanguy, J. (1985). The occurrence and possible function of hydroxylcinnamoyl acid amides in plants. *Plant Growth Regulation,* **3**, 381-399.

Mathew, S., Abraham, T.E. & Sudheesh, S. (2007). Rapid conversion of ferulic acid to 4-vinylguaiacol and vanillin metabolites by *Debaryomyces hansenii*. *Journal of Molecular Catalysis B: Enzymatic*, **44**, 48-52.

Matsubara, H., Goto, K., Matsumura, T., Mochida, K., Iwaki, M., Niwa, M. & Yamasato, K. (2002). *Alicyclobacillus acidiphilus* sp. nov., a novel thermo-acidophilic, ω-alicyclic fatty acid-containing bacterium isolated from acidic beverages.

International Journal of Systematic and Evolutionary Microbiology, **52**, 1681-1685.

Mayer, F., Czerny, M. & Grosch, W. (1999). Influence of provenance and roast degree on the composition of potent odorants in Arabica coffees. *European Food Research and Technology,* **209**, 242-250.

McIntyre, S., Ikawa, J.Y., Parkinson, N., Haglund, J. & Lee, J. (1995). Characteristics of an acidophilic bacillus strain isolated from shelf-stable juices. *Journal of Food Protection*, **58**, 319-321.

Montgomery, M.W. & Petropakis, H.J. (1980). Inactivation of bartlett pear polyphenol oxidase with hea

Murdock, D.I. (1976). Fruit drinks, juices, and concentrates. In: *Compendium of Methods for the Microbiological Examination of Foods,* 2nd ed. (edited by M.I. Speck). Pp. 563-567. Washington, D.C.: American Public Health Association.

Murray, M.B., Gurtler, J.B., Ryu, J., Harrison, M.A. & Beuchat, L.R. (2007). Evaluation of direct plating methods to enumerate Alicyclobacillus in beverages. International Journal of Food Microbiology, 115, 59-69.

Nazareth, S. & Mavinkurve, S. (1986). Degradation of ferulic acid via 4-vinylguaiacol by *Fusarium solani* (Mart.) Sacc. *Canadian Journal of Microbiology*, **32**, 494-497.

Niwa, M. (2004). Control of hazardous bacteria in acidic beverages by using a guaiacol detection kit (peroxidase method). [WWW document]. URL http://www.cosmobio.co.jp/export_e/products/kits/products_KYO_20050412/Guaiakol_detection_kit_reference.pdf. 19 February 2009.

Niwa, M. & Kawamoto, A. (2003). Development of a rapid detection method of *A. acidoterrestris*, hazardous bacteria to acidic beverage. *Fruit Processing*, **13**, 102-107.

Niwa, M. & Kuriyama, A. (2003*). A. acidoterrestris* rapid detection kit. *Fruit Processing*, **13**, 328-331.

Orr, R.V. & Beuchat, L.R. (2000). Efficiency of disinfectants in killing spores of *Alicyclobacillus acidoterrestris* and performance of media for supporting colony development by survivors. *Journal of Food Protection*, **63**, 1117-1122.

Orr, R.V., Shewfelt, R.L., Huang, C.J., Tefera, S. & Beuchat, L.R. (2000). Detection of guaiacol produced by *Alicyclobacillus acidoterrestris* in apple juice by sensory and chromatographic analyses, and comparison with spore and vegetative cell populations. *Journal of Food Protection*, **63**, 1517-1522.

Oshima, M. & Ariga, T. (1975). ω-Cyclohexyl fatty acids in acidophilic thermophilic bacteria - studies on their presence, structure and biosynthesis using precursors labeled with stable isotopes and radioisotopes. *The Journal of Biological Chemistry*, **250**, 6963-6968.

Palop, A., Álvarez, I., Raso, J. & Condón, S. (2000). Heat resistance of *Alicyclobacillus acidocaldarius* in water, various buffers and orange juice. *Journal of Food Protection*, **63**, 1377-1380.

Peleg, H., Striem, B.J., Naim, M. & Zehavi, U. (1988). Phenolic compounds contributing to off-flavors in citrus products. In: *Proceedings of the International Citrus Congress*, Tel Aviv, March 6-11 (edited by R. Goren, & K. Mendel). Weikershim: Balaban, **4**, Pp 1743-1748. (as cited by Peleg *et al.,* 1992).

Peleg, H., Naim, M., Zehavi, U., Rouseff, R.L. & Nagy, S. (1992). Pathways of 4-vinylguaiacol formation from ferulic acid in model solutions of orange juice. *Journal of Agricultural and Food Chemistry,* **40**, 764-767.

Pérez-Ilzarbe, F.J., Hernández, T. & Estrella, I. (1991). Phenolic compounds in apples: varietal differences. *Zeitschrift für Lebensmitteluntersuchung und -Forschung*, **192**, 551-554.

Pettipher, G.L. & Osmundson, M.E. (2000). Methods for the detection, enumeration and identification of *Alicyclobacillus acidoterrestris*. *Food Australia*, **52**, 293-295.

Pettipher, G.L., Osmundson, M.E. & Murphy, J.M. (1997). Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit juice-containing drinks. *Letters in Applied Microbiology*, **24**, 185-189.

Piacquadio, P., De Stefano, G. & Sciancalepore, V. (2002). Phenolic composition of apple juice treated by laccase immobilized on copper-chelate regenerable carrier. *Italian Journal of Food Science*, **14**, 291-294.

Pinhatti, M.E.M.C., Variane, S., Eguchi, S.Y. & Manfio, G.P. (1997). Detection of acidothermophilic bacilli in industrialized fruit juices. *Fruit Processing*, **9**, 350-353.

Pollnitz, A.P., Pardon, K.H., Sykes, M. & Sefton, M.A. (2004). The effects of sample preparation and gas chromatograph injection techniques on the accuracy of measuring guaiacol, 4-methylguaiacol and other volatile oak compounds in oak extracts by stable isotope dilution analyses. *Journal of Agricultural and Food Chemistry,* **52**, 3244-3252.

Pometto, A.L., Sutherland, J.B. & Crawford, D.L. (1981). *Streptomyces setonii*: catabolism of vanillic acid via guaiacol and catechol. *Canadian Journal of Microbiology,* **27**, 636-638.

Pontius, A.J., Rushing, J.E. & Foegeding, P.M. (1998). Heat resistance of Alicyclobacillus acidoterrestris spores as affected by various pH values and organic acids. Journal of Food Protection, 61, 41-46.

Poralla, K. & König, W.A. (1983). The occurrence of ω-cycloheptane fatty acids in a thermo-acidophilic bacillus. *FEMS Microbiology Letters*, **16**, 303-306.

Poralla, K., Kannenberg, E. & Blume, A. (1980). A glycolipid containing hopane isolated from the acidophilic, thermophilic *Bacillus acidocaldarius*, has a cholesterol-like function in membranes. *FEBS Letters*, **113**, 107-110.

Proestos, C., Sereli, D. & Komaitis, M. (2006). Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. *Food Chemistry,* **95**, 44-52.

Proestos, C., Kapsokefalou, M. & Komaitis, M. (2008). Analysis of naturally occurring phenolic compounds in aromatic plants by RP-HPLC and GC-MS after silylation. *Journal of food chemistry,* **31,** 402-414.

Pyysalo, T., Honkanen, E. & Hirvi, T. (1979). Volitiles of wild strawberries, *Fragaria vesca* L., compared to those of cultivated berries, *Fragaria x ananassa* cv. Senga Sengana. *Journal of Agricaltural and Food Chemistry*, **27**, 19-22.

Rahouti, M., Seigle-Murandi, F., Steiman, R. & Eriksson, K-E. (1989). Metabolism of ferulic acid by *Paecilomyces variotii* and *Pestalotia palmarum*. *Applied and Environmental Microbiology*, **55**, 2391-2398.

Reshke, A. & Herrmann, K. (1981). Occurrence of 1-O-hydroxycinnamyl 8-D-glucose derivatives in fruits. *Zeitschrift für Lebensmitteluntersuchung und –Forschung,* **173**, 458-463.

Risch, B., Herrmann, K., Wray, V. & Grotjahn, L. (1987). 2'-(E)-O-p-Coumaroylgalactaric acid and 2'-(E)-O-feruloylgalactaric in citrus. *Phytochemistry*, **26**, 509-510.

Risch, B., Herrmann, K., & Wray, V. (1988). (E)-O-p-Coumaroyl-,(E)-O-feruloyl-derivatives of glucaric acid in citrus. *Phytochemistry*, **27**, 3327-3329.

Rosazza, J.P.N., Huang, Z., Dostal, L., Volm, T. & Rousseau, B. (1995). Review: Biocatalytic transformations of ferulic acid: an abundant aromatic natural product. *Journal of Industrial Microbiology*, **15**, 457-471.

Sakho, M., Chassagne, D. & Crouzet, J. (1997). African mango glycosidically bound volatile compounds. *Journal of Agricaltural and Food Chemistry*, **45**, 883-888.

Saxby, M.J. (1993). A survey of chemicals causing taints and off-flavours in foods. In: *Food taints and off-flavours,* 1st ed. (edited by M.J. Saxby). p 40. London, UK: Blackie Academic & Professional.

Schieber, A., Keller, P. & Carle, R. (2001). Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *Journal of Chromatography A*, **910**, 265–273.

Schols, H.A., Veld, P.H., Van Deelen, W. & Vorgen, A. G. J. (1991). The effect of the manufacturing method on the characteristics of apple juice. *Zeitschrift für Lebensmitteluntersuchung und –Forschung*, **192**, 142-148.

Schultz, J.M. & Herrmann, K. (1980). Analysis of hydroxybenzoic and hydroxycinnamic acids in plant material. *Journal of Chromatography,* **195**, 85-89.

Siegmund, B. & Pöllinger-Zierler, B. (2006). Odor thresholds of microbially induced off-flavor compounds in apple juice. *Journal of Agricultural and Food Chemistry*, **54**, 5984-5989.

Simbahan, J., Drijber, R. & Blum, P. (2004). *Alicyclobacillus vulcanalis* sp. nov., a thermophilic, acidophilic bacterium isolated from Coso Hot Springs, California, USA. *International Journal of Systematic and Evolutionary Microbiology*, **54**, 1703-1707.

Simpson, R.F., Amon, J.M. & Daw, A.J. (1986). Off-flavour in wine caused by guaiacol. *Food Technology in Australia*, **38**, 31-33.

Smit, Y. (2009). Growth and guaiacol production of species of *Alicyclobacillus* isolated from the South African fruit processing environment. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Spanos, G.A. & Wrolstad, R.E. (1990). Influence of processing and storage on the phenolic composition of Thompson Seedless grape juice. *Journal of Agricultural and Food Chemistry,* **38**, 1565-1571.

Spanos, G.A. & Wrolstad, R.E. (1992). Phenolics of apple, pear, and white grape juices and their changes with processing and storage. A review. *Journal of Agricultural and Food Chemistry,* **40**, 1478-1487.

Spanos, G.A., Wrolstad, R.E. & Heatherhell, D.A. (1990). Influence of processing, and storage on the phenolic composition of apple juice. *Journal of Agricultural and Food Chemistry,* **38**, 1572-1579.

Spillman, P.J., Pollnitz, A.P., Liacopoulos, D., Skouroumounis, K. & Sefton, M.A. (1997). Accumulation of vanillin during barrel-aging of white, red, and model wines. *Journal of Agricaltural and Food Chemistry*, **45**, 2584-2589.

Splittstoesser, D.F.& Churey, J.J. (1996). Unique spoilage organisms of musts and wines. In: *Wine spoilage microbiology Confrence* (edited by T. Toland & K.C. Fugelsang). Pp. 36-41. California State University, Fresno.

Splittstoesser, D.F. & Mundt, J.O. (1974). Fruit and vegetables. In: *Compendium of Methods for the Microbiological Examination of Foods,* 2nd ed. (edited by M.I. Speck). Pp. 556-562. Washington, D.C.: American Public Health Association.

Splittstoesser, D.F., Churey, J.J. & Lee, C.Y. (1994). Growth characteristics of aciduric sporeforming bacilli isolated from fruit juices. *Journal of Food Protection*, **57**, 1080-1083.

Splittstoesser, D.F., Lee, C.Y. & Churey, J.J. (1998). Control of *Alicyclobacillus* in the juice industry. *Dairy, Food and Environmental Sanitation*, **18**, 585-587.

Sutherland, J.B., Crawford, D.L. & Pometto, A.L. (1983). Metabolism of cinnamic, *p*-coumaric and ferulic acid by *Streptomyces setonii. Canadian. Journal of Microbiology,* **29**, 1253.

Tadasa, K. (1977). Degradation of eugenol by a microorganism. Agricultural *and Biological Chemistry*, **41**, 925-929.

Toms, A. & Wood, J.M. (1969). The degradation of trans-ferulic acid by *Pseudomonas acidovorans. Biochemistry,* **9**, 337.

Topakas, E., Kalogeris, E., Kekos, D., Macris, B.J. & Christakopoulos, P. (2003). Bioconversion of ferulic acid into vanillic acid by the thermophilic fungus *Sporotrichum thermophile*. *Lebensmittel-Wissenschaft und -Technologie*, **36**, 561-565.

Treuter, D. (2001). Biosynthesis of phenolic compounds and its regulation in apple. *Plant Growth Regulation,* **34**, 71-89.

Tsuruoka, N., Isono, Y., Shida, O., Hemmi, H., Nakayama, T. & Nishino, T. (2003). *Alicyclobacillus sendaiensis* sp. nov., a novel acidophilic, slightly thermophilic species isolated from soil in Sendai, Japan. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 1081-1084.

Uchino, F. & Doi, S. (1967). Acido-thermophilic bacteria from thermal waters. *Agricultural and Biological Chemistry*, **31**, 817-822.

Van Buren, J., de Vos, L. & Pihtik, W. (1976). Polyphenols in golden delicious apple juices in relation to method of preparation. *Journal of Agricultural and Food Chemistry,* **24**, 448-51.

Vicuña, R., González, B., Mozuch, M.D. & Kirk, T.K. (1987). Metabolism of lignin model compounds of the arylglycerol-β-aryl ether type by *Pseudomonas acidovorans* D_3. *Applied and Environmental Microbiology,* **53**, 2605-2609

Walker, M. & Phillips, C.A. (2005). The effect of intermittent shaking, headspace and temperature on the growth of *Alicyclobacillus acidoterrestris* in stored apple juice. *International Journal of Food Science and Technology*, **40**, 557-562.

Walker, M. & Phillips, C.A. (2008). *Alicyclobacillus acidoterrestris:* an increasing threat to the fruit juice industry? *International Journal of Food Science and Technology,* **43**, 250-260.

Walls, I. & Chuyate, R. (1998). *Alicyclobacillus* - historical perspective and preliminary characterization study. *Dairy, Food and Environmental Sanitation*, **18**, 499-503.

Wardencki, W., Michulee, M. & Curylo, J. (2004). A review of theoretical and practical aspects of solid-phase microextraction in food analysis. *International Journal of Food Science and Technology*, **39**, 703-717.

Wasserman, A.E. (1966). Organoleptic evaluation of three phenols present in wood smoke. *Journal of Food Science*, 31, 1005-1010.

Wheaton, T.A. & Stewart, I. (1965). Feruloylputrescine: isolation and identification from citrus leaves and fruits. *Nature*, **206**, 620-621.

Whitfield, F.B. (1998). Microbiology of food taints. *International Journal of Food Science and Technology*, **33**, 31-51.

Wilson, E.L.J. (1981). High-pressure liquid chromatography of apple juice phenolic compounds. *Journal of the Science of Food and Agriculture,* **32**, 257-264.

Wilson, K. & Walker, J. (2005). Chromatographic technigues. In: *Principles and Techniques of Biochemistry and Molecular Biology,* 6th ed. Pp. 485-550. USA: Cambridge University Press.

Wisotzkey, J.D., Jurtshuk, P., Fox, G.E., Deinhard, G. & Poralla, K. (1992). Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. *International Journal of Systematic Bacteriology*, **42**, 263-269.

Wisse, C.A. & Parish, M.E. (1998). Isolation and enumeration of sporeforming, thermo-acidophilic, rod-shaped bacteria from citrus processing environments. *Dairy, Food and Environmental Sanitation,* **18**, 504-509.

Witthuhn, R.C., Duvenhage, W. & Gouws, P.A. (2007). Evaluation of different growth media for the recovery of the species of *Alicyclobacillus*. *Letters in Applied Microbiology*, **45**, 224-229.

Yamazaki, K., Teduka, H. & Shinano, H. (1996). Isolation and identification of *Alicyclobacillus acidoterrestris* from acidic beverages. *Bioscience, Biotechnology, and Biochemistry*, **60**, 543-545.

Yamazaki, K., Murakami, M., Kawai, Y., Inoue, N. & Matsuda, T. (2000). Use of nisin for inhibition of *Alicyclobacillus acidoterrestris* in acidic drinks. *Food Microbiology*, **17**, 315-320.

Zierler, B., Siegmund, B. & Pfannhauser, W. (2004). Determination of off-flavour compounds in apple juice caused by microorganisms using headspace solid phase microextraction-gas chromatography-mass spectrometry. *Analytica Chimica Acta*, **520**, 3-11.

CHAPTER 3

SEPARATION AND QUANTIFICATION OF GUAIACOL, VANILLIC ACID, VANILLIN AND FERULIC ACID WITH HPLC

ABSTRACT

Guaiacol is accepted as the predominant metabolite associated with taint production in fruit juices through the metabolic activity of *Alicyclobacillus* spp. According to the suggested pathway, the phenolic acids vanillic acid, vanillin and ferulic acid are the precursors of guaiacol production. The aim of this study was to develop a high performance liquid chromatography (HPLC) method to quantify guaiacol and the phenolic acid compounds. Different elution programs were tested to obtain a method that could simultaneously separate the three precursors and guaiacol present in *Bacillus acidoterrestris* (BAT) broth. A HPLC system with UV-diode array detection (DAD) fitted with a silica C_{18} column (25 cm x 4.6 mm x 5 µm) was used. The injection volume was set at 2 µL, the flow rate at 1 mL min^{-1}, loop injection at 50 µL and the run pressure at 3 000 psi. The column was operated at a temperature of 40 °C. The mobile phase consisted of 5 % (v/v) formic acid in water (solvent A) and methanol as solvent B. Different elution programs were tested and the best results were obtained with the mobile phase consisting of 50 % solvent A and 50 % solvent B. Simultaneous UV detection was performed at three wavelengths, namely 272 nm, 312 nm and 328 nm. Vanillic acid had a retention time of 4.8 - 4.9 min (272 nm), followed by vanillin with a retention time of 5.7 - 5.8 min (312 nm), ferulic acid at 6.2 - 6.5 min (328 nm) and guaiacol at 8.1 - 8.3 min (272 nm). This optimised HPLC method was able to successfully separate and quantify the 3 precursors and guaiacol known to be involved in the spoilage of fruit juice products by *Alicylobacillus* spp.

INTRODUCTION

The taint chemical guaiacol is accepted as the predominant metabolite produced during the metabolic activity of *Alicyclobacillus* spp. present in contaminated fruit juices (Pettiper *et al.,* 1997; Jensen, 2000; Jensen & Whitfield, 2003). According to the proposed pathway for guaiacol production, phenolic acids vanillic acid, vanillin and ferulic acid are precursors of guaiacol (Crawford & Olsen, 1978; Pometto *et al.,* 1981; Huang *et al.,* 1993; Rosazza *et al.,* 1995; Niwa & Kawamoto, 2003). Guaiacol and can

be detected by using sensory (Orr *et al.,* 2000; Eisele & Semon, 2005) and chemical (Bahçeci & Acar, 2007) methods, while both guaiacol and the precursors can be detected using contemporary analytical methodologies (Spanos *et al.,* 1990; Kermasha *et al.,* 1995; Hernandez *et al.,* 1997; Zierler *et al*., 2004; Bahçeci *et al*., 2005).

Chemical and sensory detection is generally applied as rapid, inexpensive methods for guaiacol screening (Chang & Kang, 2004). The chemical method, also described as the peroxidase method, is based on a chemical reaction between the peroxidase enzyme and hydrogen peroxide in the presence of guaiacol yielding a colour or absorbance change (Niwa, 2004; Bahçeci & Acar, 2007). Sensory methods make use of olfactory senses to detect the presence of guaiacol (Chang & Kang, 2004). Experienced panels are used to determine the recognition threshold of guaiacol odour (Orr *et al.,* 2000) and taste by using a forced-choice ascending concentration method of limits (Eisele & Semon, 2005). The sensory threshold of guaiacol is low and consumers without formal sensory training will generally not be able to detect the presence of guaiacol in fruit juices (Chang & Kang, 2004).

Analytical methods used for the quantification of guaiacol (Gökmen *et al*., 2001; Chang & Kang, 2004; Bahçeci & Acar, 2007) usually include extensive sample preparation, chromatographic separation and detection. The most common chromatographic procedures for guaiacol separation are liquid chromatography (Bahçeci *et al*., 2005; Bahçeci & Acar, 2007) and gas chromatography (GC) (Pettipher *et al*., 1997; Jensen *et al*., 2001; Pollnitz *et al*., 2004; Zierler *et al*., 2004; Gocmen *et al*., 2005). High performance liquid chromatography (HPLC) is a precise, accurate and sensitive method for the detection of guaiacol in fruit juices (Bahçeci & Acar, 2007). Zierler *et al*. (2004) developed a headspace solid phase microextraction GC mass spectrometry (HS-SPME GC-MS) method for the detection of guaiacol produced by *A. acidoterrestris* in apple juice. In this procedure, volatile compounds are collected in the headspace of the container before absorption to a SPME fiber. The fiber is then transferred directly to the injection port of the GC–MS system to analyse the volatile compounds (Zierler *et al*., 2004). A HS GC-MS method was developed by Smit (2009) for the detection of guaiacol in *Bacillus acidoterrestris* (BAT) broth. HS GC-MS requiring minimal sample preparation and provide a very accurate and sensitive detection system.

Gas chromatography (Proestos *et al.,* 2008) and HPLC methods (Spanos & Wrolstad, 1990; Spanos *et al.,* 1990; Kermasha *et al.,* 1995; Bengoechea *et al.,* 1997; Proestos *et al.,* 2006; Bahçeci & Acar, 2007) are also used for the analysis of the

phenolic acids, ferulic acid, vanillin and vanillic acid. In the identification of phenolic acids with GC-MS a silylation or trifluoroacetylation procedure is incorporated to analyse non-volatile and thermolable compounds. The silylation is very sensitive and quantification of compounds may not be easy. Reverse-phase (RP) HPLC coupled with UV spectrophotometry is also widely used to separate and quantify phenolic compounds, including caffeic acid, *p*-coumaric acid, ferulic acid and vanillic acid (Proestos *et al.,* 2008). Therefore, with RP-HPLC the phenolic compounds can be detected without derivatisation and the results are reproducible (Proestos, *et al.,* 2008). Reverse-phase (RP) HPLC with UV-diode array detection (DAD) has been used to separate and quantify the phenolic compounds caffeic acid, *p*-coumaric acid and ferulic acid in fruit juices, such as grape juice (Spanos & Wrolstad, 1990), apple and peach juices (Spanos *et al.,* 1990; Kermasha *et al.,* 1995; Hernandez *et al.,* 1997) and the purée and concentrates of peach and apple (Bengoechea *et al*., 1997).

Little information exists on the metabolic pathway of guaiacol production by *Alicyclobacillus* spp. In the evaluation of the substrates required for guaiacol production an analytical method is required to quantify guaiacol and the precursors. A HPLC-DAD method was used by Bahçeci *et al.* (2005) and by Bahçeci and Acar (2007) to analyse the vanillin and guaiacol concentrations in apple juice and the same HPLC method was used by Gökmen *et al.* (2001) to identify and quantify ferulic acid and other phenolic compounds. The aim of this study was to optimise a HPLC method to simultaneously separate and quantify guaiacol and the phenolic acid compounds that are possible precursors of guaiacol production by *A. acidoterrestris.*

MATERIALS AND METHODS

Bacterial cultures

Alicyclobacillus acidoterrestris FB2 isolated from pear concentrate (Department of Food Science Culture Collection, Stellenbosch University) (Groenewald *et al*., 2009) was used in this study. A pure culture of *A. acidoterrestris* FB2 (vegetative cells and endospores) was inoculated into *Bacillus acidoterrestris* (BAT) broth (IFU, 2007) adjusted to pH 4 using 1 M H_2SO_4 (Merck) and sterilised by autoclaving. The culture was incubated at 45°C for 2 d and 1 mL of the 48 h culture was aseptically removed and added to sterile BAT broth (pH 4), followed by incubation at 45 °C for 5 d. After incubation the inoculated broth was centrifuged in a Beckman Coulter TJ-25 centrifuge (Beckman Coulter Inc., Fullerton, California, USA) at 5 500 *g* for 10 min and the

supernatant discarded while the pellet was resuspended in sterile saline solution (SSS) (0.85% (m/v) NaCl (Merck)). The optical density (OD) of the culture was determined with a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA) at 540 nm and the approximate cell concentration of *A. acidoterrestris* FB2 was determined using a standard curve of cell concentration and optical density (540 nm).

Identification of ferulic acid, vanillin, vanillic acid and guaiacol

Sample preparation

Three hundred mL BAT broth (pH 4) was supplemented with either 100 mg L^{-1} vanillin (Merck), 100 mg L^{-1} vanillic acid (Sigma-Aldrich) or 5.67 mg L^{-1}or 66.67 mg.L^{-1} ferulic acid (Sigma-Aldrich) in 1 L sealed Schott bottles. The samples were inoculated with *A. acidoterrestris* FB2 to a final cell concentration of 10^6 cfu mL^{-1}, incubated at 45 °C for 7 d and analysed every 24 h to evaluate the supplemented precursors and the production of guaiacol. Three hundred mL sterilised BAT broth (pH 4) inoculated with *A. acidoterrestris* FB 2 to a final cell concentration of 10^6 cfu mL^{-1} was used as the *Alicyclobacillus* control sample. Samples were filtered using a 0.8/0.2 µm Supor Membrane Low Protein binding Non-pyrogenic filter (Pall, USA) prior to analysis with high performance liquid chromatography with UV-diode array detection (HPLC-DAD).

Standard solutions

Guaiacol standard solutions were prepared in the range of 0.0125 - 0.5 mM and ferulic acid standard solutions were prepared in the range of 0.1 - 100 mg.L^{-1} (0.0005 - 0.515 mM). Vanillin (0.007 - 0.789 mM) and vanillic acid (0.006 - 0.714 mM) standard solutions were prepared in the range of 1 - 120 mg.L^{-1}. Each standard solution was filtered using a 0.8/0.2 µm Supor Membrane Low Protein binding Non-pyrogenic filter prior to HPLC analysis.

High performance liquid chromatography

A HPLC-DAD system (Spectra Physics) fitted with a silica C_{18} column (Supelco, South Africa) with dimensions 25 cm x 4.6 mm x 5 µm was used to analyse a filtered extract consisting of a combination of all the standard compounds. These standard solutions included ferulic acid, vanillin, vanillic acid and guaiacol, as well as the phenolic compounds chlorogenic acid (Sigma-Aldrich), *p*-coumaric acid (Sigma-Aldrich) and

caffeic acid (Sigma-Aldrich) commonly found in apple juice. The injection volume was set at 10 µL, the flow rate at 1 mL min^{-1}, loop injection at 50 µL, the max run pressure at 3 000 psi with spectra scanned from 190 - 350 nm. The column was operated at a temperature of 40 °C. The mobile phase consisted of 5 % (v/v) formic acid (Merck) in water (solvent A) and (v/v) methanol (HPLC grade) (Merck) (solvent B). Different elution programs were tested to obtain a method that could separate all the standard compounds. The first separation method was based on the method used by Gökmen *et al.* (2001) for the analysis of phenolic compounds in apple juice, in which a gradient elution program was followed (Table 1). In the second separation method the elution program consisted of 40 % solvent A and 60 % solvent B for 10 min and in the third method the elution program was adjusted to 50 % solvent A and 50 % solvent B for 10 min.

RESULTS & DISCUSSION

Method development and identification of standard compounds

The first separation method consisting of the gradient elution program and the second separation method consisting of 40 % solvent A and 60 % solvent B did not give satisfactory results for the separation of the compounds evaluated in this study. There was limited differentiation between the standard compounds in the first separation method and only four compounds could be identified using the second method. All the compounds eluded within the first 10 min. All the standard compounds in the combined filtered extract were separated with the elution program consisting of 50 % solvent A and solvent B (Fig. 1). The different standard solutions were, therefore, analysed using this method. The retention time of the standard compounds changed slightly due to increase in the concentration of the standard compounds and the amount of compounds present in the solutions. Chlorogenic acid (1) and caffeic acid (2) were first separated with a retention time of 3.4 - 3.6 and 4.3 - 4.6 min, respectively. Vanillic acid (3) had a retention time of 4.8 - 4.9 min, followed by vanillin (4) with a retention time of 5.7 - 5.8 min, *p*-Coumaric acid (5) at 6.1 - 6.3 min, ferulic acid (6) at 6.2 - 6.5 min and guaiacol at 8.1 - 8.3 min (8) (Fig. 1). In the mixture of the standard compounds, an unknown compound (7) was detected at a retention time of 7 min. The same peak was also present at a retention time of 7.3 - 7.6 min during the analysis of the ferulic acid standard solutions. Therefore, the compound that appeared between ferulic acid and

Table 1 The gradient program based on the separation method used by Gökmen *et al.* (2001)

Time	Percentage of solvent A	Percentage of solvent B
5	85	15
15	75	25
10	60	40
10	50	50
10	85	15

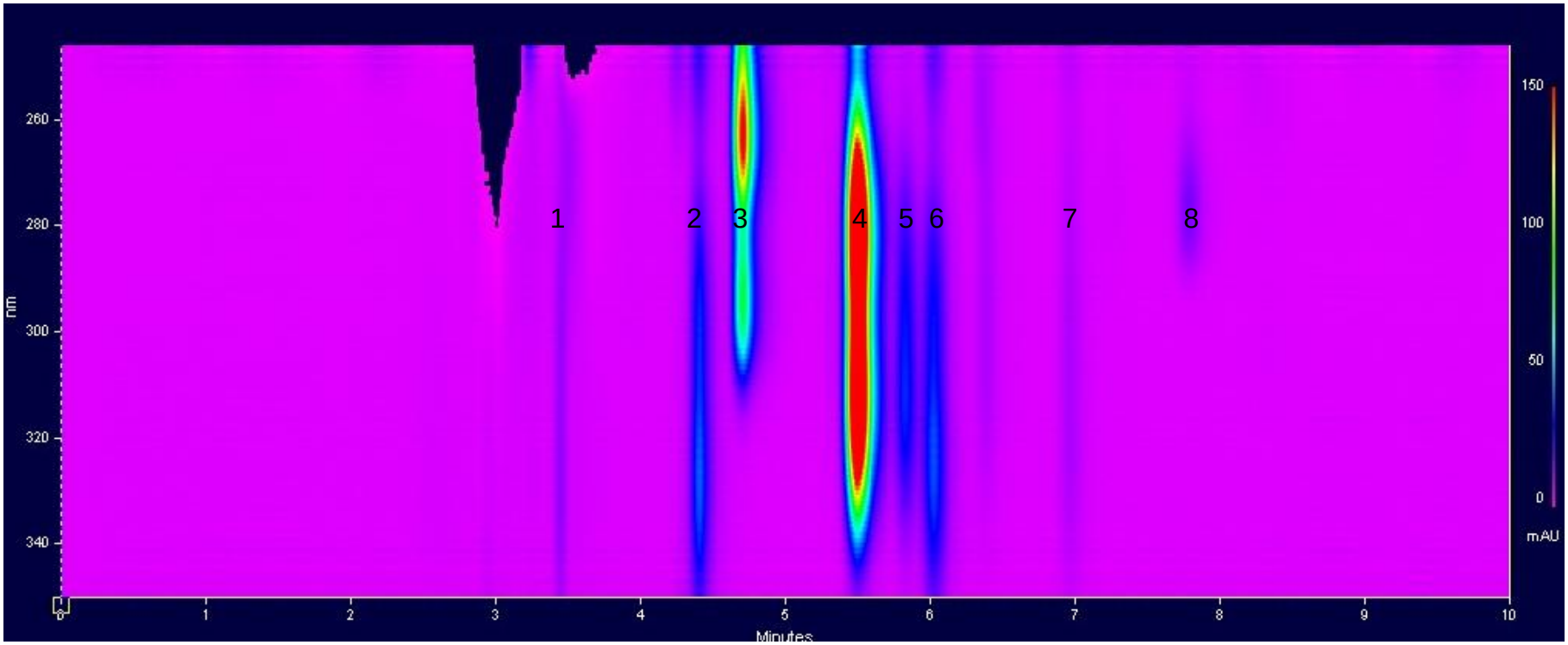

Figure 1 Contour plot (250 – 350 nm) of the standard compound mixture.

guaiacol may be an enantiomer of ferulic acid. The three wavelengths, namely 272, 312 and 328 nm were selected from the spectral data of the standard compounds for the detection and quantification of ferulic acid, vanillin, vanillic acid and guaiacol in the standard solutions and the samples. Vanillic acid and guaiacol were monitored at 272 nm, vanillin was monitored at 312 nm and ferulic acid at 328 nm.

Samples

The samples were analysed to identify the supplemented precursors and the produced guaiacol. Due to the satisfactory results of the final separation method, this method was used to analyse the samples. Computerised integration was performed using Chromquest v. 3.0 to calculate the peak area of a specific compound at a specific retention time. Identification of ferulic acid, vanillin, vanillic acid and guaiacol in all samples were carried out by comparing retention times and spectral data with those of the appropriate standards. Compounds present at retention time 0 – 4.2 min on the chromatograms was identified as UV active constituents of the BAT broth present in each sample (Fig. 2). The identification of ferulic acid (Fig. 3), vanillin (Fig. 4), vanillic acid (Fig. 5) and guaiacol (Fig. 6) in the samples were done by comparing retention times and spectral data with those of the standards. The peak at 7.4 min (Fig. 3), presumed to be the enantiomere of ferulic acid, was always present on the chromatogram when ferulic acid was supplemented. Each compound separated a fraction later than their standard. This is due to the presence of compounds in the BAT broth, as well as the supplemented precursors in each sample.

CONCLUSION

The stationary and mobile phase used in this study displayed excellent separation of guaiacol and the phenolic compounds supplemented in the BAT broth. The mobile phase consisting of 5 % (v/v) formic acid in water (solvent A) and methanol (solvent B) with the elution program of 50 % solvent A and 50 % solvent B gave satisfactory results and will be used as the HPLC-DAD method in further studies to evaluate the guaiacol production from ferulic acid, vanillin and vanillic acid and to evaluate the spoilage potential of apple juice supplemented with the precursors.

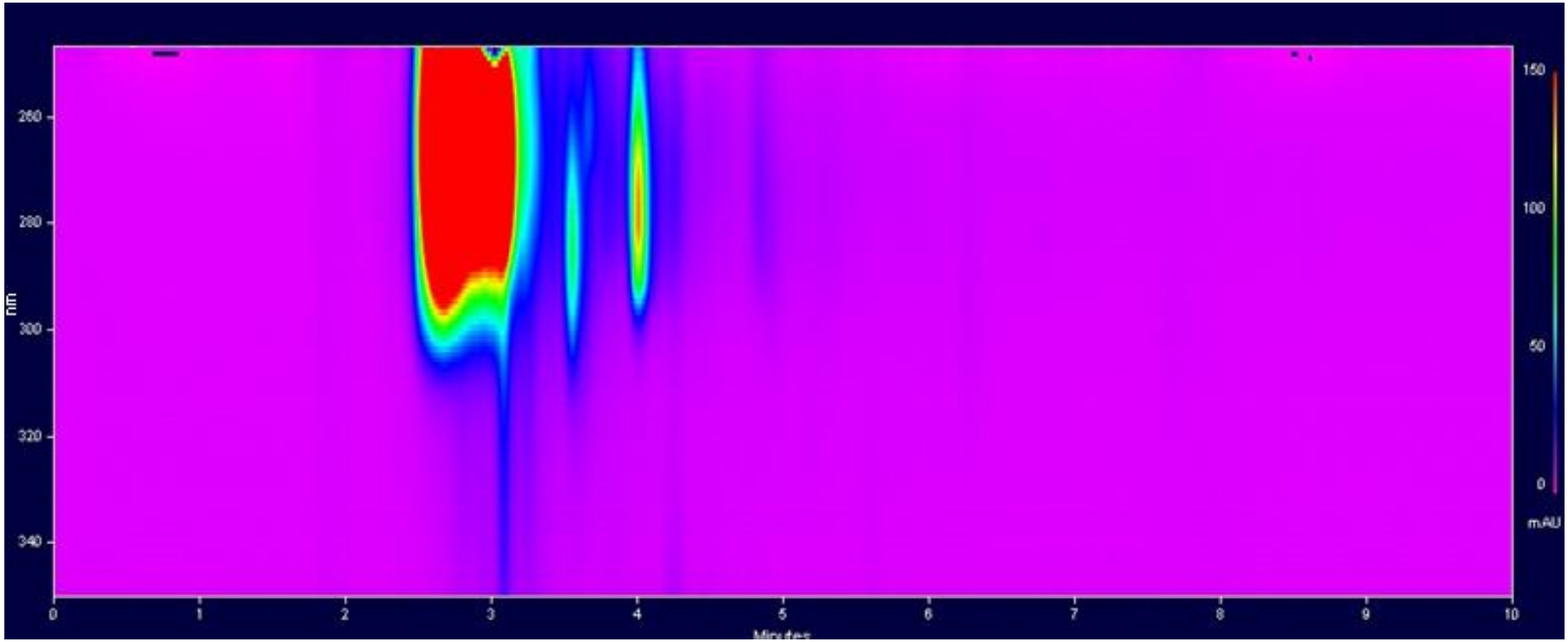

Figure 2 The contour plot (250 – 350 nm) of the BAT broth, inoculated with *A. acidoterrestris* without precursors added (*Alicyclobacillus* control).

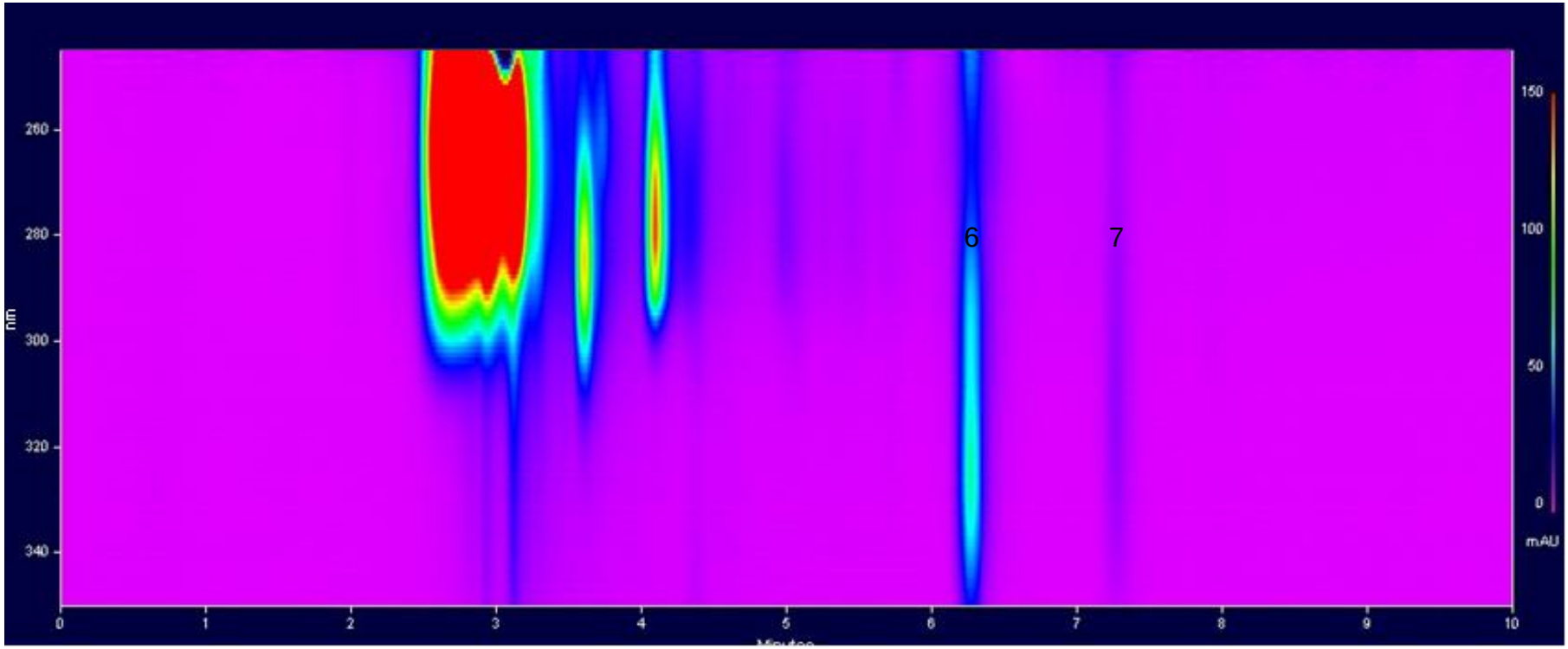

Figure 3 The contour plot (250 – 350 nm) of the ferulic acid sample analysed with HPLC-DAD. Ferulic acid peak eluted between 6 and 7 min.

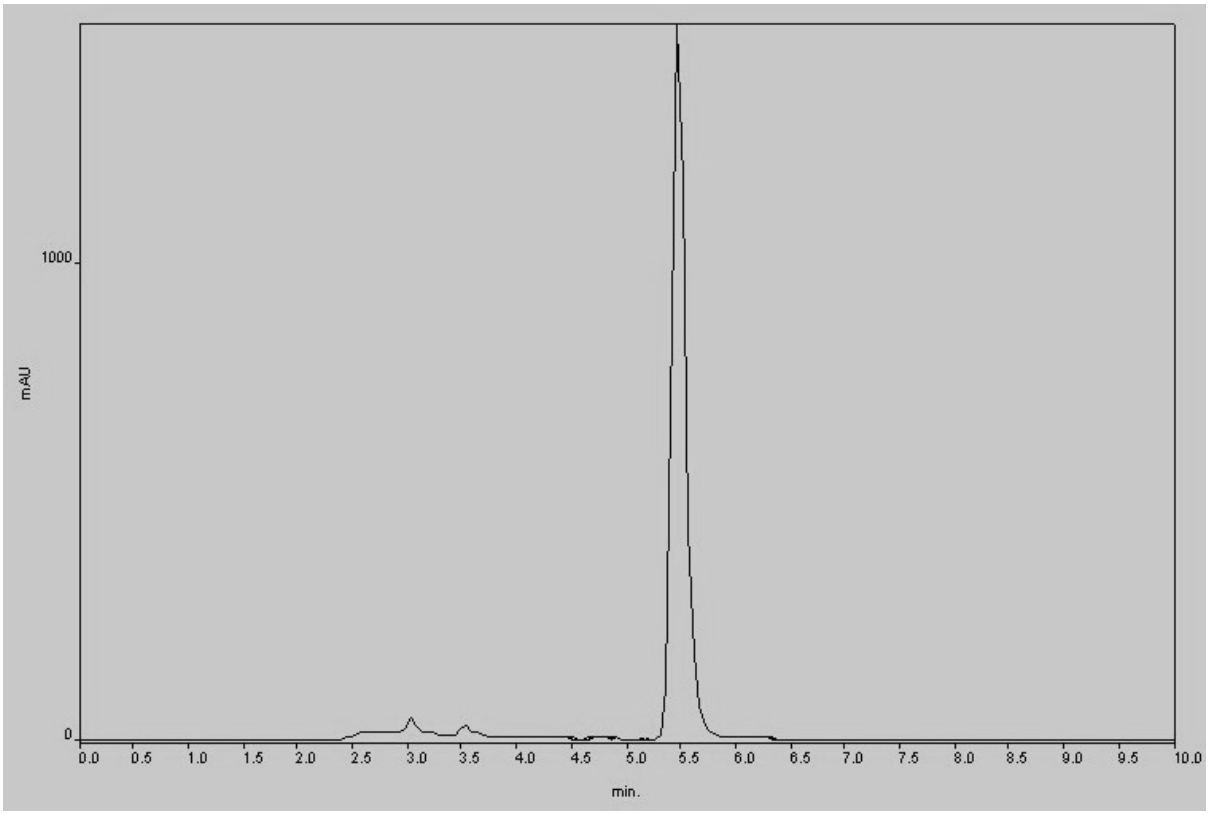

Figure 4 Chromatogram at 312 nm indicating the vanillin peak.

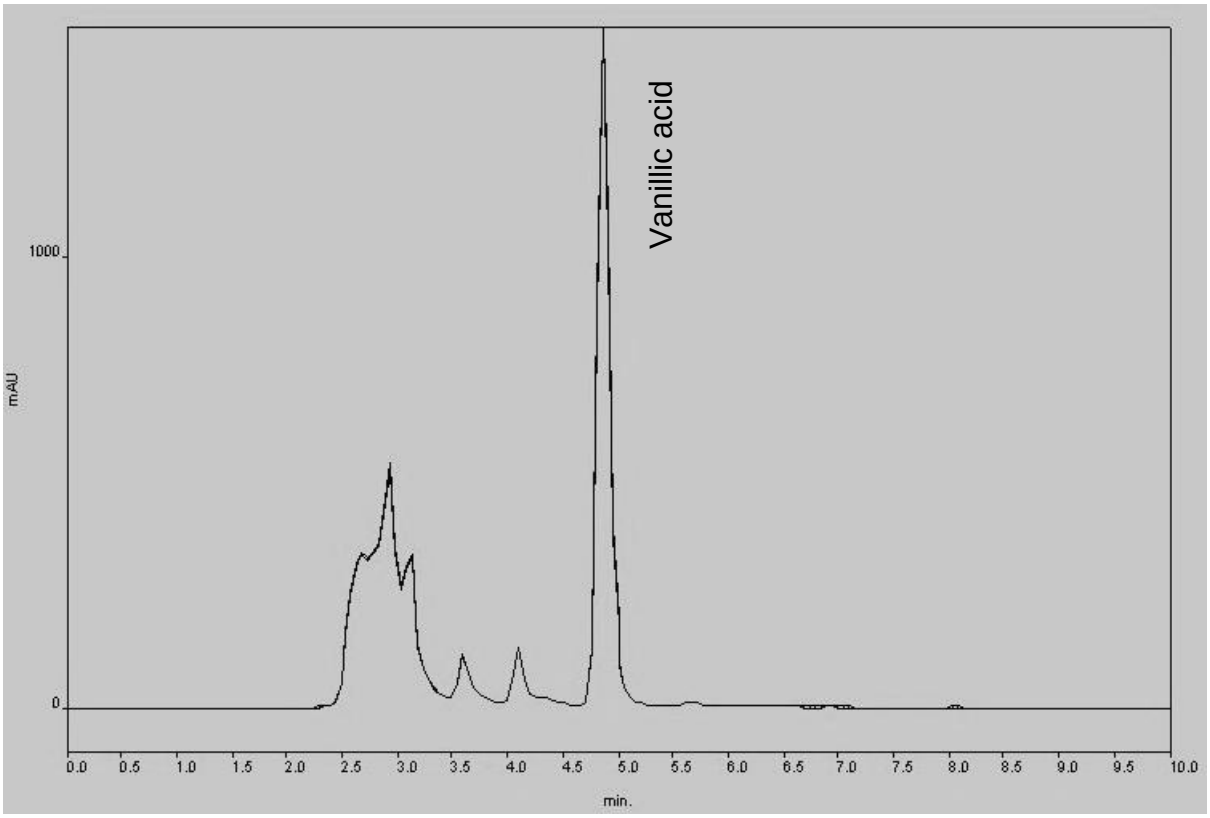

Figure 5 Chromatogram at 272 nm indicating the vanillic acid peak.

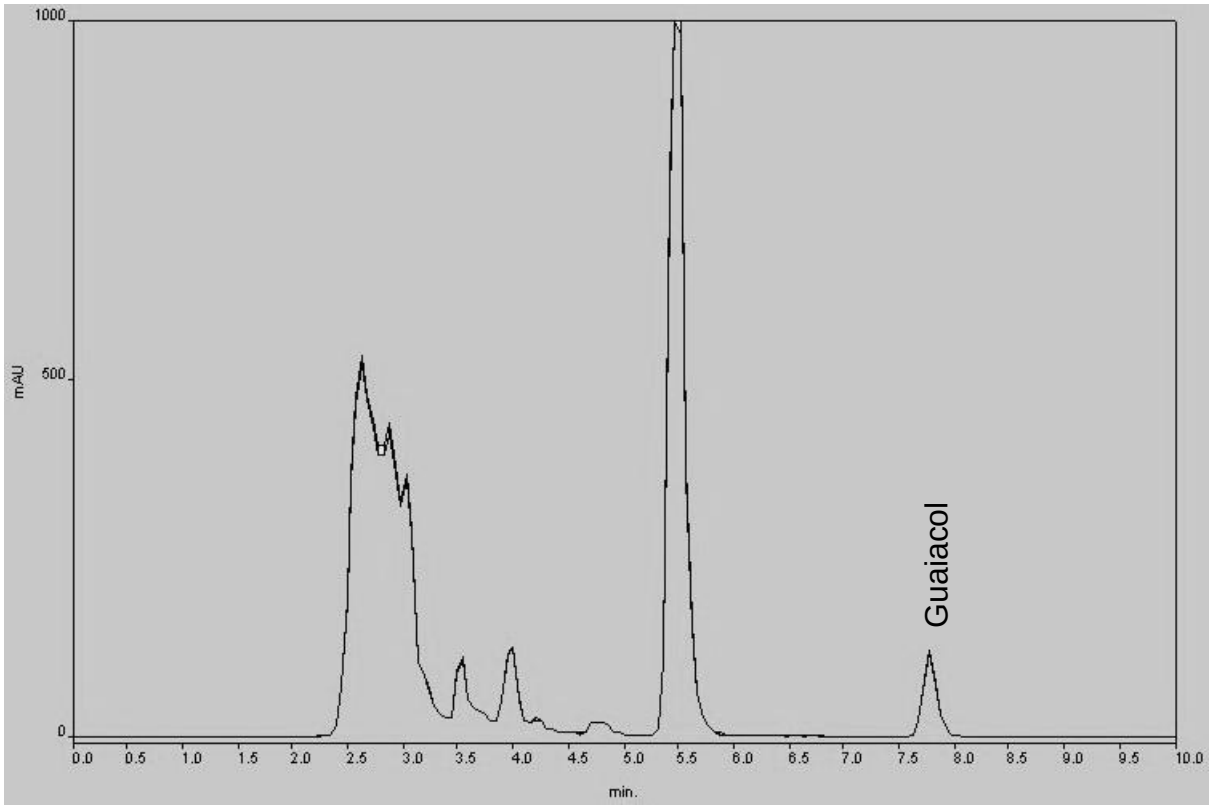

Figure 6 Chromatogram at 272 nm indicating the guaiacol peak.

REFERENCES

Bahçeci, K.S. & Acar, J. (2007). Determination of guaiacol produced by *Alicyclobacillus acidoterrestris* in apple juice by using HPLC and spectrophotometric methods, and mathematical modeling of guaiacol production. *European Food Research and Technology*, **225**, 873-878.

Bahçeci, K.S., Gökmen, V. & Acar, J. (2005). Formation of guaiacol from vanillin by *Alicyclobacillus acidoterrestris* in apple juice: a model study. *European Food Research and Technology*, **220**, 196-199.

Bengoechea, M.L., Sancho, A.I., Bartolom, B., Estrella, I., Gómez-Cordovés, C & Hernandez, M.T. (1997). Phenolic composition of industrially manufactured purées and concentrates from peach and apple fruits. *Journal of Agricultural and Food Chemistry,* **45**, 4071-4075.

Chang, S.S. & Kang, D.H. (2004). *Alicyclobacillus* spp. in the fruit juice industry: history, characteristics, and current isolation/detection procedures. *Critical Reviews in Microbiology*, **30**, 55-74.

Crawford, R.L. & Olson, P.P. (1978). Microbial catabolism of vanillate: decarboxylation to guaiacol. *Applied and Environmental Microbiology*, **36**, 539-543.

Eisele, T.A. & Semon, M.J. (2005). Best estimated aroma and taste detection threshold for guaiacol in water and apple juice. *Journal of Food Science*, **70**, 267-269.

Gocmen, D., Elston, A., Williams, T., Parish, M. & Rouseff, R.L. (2005). Identification of medicinal off-flavours generated by *Alicyclobacillus* species in orange juice using GC-olfactometry and GC-MS. *Letters in Applied Microbiology*, **40**, 172-177.

Gökmen, V., Artik, N., Acar, J., Kahraman, N. & Poyrazoğlu, E. (2001). Effect of various clarification treatments on patulin, phenolic compounds and organic acid compositions of apple juice. *European Food Research and Technology*, **213**, 194-199.

Groenewald, W.H., Gouws, P.A., & Witthuhn, R.C. (2009). Isolation, identification and typification of *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius* strains from orchard soil and the fruit processing environment in South Africa. *Food Microbiology*, **26**, 71-76.

Hernandez, T., Ausin, N., Bartolomé, B., Bengoechea, L., Estrella, I. & Gómez-Cordovés, C. (1997). Variations in the penolic composition of fruit juices with different treatments. *Zeitschrift für Lebensmitteluntersuchung und –Forschung A,* **204**, 151-155.

Huang, Z., Dostal, L. & Rosazza, J.P.N. (1993). Mechanisms of ferulic acid conversions to vanillic acid and guaiacol by *Rhodotorula rubra*. *The Journal of Biological Chemistry*, **268**, 23954-23958.

International Federation of Fruit Juice Producers (IFU). (2007). Method on the detection of taint producing *Alicyclobacillus* in fruit juices. *IFU Method No. 12*. Pp. 1-11. IFU, Paris.

Jensen, N. (2000). *Alicyclobacillus* in Australia. *Food Australia*, **52**, 282-285.

Jensen, N. & Whitfield, F.B. (2003). Role of *Alicyclobacillus acidoterrestris* in the development of a disinfectant taint in shelf-stable fruit juice. *Letters in Applied Microbiology*, **36**, 9-14.

Jensen, N., Varelis, P. & Whitfield, F.B. (2001). Formation of guaiacol in chocolate milk by the psychrotrophic bacterium *Rahnella aquatilis*. *Letters in Applied Microbiology*, **33**, 339-343.

Kermasha, S, Goetghebeur, M., Dumod, J. & Couturec, R. (1995). Analyses of phenolic and furfural compounds in concentrated and non-concentrated apple juices. *Food Research International,* **28**, 245-252.

Niwa, M. (2004). Control of hazardous bacteria in acidic beverages by using a guaiacol detection kit (peroxidase method). [WWW document]. URL http://www.cosmobio.co.jp/export_e/products/kits/products_KYO_20050412/Guaiakol_detection_kit_reference.pdf. 19 February 2009.

Niwa, M. & Kawamoto, A. (2003). Development of a rapid detection method of *A. acidoterrestris*, hazardous bacteria to acidic beverage. *Fruit Processing*, **13**, 102-107.

Orr, R.V., Shewfelt, R.L., Huang, C.J., Tefera, S. & Beuchat, L.R. (2000). Detection of guaiacol produced by *Alicyclobacillus acidoterrestris* in apple juice by sensory and chromatographic analyses, and comparison with spore and vegetative cell populations. *Journal of Food Protection*, **63**, 1517-1522.

Pettipher, G.L., Osmundson, M.E. & Murphy, J.M. (1997). Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit juice-containing drinks. *Letters in Applied Microbiology*, **24**, 185-189.

Pollnitz, A.P., Pardon, K.H., Sykes, M. & Sefton, M.A. (2004). The effects of sample preparation and gas chromatograph injection techniques on the accuracy of measuring guaiacol, 4-methylguaiacol and other volatile oak compounds in oak extracts by stable isotope dilution analyses. *Journal of Agricultural and Food Chemistry,* **52**, 3244-3252.

Pometto, A.L., Sutherland, J.B. & Crawford, D.L. (1981). *Streptomyces setonii*: catabolism of vanillic acid via guaiacol and catechol. *Canadian Journal of Microbiology,* **27**, 636-638.

Proestos, C., Sereli, D. & Komaitis, M. (2006). Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. *Food Chemistry,* **95**, 44-52.

Proestos, C., Kapsokefalou, M. & Komaitis, M. (2008). Analysis of naturally occurring phenolic compounds in aromatic plants by RP-HPLC and GC-MS after silylation. *Journal of food chemistry,* **31,** 402-414.

Rosazza, J.P.N., Huang, Z., Dostal, L., Volm, T. & Rousseau, B. (1995). Review: Biocatalytic transformations of ferulic acid: an abundant aromatic natural product. *Journal of Industrial Microbiology*, **15**, 457-471.

Smit, Y. (2009). Growth and guaiacol production of species of *Alicyclobacillus* isolated from the South African fruit processing environment. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Spanos, G.A. & Wrolstad, R.E. (1990). Influence of processing and storage on the phenolic composition of Thompson Seedless grape juice. *Journal of Agricultural and Food Chemistry,* **38**, 1565-1571.

Spanos, G.A., Wrolstad, R.E. & Heatherhell, D.A. (1990). Influence of processing, and storage on the phenolic composition of apple juice. *Journal of Agricultural and Food Chemistry,* **38**, 1572-1579.

Zierler, B., Siegmund, B. & Pfannhauser, W. (2004). Determination of off-flavour compounds in apple juice caused by microorganisms using headspace solid phase microextraction-gas chromatography-mass spectrometry. *Analytica Chimica Acta*, **520**, 3-11.

CHAPTER 4

GUAIACOL PRODUCTION FROM FERULIC ACID, VANILLIN AND VANILLIC ACID BY *ALICYCLOBACILLUS ACIDOTERRESTRIS*

ABSTRACT

Alicyclobacilli are thermophilic, acidophilic bacteria (TAB) that spoil fruit juice products by producing guaiacol. The precursors of guaiacol production by *Alicyclobacillus* are not fully understood and it is currently believed that guaiacol form as a product of ferulic acid metabolism. The aim of this study was to confirm the precursors that can be metabolised by *Alicyclobacillus acidoterrestris* to produce guaiacol and to evaluate the pathway of guaiacol production. *Alicyclobacillus acidoterrestris* FB2 was incubated at 45 °C for 7 d in *Bacillus acidoterrestris* (BAT) broth supplemented with ferulic acid, vanillin or vanillic acid, respectively. The samples were analysed every 24 h to determine the cell concentration, the supplement concentration using high performance chromatography with UV-diode array detection (HPLC-DAD) and the guaiacol concentration, using both the peroxidase enzyme colourimetric assay (PECA) and HPLC-DAD. The cell concentration of *A. acidoterrestris* FB2 during the 7 d in all samples were above the critical cell concentration of 10^5 cfu mL^{-1} reportedly required for guaiacol production. The guaiacol production by *A. acidoterrestris* FB2 increased with an increase in vanillin or vanillic acid concentration and a metabolic pathway of *A. acidoterrestris* FB2 directly from vanillin to guaiacol was established. High concentration vanillic acid (1 000 mg L^{-1}) had an initial inhibitory effect on the cells, but the cell concentration increased after day 2. The high concentrations of vanillin and vanillic acid caused a pale yellow discolouration with the PECA that influenced the guaiacol measurement, resulting in higher guaiacol concentrations. Guaiacol production did not occur in the absence of either a precursor or *A. acidoterrestris* FB2 and guaiacol was not produced by *A. acidoterrestris* FB2 in the samples supplemented with ferulic acid. The presence of *Alicyclobacillus* spp. that has the ability to produce guaiacol, as well as the substrates vanillin or vanillic acid is prerequisites for production of guaiacol. Knowledge of these precursors in fruit juice is of importance to indicate the spoilage potential of the different fruit juices by *Alicyclobacillus* spp.

INTRODUCTION

The endospores of *Alicyclobacillus* can survive the low pH and pasteurisation process of fruit juice to germinate, grow and spoil the product (Splittstoesser *et al.,* 1994; Pettipher *et al.,* 1997; Walls & Chuyate, 1998) by the production of the taint compound, guaiacol (Jensen, 2000; Jensen & Whitfield, 2003). Guaiacol causes an undesirable odour in fruit juice with an unpleasant smoky, medicinal or phenolic-like taint (Pettipher *et al.,* 1997; Jensen, 2000).

The production of guaiacol by *Alicyclobacillus acidoterrestris* has been reported as a flat-sour type spoilage in various acidic juices, isotonic water, lemonade, fruit juice blends and fruit-carrot juice blends (Yamazaki *et al.*, 1996; Pettipher *et al.,* 1997). *Alicyclobacillus acidiphilus, A. herbarius, A. hesperidum* (Matsubara *et al.,* 2002; Goto *et al.,* 2008), *A. cycloheptanicus* (Gocmen *et al.,* 2005) and *A. acidocaldarius* (Smit, 2009) have the ability to produce guaiacol, however, *A. acidoterrestris* are primarily responsible for spoilage and the production of guaiacol.

Guaiacol may be produced from vanillic acid by a number of microbes, including *Bacillus megaterium* (Crawford & Olson, 1978)*, Bacillus subtilis* (Álvarez-Rodríguez *et al*., 2003), *Streptomyces setonii* and *Streptomyces* strains (Crawford & Olson, 1978). Guaiacol can also be formed from ferulic acid by *Paecilomyces variotii* (Rahouti *et al*., 1989)*, Rhodotorula rubra* (Huang *et al*., 1993) and *Sporotrichum thermophile* (Topakas *et al*., 2003). Although the metabolic pathway of guaiacol production by *Alicyclobacillus* has not been fully elucidated, it is currently believed that guaiacol is produced as a product of ferulic acid metabolism (Jensen, 2000; Niwa & Kawamoto, 2003; Bahçeci *et al*., 2005). Guaiacol is produced through the non-oxidative decarboxilation of vanillic acid, the oxidation product of vanillin, a derivative of ferulic acid (Chang & Kang, 2004).

The possible precursors of guaiacol, namely ferulic acid, vanillin and vanillic acid are ubiquitous in nature. Ferulic acid is an aromatic phenolic compound (Rosazza *et al.,* 1995) found in fruits, vegetables, grains, beans, leaves, seeds, nuts, grasses, flowers (Herrmann, 1989) and form the precursor of lignin biosynthesis or the product of lignin degradation (Ishikawa *et al.,* 1963). Ferulic acid can also be metabolised to vanillin and vanillic acid by various bacteria (Sutherland *et al.,* 1983) and fungi (Henderson, 1961; Ishikawa *et al.,* 1963; Toms & Wood, 1970; Tadasa, 1977). Vanillic acid can be naturally present in fruit juice as a derivative of lignin or as a metabolite of several microbes (Piacquadio *et al.,* 2002; Chang & Kang, 2004) and vanillin, a derivative of ferulic acid is present in several fruit and fruit products, including strawberries (Pyysalo *et al.,* 1979), orange juice (Martin *et al.,* 1992), appel cider brandy

(Mangas *et al*., 1997), mango (Sakho *et al.,* 1997), wines (Spillman *et al.,* 1997) and citrus juice (Goodner *et al.,* 2000).

The presence of *Alicyclobacillus* in the fruit processing environment is unavoidable. The production of guaiacol in fruit juice products by *Alicyclobacillus* spp. is of great concern, however, little information currently exist on the precursors in fruit juice required for the production of guaiacol by *Alicyclobacillus* spp. The aim of this study was to confirm the precursors that can be metabolised by *A. acidoterrestris* to produce guaiacol and to confirm the anabolic pathway of guaiacol production.

MATERIALS AND METHODS

Bacterial inoculation cultures

Alicyclobacillus acidoterrestris FB2 isolated from pear concentrate (Groenewald *et al*., 2009) was used in this study. A pure culture of *A. acidoterrestris* FB2 was inoculated into *Bacillus acidoterrestris* (BAT) (pH 4) (IFU, 2007) and incubated at 45 °C. After 2d incubation 1 mL of the culture was aseptically removed and added to sterile BAT broth (pH 4) followed by incubation at 45 °C for 5 d. The culture was then centrifuged in a Beckman Coulter TJ-25 centrifuge (Beckman Coulter Inc., Fullerton, California, USA) at 5 500 *g* for 10 min and the pellet was resuspended in sterile saline solution (SSS) (0.85% (m/v) NaCl (Merck)). The optical density (OD) of the culture was determined at 540 nm with a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA) and the approximate cell concentration was determined using a standard curve.

Guaiacol production and the growth of *A. acidoterrestris* FB 2

Three hundred mL BAT broth (pH 4) was supplemented with either 100 mg L^{-1} or 1 000 mg L^{-1} vanillin (Merck), 100 mg L^{-1} or 1 000 mg L^{-1} vanillic acid (Sigma-Aldrich), or 5.67 mg L^{-1}, 66 67 mg.L^{-1} or 100 mg L^{-1} ferulic acid (Sigma-Aldrich) in sealed Schott bottles. A concentration of 100 mg L^{-1} vanillin or vanillic acid was supplemented in the samples to provide *A. acidoterrestris* FB2 with an optimum concentration of the possible precursors and a concentration of 1 000 mg L^{-1} vanillin or vanillic acid was supplemented in the samples to evaluate the effect of higher concentrations vanillin and vanillic acid on the growth of *A. acidoterrestris* FB2 and production of guaiacol. The low concentration of ferulic acid was used to evaluate the guaiacol production from ferulic acid at a concentration possibly present in fruit juice (Kermasha *et al.,* 1995;

Piacquadio *et al.,* 2002) and the two higher concentrations of ferulic acid were supplemented in the samples to evaluate the effect of increased amounts of ferulic acid. The samples were inoculated with *A. acidoterrestris* FB2 (10^6 cfu mL^{-1}), incubated at 45 °C for 7 d and analysed every 24 h to evaluated the guaiacol production from the supplemented precursors. The vegetative cell concentration was determined, as well as the endospore concentration after a heat shock treatment (80 °C for 10 min). The guaiacol concentration was determined using the peroxidase enzyme colourimetric assay (PECA), as well as high performance liquid chromatography with UV-diode array detection (HPLC-DAD). The vanillin, vanillic acid and ferulic acid concentrations in the samples were also determined by HPLC analysis. All experiments were performed in triplicate. Three hundred mL BAT broth (pH 4) supplemented with either 100 mg L^{-1} or 1 000 mg L^{-1} vanillic acid, or 100 mg L^{-1} or 1 000 mg L^{-1} vanillin served as supplement control samples (without *A. acidoterrestris* FB2). Three hundred mL sterilised BAT broth (pH 4) inoculated with *A. acidoterrestris* FB 2 to a final cell concentration of 10^6 cfu mL^{-1} served as the *Alicyclobacillus* control sample (without precursors).

Vegetative cell and endospore concentration

The cell concentration during the 7 d incubation period was determined by serially diluting (10^0 – 10^6) each sample in SSS and enumeration with Potato dextrose agar (PDA) (Merck) (pH 4) using the pour plate technique, followed by incubation at 45 °C for 4 d. The endospore concentration was determined by subjecting 1 mL of each sample to a heat shock treatment in a water bath at 80 °C for 10 min to eliminate all the viable vegetative cells followed by serial dilution (10^0 – 10^6) and enumeration. The pour plate technique, using PDA (Merck) (pH 4), followed by incubation at 45 °C for 4 d was used to enumerate the endospores of *Alicyclobacillus*. Control plates of PDA (pH 4) incubated at 45 °C for 4 d had no growth. All experiments were conducted in duplicate.

Peroxidase enzyme colourimetric assay (PECA)

The peroxidase enzyme colourimetric assay (Sheu & Chen, 1991; Bahçeci & Acar 2007) was used to determine the guaiacol concentration in the control samples and the inoculated samples supplemented with vanillic acid, vanillin and guaiacol. One mL of each sample was centrifuged at 2 300 *g* for 10 min (Eppendorf 5415 D centrifuge, Hamburg, Germany) and 300 µL of the supernatant was vortexed with 2 mL 0.2 M potassium phosphate buffer (4.18 g L^{-1} K_2HPO_4 (Merck) and 23.95 g L^{-1} KH_2PO_4 (Merck), pH 6), 300 µL (5 U) peroxidase enzyme (Merck) and 300 µL 0.5% (m/v) H_2O_2

(Merck). A blank was prepared by vortexing 2.3 mL 0.2 M potassium phosphate buffer, 300 µL (5 U) peroxidase enzyme and 300 µL 0.5% (m/v) H_2O_2. A Beckman Coulter DU 530 Life science UV/Vis spectrophotometer was used to measure the colour changes at 420 nm. The guaiacol concentration was calculated using a standard curve of absorbance at 420 nm versus guaiacol (Sigma-Aldrich, USA) concentration (Fig.1A, Appendix).

High performance liquid chromatography

Standard solutions

Guaiacol standard solutions for HPLC analysis were prepared in the range of 0.0125 - 5 mM and ferulic acid standard solutions were prepared in the range of 0.1 - 100 mg L^{-1} (0.0005 - 0.515 mM). Vanillin (0.007 - 6.572 mM) and vanillic acid (0.006 - 5.947 mM) standard solutions were prepared in the range of 1 - 1000 mg L^{-1}. Each standard solution was filtered using a 0.8/0.2 µm Supor Membrane Low Protein binding Non-pyrogenic filter (Pall, USA) prior to HPLC analysis.

HPLC analysis

A HPLC system (Spectra Physics) with UV-diode array detection was used to determine the ferulic acid, vanillin, vanillic acid and guaiacol concentrations present in each sample. The filtered extract was injected into the HPLC fitted with a silica C_{18} column (Supelco, SA) with dimensions of 25 cm x 4.6 mm x 5 µm. The elution program was carried out at 40 °C. The mobile phase consisted of 50 % solvent A, a mixture of formic acid (Merck) and water (5:95, v/v) and 50 % solvent B, consisting of methanol (HPLC grade) (Merck). The flow rate was set at 1 mL min^{-1}, loop injection at 50 µL, the injection volume at 2 µL and the max run pressure at 3 000 psi. UV detection was performed at three wavelengths, 272 nm, 312 nm and 328 nm. Computerised integration was performed using Chromquest v. 3.0 to calculate the peak area of a specific compound at a specific retention time. Identification of ferulic acid, vanillin, vanillic acid and guaiacol in the samples were carried out by comparing retention times and spectral data with those of the appropriate standards. A standard curve of each standard was used to quantify the compounds in the samples.

Statistical analysis

Repeated measures ANOVA with a mixed model approach were used to investigate guaiacol production from possible precursors, as well as the effect of different

precursors on the growth of *A. acidoterrestris.* Correlation analyses were done to determine the strength of relationships between measurements. Statistical analyses were performed using Statistica 9. A 95 % confidence interval was used as a guideline for indicating significant differences.

Rapid guaiacol production by *A. acidoterrestris* FB2 from vanillic acid

The production of guaiacol from vanillic acid was further evaluated due to the fast conversion of vanillic acid to guaiacol by *A. acidoterrestris*. Experiments were conducted to establish the onset of guaiacol production from vanillic acid when *A. acidoterrestris* was present at a cell concentration of 10^6 cfu mL^{-1}. Three hundred mL BAT broth (pH 4) was supplemented with either 100 mg L^{-1} or 50 mg L^{-1} vanillic acid in sealed Schott bottles. The samples were inoculated with *A. acidoterrestris* FB2 to a final cell concentration of 10^6 cfu mL^{-1}. The samples were incubated at 45 °C for 4 h and the peroxidase enzyme colourimetric assay (PECA) was performed every hour to determine the start of guaiacol production. The vegetative cell concentration, as well as the endospore concentration was determined every 2 h. Vanillic acid and guaiacol concentration in the samples were also determined every 2 h by HPLC-DAD analysis. Three hundred mL sterilised BAT broth (pH 4), inoculated with *A. acidoterrestris* FB2 was used as the *Alicyclobacillus* control sample. This experiment was performed in duplicate.

RESULTS AND DISCUSSION

Influence of precursors on growth of A. acidoterrestris FB 2

A 10^6 cfu mL^{-1} *A. acidoterrestris* inoculum was used to ensure that the concentration of cells present in the medium was above the concentrations of 10^4 to 10^5 cfu mL^{-1} that is reportedly required for guaiacol production (Pettipher *et al.,* 1997; Komitopoulou *et al.,* 1999), therefore, reducing the lag phase of guaiacol production due to low cell concentrations (Smit, 2009).

All of the control samples supplemented with either vanillin or vanillic acid were sterile, without any growth. The samples supplemented with 100 mg L^{-1} vanillin and 5.67 mg L^{-1} and 66.67 mg L^{-1} ferulic acid (Figs. 1 & 2) followed a similar *Alicyclobacillus* growth trend when compared to the *Alicyclobacillus* control sample (without supplementation) (Fig. 1), where a significant ($p < 0.05$) increase in vegetative cell concentration was observed for the first 24 h with a constant and sustained growth

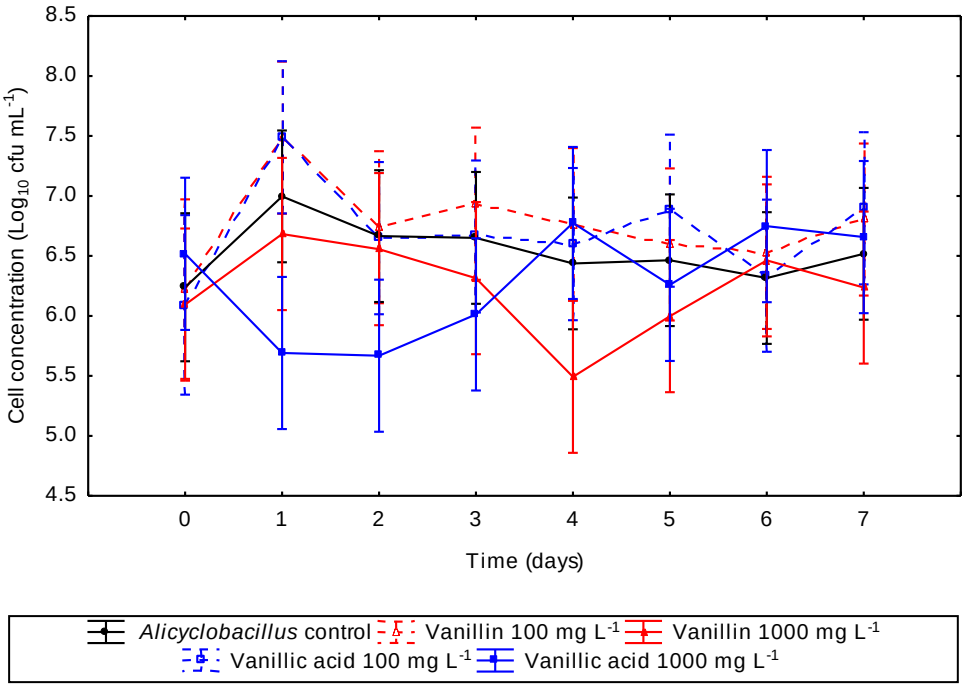

Figure 1 Changes in vegetative cell concentration of *A. acidoterrestris* FB2 at different concentrations of vanillin and vanillic acid (100 mg L^{-1} and 1000 mg L^{-1}) in BAT broth incubated at 45 °C for 7 d. Vertical bars denote 0.95 confidence intervals.

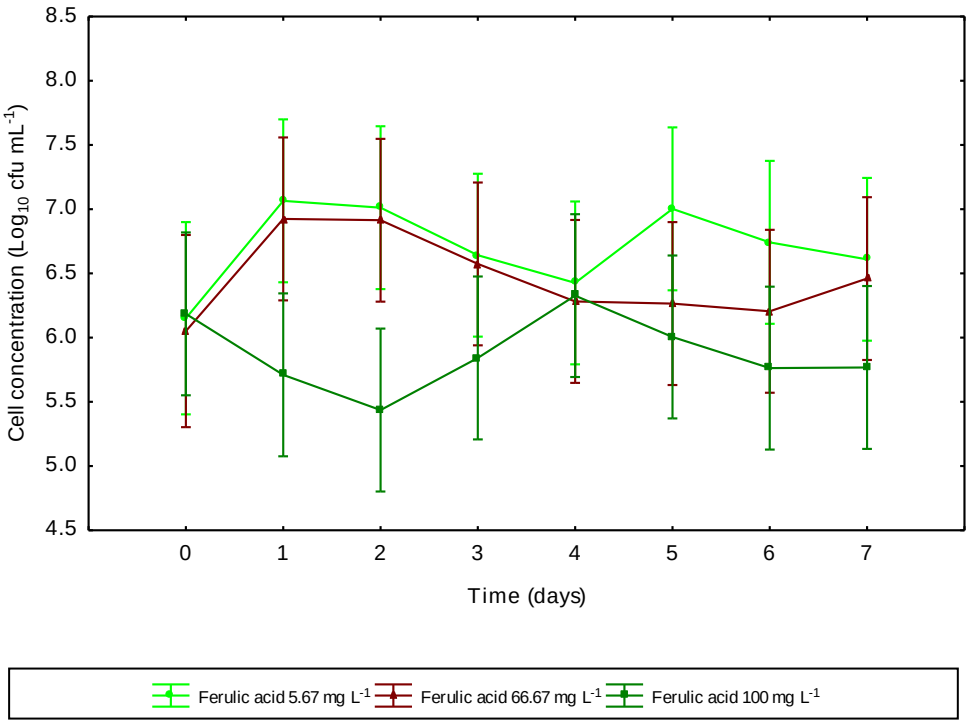

Figure 2 Changes in vegetative cell concentration of *A. acidoterrestris* FB 2 at different concentrations of ferulic acid in BAT broth incubated at 45 °C for 7 d. Vertical bars denote 0.95 confidence intervals.

thereafter. The increase in cell concentration in the first 24 h is possibly due to the introduction of the cells into fresh media containing an abundant of substrate. Although an increase in cell concentration during the first 24 h was also observed for the 100 mg L^{-1} vanillic acid sample (Fig. 1), the vegetative cell concentration decreased significantly ($p < 0.05$) from day 1 to 2. However, the vegetative cell concentration on day 2 in this sample did not differ significantly ($p \geq 0.05$) from the initial cell concentration. The vegetative cell concentration decreased significantly ($p < 0.05$) in the 1 000 mg L^{-1} vanillic acid sample (Fig. 1) in the first 24 h with a significantly ($p < 0.05$) lower cell concentration on day 1 and 2 than in the *Alicyclobacillus* control sample (without precursors) (Fig. 1). This is due to the introduction of the cells to a high concentration of vanillic acid that may have an inhibitory effect on the growth of *Alicyclobacillus*. Different concentrations of vanillic acid, ranging from 200 to 1 682 mg L^{-1} had an inhibitory or antimicrobial effect on different microbes, including *Listeria* spp. (Delaqui *et al.,* 2006), *Escherichia coli* O157:H7 (Moon *et al*., 2005), *Klebsiella pneumoniae, Bacillus cereus, Aspergillus* spp. (Aziz *et al.,* 1998) and *Campylobacter jejuni* (Gañan *et al.,* 2006). The cell concentration did, however, increase from day 2 to 4 and this may possibly be due to the cells adapting to the high concentration of vanillic acid or the reduction of vanillic acid due to guaiacol production.

The vegetative cell concentrations in the 100 mg L^{-1} ferulic acid (Fig. 2) and 1 000 mg L^{-1} vanillic acid sample on day 1 and 2 and the cell concentration in the 1 000 mg L^{-1} vanillin sample on day 4 (Fig. 1) were significantly ($p < 0.05$) lower than the cell concentration in the *Alicyclobacillus* control sample (Fig. 1). However, none of the cell concentrations on day 7 in any of the samples were significantly ($p < 0.05$) different compared to the initial vegetative cell concentrations on day 0. Therefore, the growth of *A. acidoterrestris* FB2 was in the stationery phase and ferulic acid, vanillin and vanillic acid added to the samples did not have an inhibitory effect on the growth of *A. acidoterrestris* FB2 after 7 d incubation. Although significant ($p \geq 0.05$) growth did not occur, a concentration of above 10^5 cfu mL^{-1} was sustained in each sample during the 7 d which would suffice for guaiacol production (Pettipher *et al.,* 1997; Komitopoulou *et al.,* 1999).

Although endospores are the differentiated form of the vegetative stage (growth) and not known to be responsible for the metabolism of the precursors to guaiacol (Chang & Kang, 2004), the endospore concentrations were determined in all samples to indicate the presence of endospores (Fig. 3 & 4). The endospore count in the

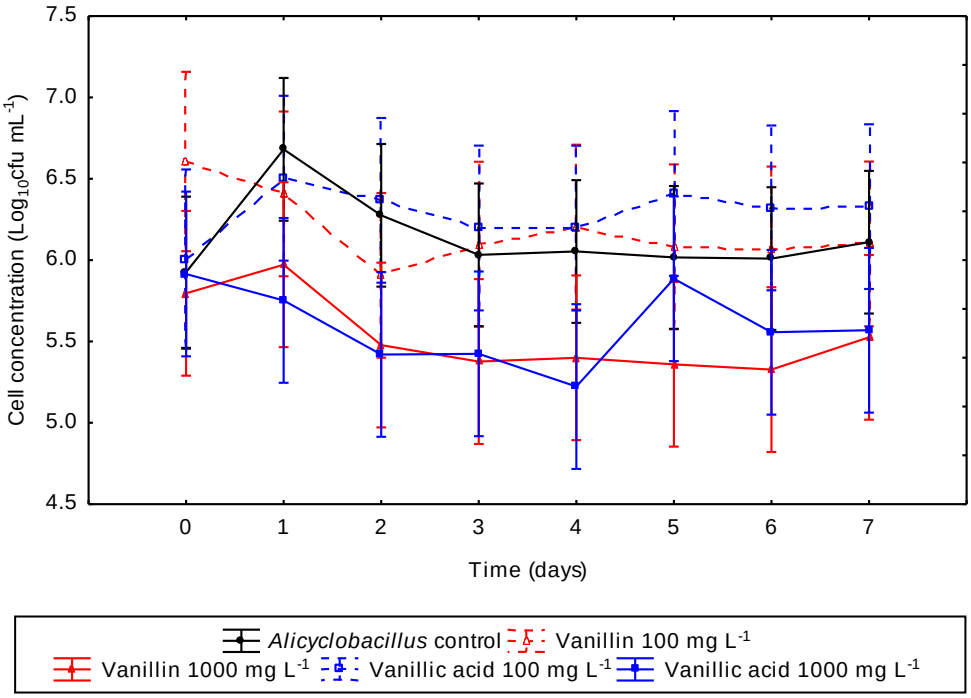

Figure 3 Changes in endospore concentration of *A. acidoterrestris* FB 2 at different concentrations of vanillin and vanillic acid (100 mg L^{-1} and 1000 mg L^{-1}) in BAT broth incubated at 45 °C for 7 d. Vertical bars denote 0.95 confidence intervals.

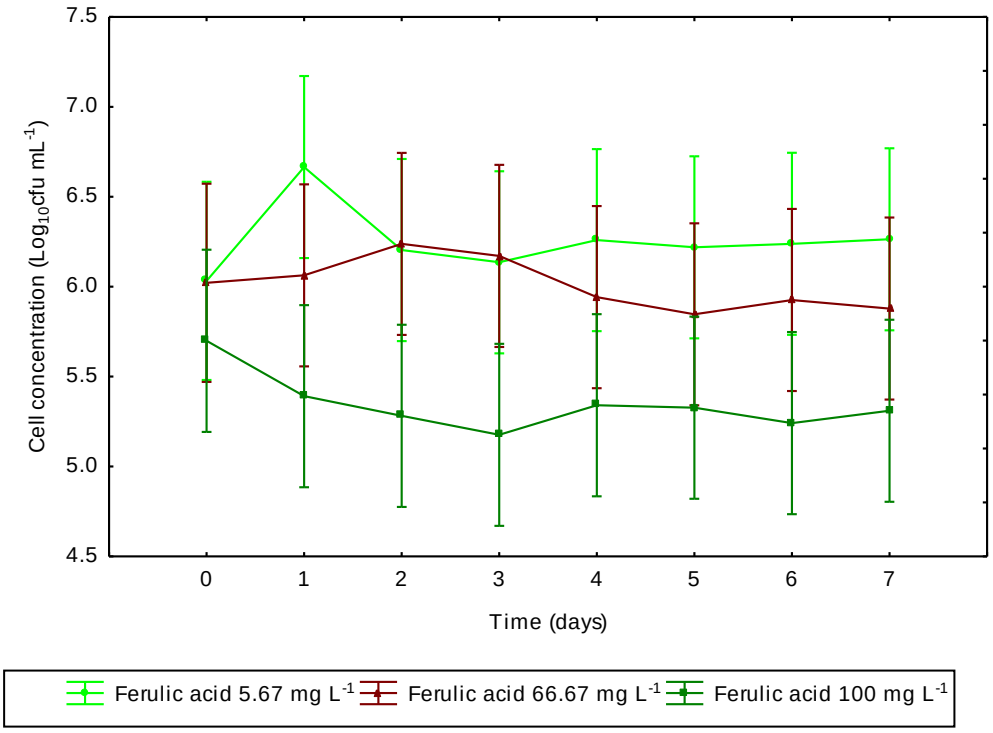

Figure 4 Changes in endospore concentration of *A. acidoterrestris* FB 2 at different concentrations of ferulic acid in BAT broth incubated at 45 °C for 7 d. Vertical bars denote 0.95 confidence intervals.

Alicyclobacillus control (Fig. 3) and 5.67 mg L^{-1} ferulic acid sample (Fig. 4) follow a similar trend with a significant ($p < 0.05$) increased in endospores in the first 24 h and a decrease from day 1 to 2. In the 100 mg L^{-1} vanillic acid sample, the endospore concentration also increased significantly ($p < 0.05$) in the first 24 h, while the endospore count in the 100 mg L^{-1} vanillin and 1 000 mg L^{-1} vanillin samples decreased significantly ($p < 0.05$) from day 1 to 2 (Fig. 3). The endospore concentrations in all of the samples remained constant from day 2 until day 7 (Figs. 3 & 4).

Guaiacol production measured with PECA

The PECA method was used as a rapid method to qualify and quantify guaiacol production in all samples. No colour change was observed in the BAT media inoculated with *A. acidoterrestris* FB2 without precursor supplementation (*Alicyclobacillus* control) (Fig. 5), in the control samples supplemented with either vanillin or vanillic acid without *A. acidoterrestris* FB2 or in the samples supplemented with ferulic acid and inoculated with *A. acidoterrestris* FB2 (Fig. 5). No guaiacol formation could thus be confirmed in these samples using the enzymatic assay (PECA).

The guaiacol concentration in the sample supplemented with 100 mg L^{-1} vanillin increased steadily during the 7 d (Fig. 5) with a significantly ($p < 0.05$) higher guaiacol concentration detected on days 3 to 7 compared to the guaiacol concentration of the *Alicyclobacillus* control sample (without precursors) on day 0 to 7. The maximum guaiacol concentration in the 100 mg L^{-1} vanillin sample was 0.439 mM (54.5 mg L^{-1}). The guaiacol concentration also increased significantly ($p < 0.05$) during the 7 d in the sample supplemented with 1 000 mg L^{-1} vanillin with a maximum guaiacol concentration of 2.009 mM (249.4 mg L^{-1}).

The guaiacol concentration in the 100 mg L^{-1} vanillic acid sample increased significantly ($p < 0.05$) in the first 24 h and then stabilised (Fig. 5). The maximum guaiacol concentration detected in these samples was 0.484 mM (60.1 mg L^{-1}). Guaiacol was also produced in the sample supplemented with 1 000 mg L^{-1} vanillic acid. The guaiacol concentration increased during the 7 d with a maximum guaiacol concentration of 3.482 mM (585.5 mg L^{-1}) on day 7. However, in the 1 000 mg L^{-1} vanillic acid samples an initial lag phase in guaiacol production was observed. This observation, together with the decrease in cell concentration in the first 24 h possibly indicates that cells that were introduced to the new environment was in an adaption phase after inoculation and the production of guaiacol did not start immediately.

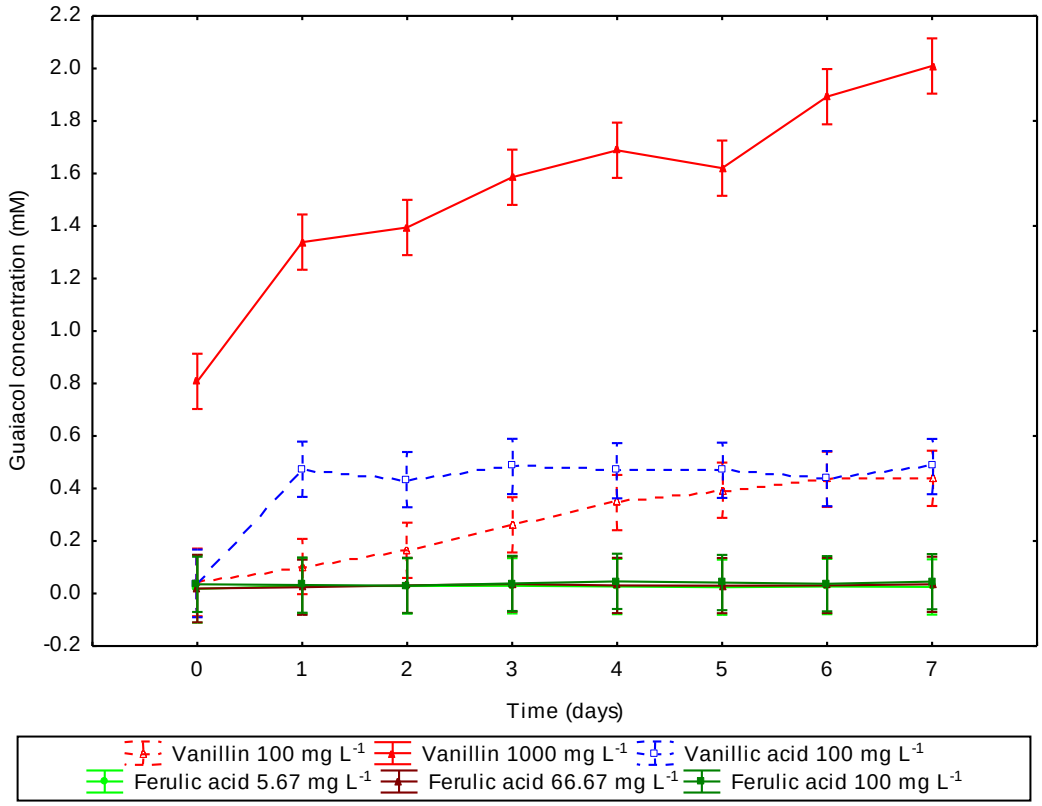

Figure 5 Guaiacol production in BAT broth supplemented with ferulic acid, vanillin and vanillic acid in the presence of *A. acidoterrestris* during the 7 d incubation measured by the peroxidase enzyme colourimetric assay (PECA). Vertical bars denote 0.95 confidence intervals.

The guaiacol concentration in the 1 000 mg L^{-1} vanillin sample (Fig. 5) was much higher compared to the maximum guaiacol concentration produced in the sample supplemented with 100 mg L^{-1} vanillin. The guaiacol production in the 1 000 mg L^{-1} vanillic acid sample (Fig. 5) is also higher compared to the guaiacol concentration in the 100 mg L^{-1} vanillic acid sample. This observation, therefore, indicates that the increase concentration of vanillin or vanillic acid available to *A. acidoterrestris* FB2, resulting in an increased concentration of guaiacol.

Although no colour change (PECA) during the 7 d was observed in the 1 000 mg L^{-1} vanillin control sample, the guaiacol concentration was significantly ($p < 0.05$) higher than the *Alicyclobacillus* control sample (without precursors), even on day 0. Guaiacol concentration in the1 000 mg L^{-1} vanillin sample on day 0 (0.808 mM) was also significantly ($p < 0.05$) higher than the guaiacol concentration measured with the PECA in the *Alicyclobacillus* control sample (without precursors) (0.016 mM) and the 100 mg L^{-1} vanillin sample (0.209 mM). The same observation was made in the 1 000 mg L^{-1} vanillic acid sample and 1 000 mg L^{-1} vanillic acid control sample. The guaiacol concentration in the 1 000 mg L^{-1} vanillic acid control sample was significantly ($p < 0.05$) higher than the *Alicyclobacillus* control sample (without precursors), even on day 0 (0.242 mM). It should be mentioned that the high concentration of 1 000 mg L^{-1} vanillin and vanillic acid that was present in these samples could cause a colour change that might result in a false positive measurement (guaiacol) with the enzymatic assay. According to Doerge *et al.* (1997) vanillic acid has been observed to cause a pale yellow discolouration with the PECA. In this study, this was also the case when high concentration of vanillin was present in the samples.

HPLC analysis

Concomitant to PECA guaiacol levels were measured with HPLC. The peroxidase enzyme colourimetric assay is based on colour change that donates guaiacol production, while the HPLC-DAD method identifies and quantifies the actual compound. The HPLC-DAD method is thus regarded as a more accurate method for the quantification of guaiacol.

The standard curves used for the quantification of ferulic acid (Fig. 2A), vanillin (Fig. 3A), vanillic acid (Fig. 4A) and guaiacol (Fig. 5A) are illustrated in the appendix of this chapter. The ferulic acid concentration in the samples supplemented with 5.67 mg L^{-1}, 66.67 mg L^{-1} or 100 mg L^{-1} ferulic acid did not decrease significantly

($p \geq 0.05$) during the 7 d (Fig. 7) and guaiacol was not produced in any of these samples (Fig. 6). Therefore, ferulic acid could not be considered a direct precursor for guaiacol production by *A. acidoterrestris* FB2. Guaiacol was also not detected in the *Alicyclobacillus* control sample (without precursors) or in the supplement control samples (without *A. acidoterrestris* FB2). Therefore, *A. acidoterrestris* FB2 could not produce guaiacol in the absence of the precursors nor could guaiacol be formed spontaneously in the presence of ferulic acid, vanillin or vanillic acid without the metabolic action of *A. acidoterrestris* FB2.

In the 100 mg L^{-1} vanillin sample a significant ($p < 0.05$) increase in guaiacol concentration was observed from day 0 to 5, with no significant ($p \geq 0.05$) guaiacol production after day 5. The vanillin in the 100 mg L^{-1} vanillin sample (Fig. 8) decreased during the 7 d and the supplemented vanillin was almost depleted on day 5 (Fig. 9). The maximum guaiacol concentration measured in this sample was 0.497 mM (61.7 mg L^{-1}) on day 7 (Fig 4) (PECA, 0.439 mM). The samples were incubated in sealed containers and the guaiacol concentration remained unchanged when precursors were no longer present.

In the 1 000 mg L^{-1} vanillin sample the guaiacol concentration increased significantly ($p < 0.05$) from day 0 to 7 (Fig 4) and guaiacol concentration on day 7 was measured at 1.376 mM (170.8 mg L^{-1}) (PECA, 2.009 mM). The vanillin supplemented in this sample was converted to guaiacol (Fig. 10), however, the vanillin was not depleted after 7 d (Fig. 8) and the guaiacol concentration may still increase until the supplemented vanillin is depleted.

The vanillic acid concentration in the samples supplemented with vanillin was also measured during the 7 d to identify the pathway of guaiacol production. Vanillic acid was not detected in this study, indicating that *A. acidoterrestris* FB2 can possibly produce guaiacol directly from vanillin (Fig. 11).

Guaiacol concentration in the 100 mg L^{-1} vanillic acid sample increased significantly ($p < 0.05$) in the first 24 h and remained unchanged from day 1 to 7 (Fig. 6). The maximum guaiacol concentration measured in the 100 mg L^{-1} vanillic acid sample was 0.543 mM (67.4 mg L^{-1}) (PECA, 0.484 mM). The vanillic acid in the 100 mg L^{-1} vanillic acid sample decreased significantly ($p < 0.05$) and was depleted in the first 24 h (Fig. 9), thus no vanillic acid was available to *A. acidoterrestris* for further guaiacol production (Fig. 12). The depletion of 100 mg L^{-1} vanillic acid (Fig. 12) was faster than the depletion of 100 mg L^{-1} vanillin (Fig. 9), indicating that the metabolic conversion of

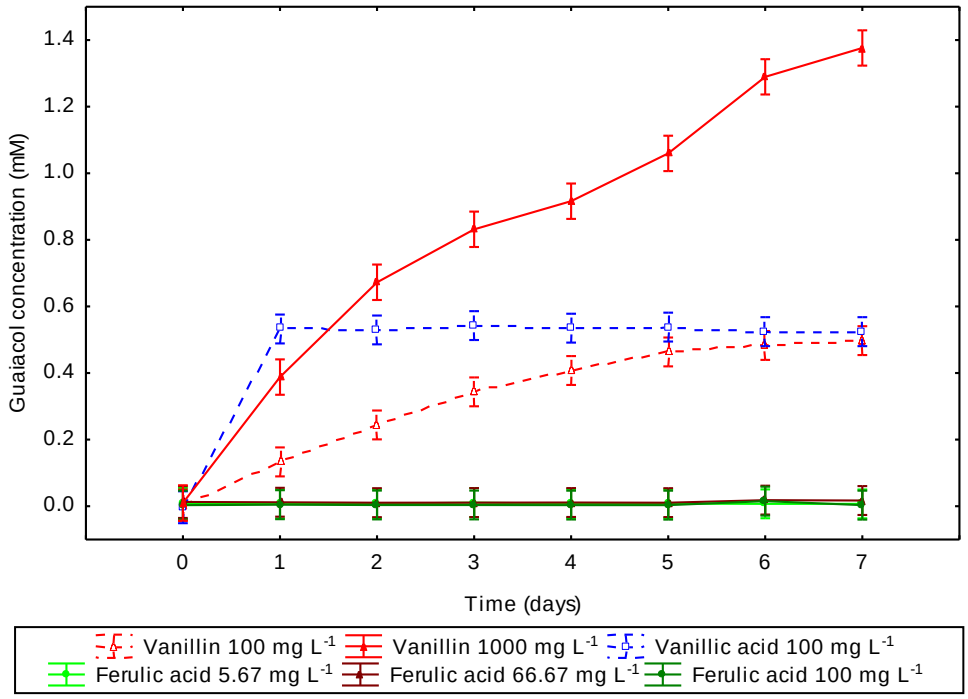

Figure 6 Guaiacol production in BAT broth supplemented with ferulic acid, vanillin and vanillic acid in the presence of *A. acidoterrestris* during the 7 d incubation period measured with high performance liquid chromatography (HPLC). Vertical bars denote 0.95 confidence intervals.

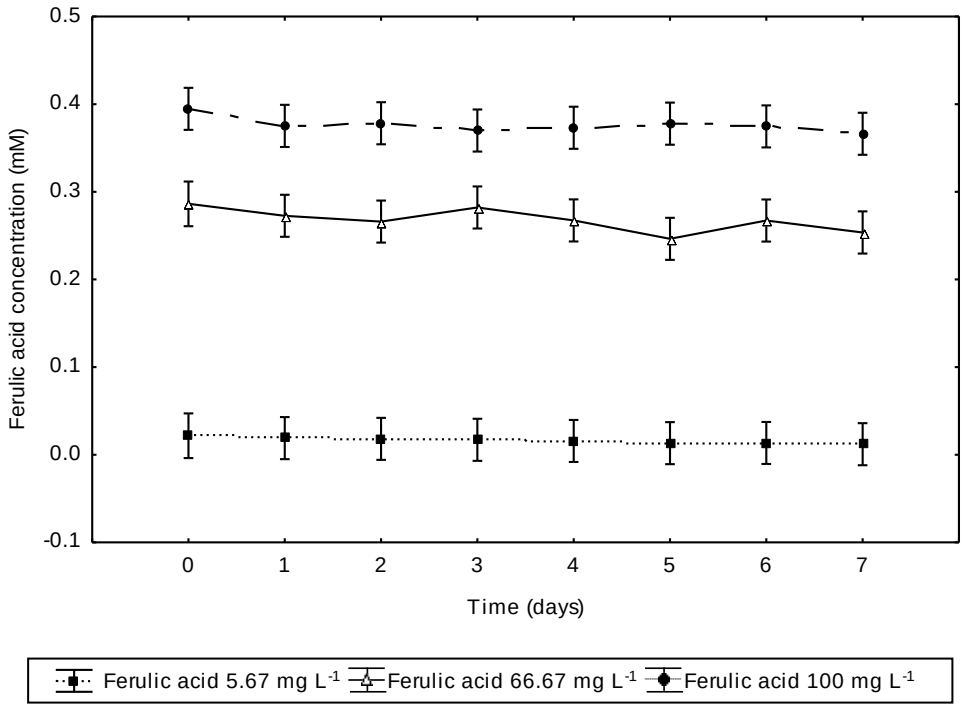

Figure 7 The ferulic acid concentrations in the BAT broth samples supplemented with ferulic acid in the presence of *A. acidoterrestris* during the 7 d incubation period measured with HPLC. Vertical bars denote 0.95 confidence intervals.

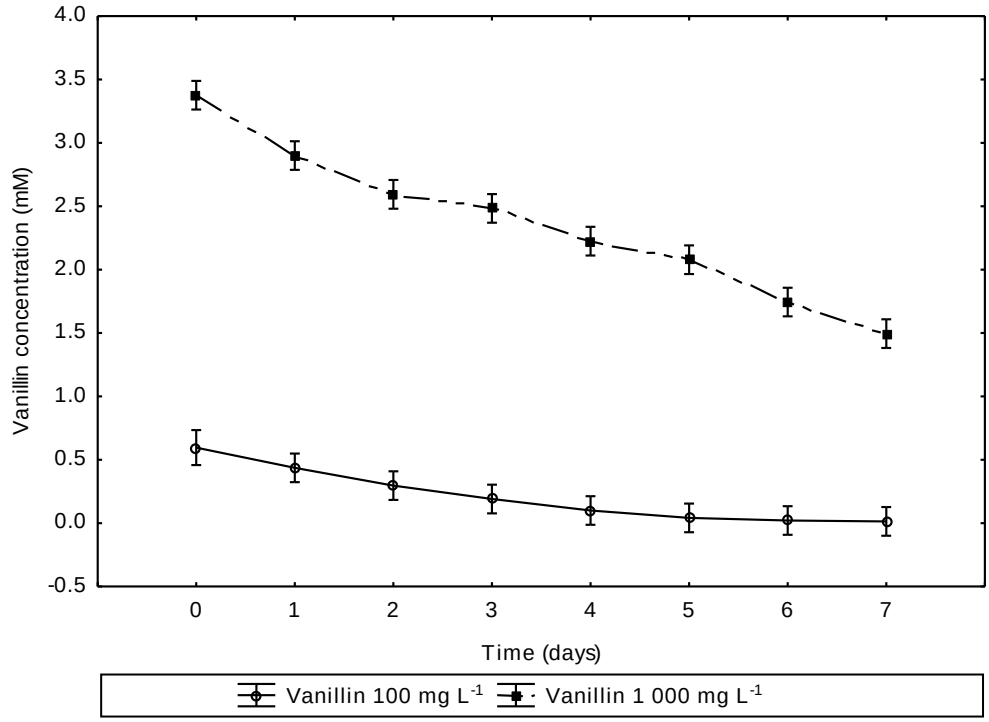

Figure 8 The decrease in vanillin concentrations (100 mg L^{-1} and 1000 mg L^{-1}) in BAT broth in the presence of *A. acidoterrestris* during the 7 d incubation period measured with HPLC. Vertical bars denote 0.95 confidence intervals.

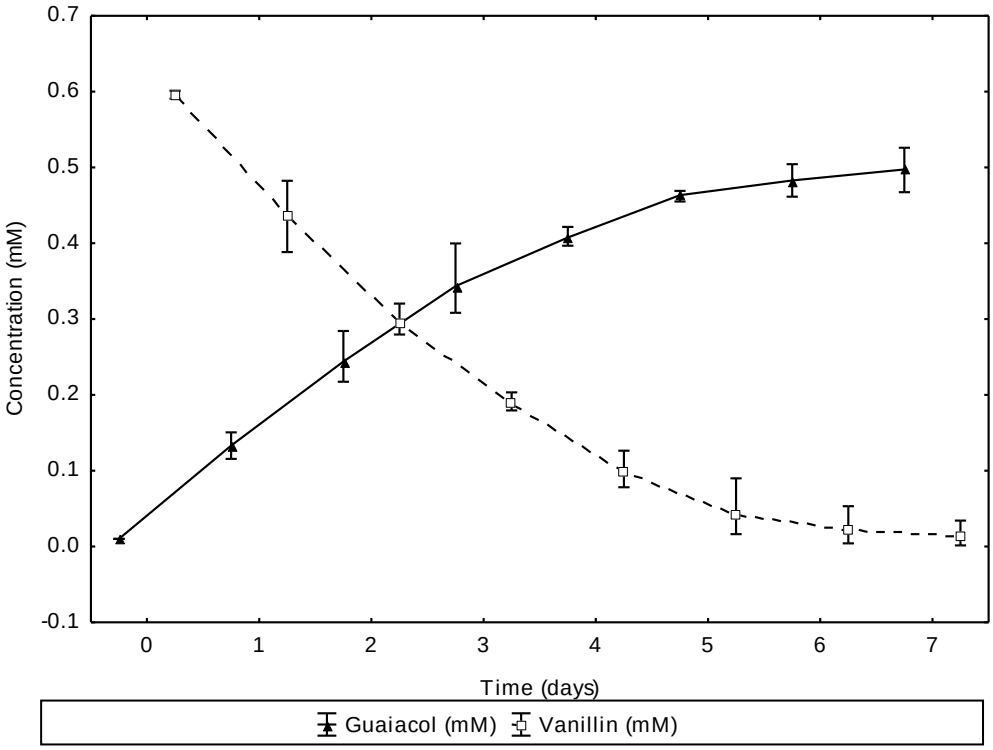

Figure 9 The guaiacol and vanillin concentrations of the 100 mg L^{-1} vanillin sample during the 7 d incubation period measured with HPLC. Vertical bars indicate minimum and maximum concentration of the triplicate experiments.

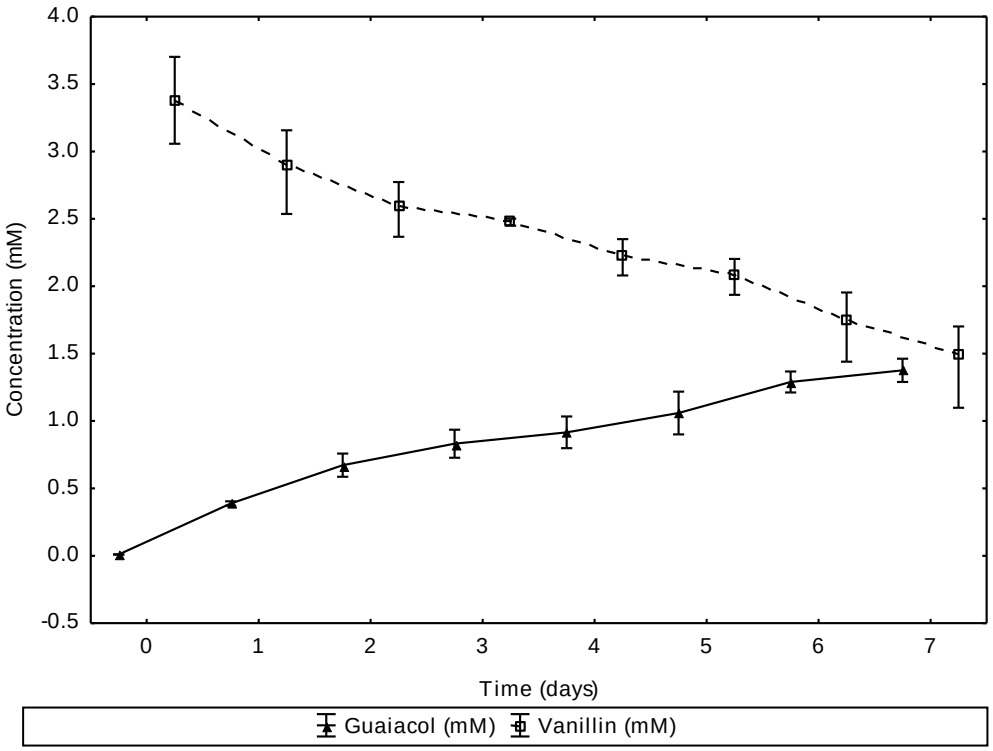

Figure 10 The guaiacol and vanillin concentrations of the 1 000 mg L^{-1} vanillin sample during the 7 d incubation period measured with HPLC. Vertical bars indicate minimum and maximum concentration of the triplicate experiments.

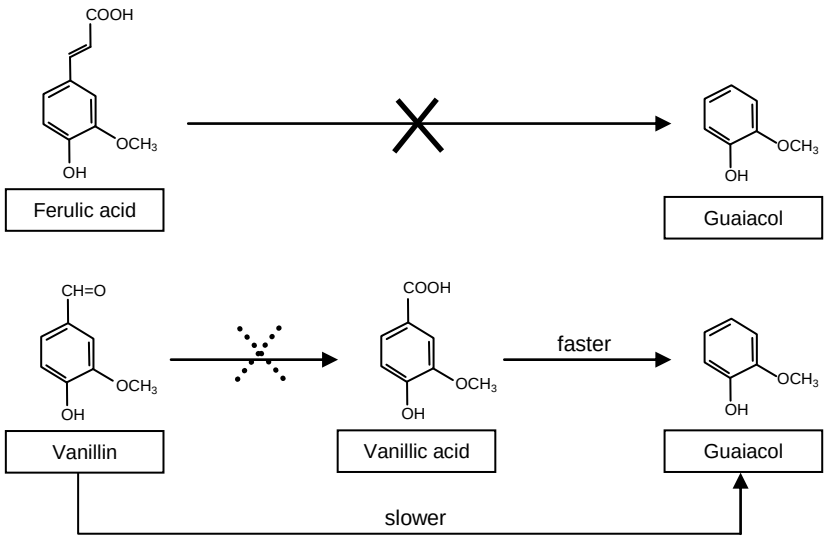

Figure 11 The pathway of guaiacol production by *A. acidoterrestris* FB2.

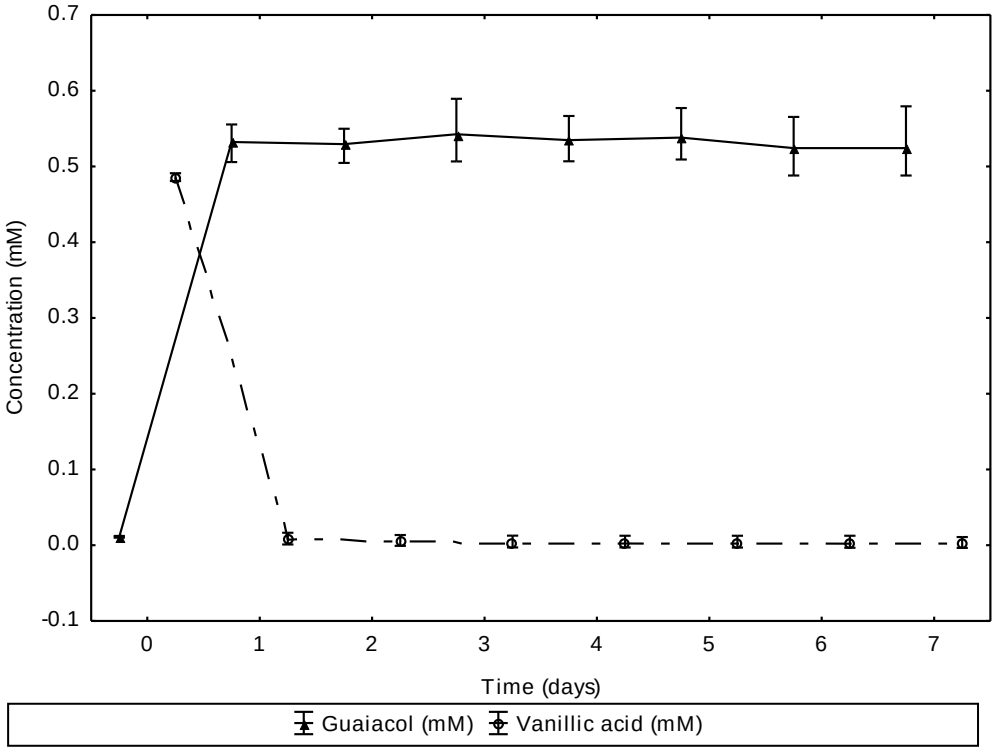

Figure 12 The guaiacol and vanillic acid concentrations of the 100 mg L^{-1} vanillic acid sample during the 7 d incubation period measured with HPLC. Vertical bars indicate minimum and maximum concentration of the triplicate experiments.

vanillic acid (at 100 mg L^{-1}) to guaiacol by *A. acidoterrestris* was faster than the conversion of vanillin to guaiacol (Fig. 11).

Guaiacol production in the 1 000 mg L^{-1} vanillic acid samples had an initial lag phase. However, the maximum concentration of guaiacol produced in this sample was higher (4.920 mM) than the guaiacol concentration produced in the 100 mg L^{-1} vanillic acid (0.543 mM) and the 1 000 mg L^{-1} vanillin (1.376 mM) samples. The initial lag phase in guaiacol production, together with the decrease in vegetative cell concentration in the first 24 h indicated that the high concentration of vanillic acid had an inhibitory effect on the cells of *A. acioterrestris* FB2 and on the initial guaiacol production (Aziz *et al.,* 1998; Moon *et al*., 2005; Delaqui *et al.,* 2006; Gañan *et al.,* 2006).

Guaiacol concentration in the 1 000 mg L^{-1} vanillin sample was higher than the maximum guaiacol concentration in the 100 mg L^{-1} vanillin sample (Fig. 6) and the guaiacol produced in 1 000 mg L^{-1} vanillic acid sample was higher (4.920 mM) compared to the guaiacol concentration produced in the 100 mg L^{-1} vanillic acid (0.543 mM). This clearly indicates that the concentration of vanillin or vanillic acid that is available to *A. acidoterrestris* influences the amount of guaiacol that is produced and, therefore, influences the spoilage potential of the sample. This observation is in agreement with the findings of Smit (2009), who indicated that guaiacol production increased when vanillin concentration increased. Contrary to the findings of Smit (2009), a lag phase in guaiacol production was not observed at a vanillin concentration of 1 000 mg L^{-1} and growth inhibition was also not observed. Growth of *A. acidoterrestris* FB2 was in the stationary phase during the 7 d at a concentration above 10^5 cfu mL^{-1} which would ensure guaiacol production (Pettipher *et al.,* 1997; Komitopoulou *et al.,* 1999).

Comparison of PECA with HPLC-DAD

An interclass correlation (Fig. 13) was used to indicate the correlation between the PECA and HPLC-DAD results that were used to measure the guaiacol concentration in all samples. A high agreement and consistency value of 0.86 was observed between the two methods, indicating that the trends of the data obtained with the two methods were similar. However, the sensitivity of the two methods was found to be different. All the samples supplemented with 1 000 mg L^{-1} vanillin where below the x = y line, possibly indicating that the high concentration of vanillin present in the samples

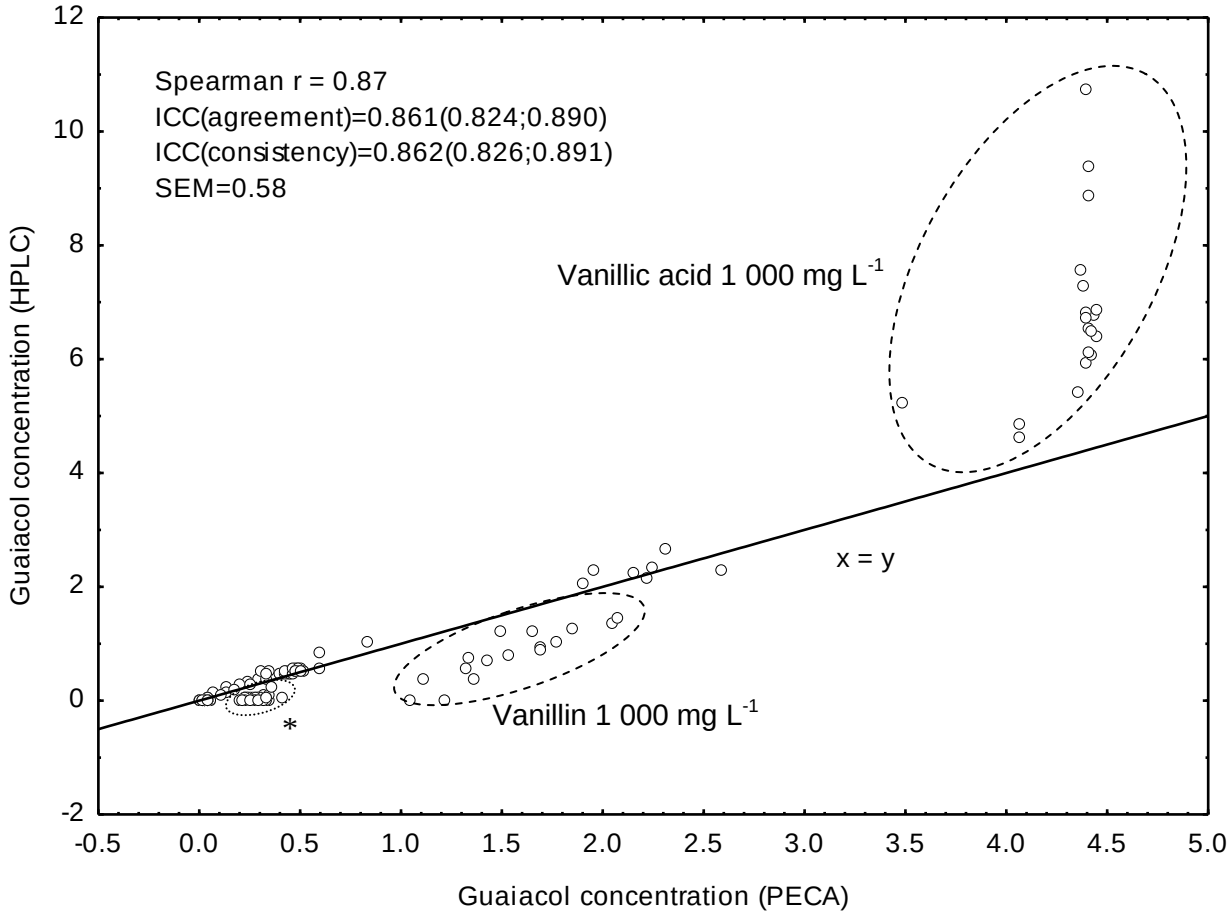

Figure 13 Interclass correlation of the HPLC-DAD method and PECA use to measure the guaiacol concentration.

influenced the colour change and absorbance measured with the PECA. The higher concentrations of guaiacol measured with the PECA in the 1 000 mg L^{-1} vanillic acid sample before the production of guaiacol or in the 1 000 mg L^{-1} vanillic acid control sample (indicated with a star on Fig. 13) are due to the high concentration of vanillic acid in the samples that also caused a discolouration of the test buffer. The samples supplemented with 1 000 mg L^{-1} vanillic acid resulting in a high concentration of guaiacol produced, where above the x = y line, indicating that the guaiacol concentration measured with the HPLC is higher compared to the concentration measured by the enzymatic assay (PECA). This is possibly an indication that the PECA cannot accurately quantify high concentrations of guaiacol. PECA can thus be used as a screening tool to indicate the presence of guaiacol and the guaiacol concentration will follow the same trend as that of the HPLC, however, HPLC analysis will be more accurate for the quantification of guaiacol.

Guaiacol production from vanillic acid

Due to the rapid conversion of vanillic acid by *A. acidoterrestris*, experiments were conducted to establish the onset of guaiacol production from vanillic acid when the critical cell concentration of *A. acidoterrestris* for guaiacol production was present. Guaiacol concentration measured with the PECA in the samples supplemented with vanillic acid, increased significantly ($p < 0.05$) 2 h after inoculation (Fig. 14). Therefore, guaiacol production started between the first and second hour after inoculation (Figs. 14 & 15). The concentration of guaiacol produced in the 100 mg L^{-1} vanillic acid sample was significantly ($p < 0.05$) higher than the concentration of guaiacol produced in the 50 mg L^{-1} vanillic acid sample (Fig. 15). Guaiacol production in the 50 mg L^{-1} vanillic acid sample remained unchanged after 2 h with no significant ($p \geq 0.05$) guaiacol production thereafter. In all the samples vanillic acid decreased significantly ($p < 0.05$) with the production of guaiacol (Fig. 15). The 100 mg L^{-1} vanillic acid was almost depleted after 4 h, while the vanillic acid in the 50 mg L^{-1} vanillic acid sample was depleted after 2 h. Therefore, the spoilage of fruit juice with vanillic acid can occur within 2 h if *A. acidoterrestris* is present at the critical cell concentration reportedly needed for guaiacol production.

CONCLUSION

The presence of *Alicyclobacillus* spp. that have the ability to produce guaiacol, as well

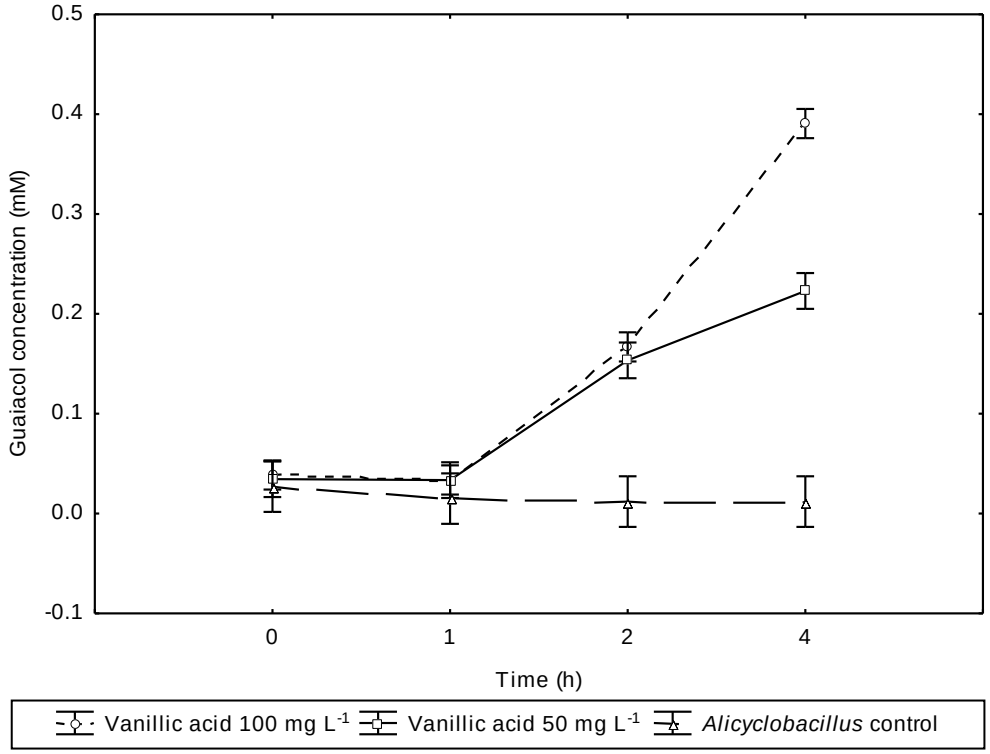

Figure 14 Guaiacol production in the first hours after incubation in the BAT broth samples supplemented with vanillic acid, measured with the peroxidase enzyme colourimetric assay (PECA). Vertical bars denote 0.95 confidence intervals.

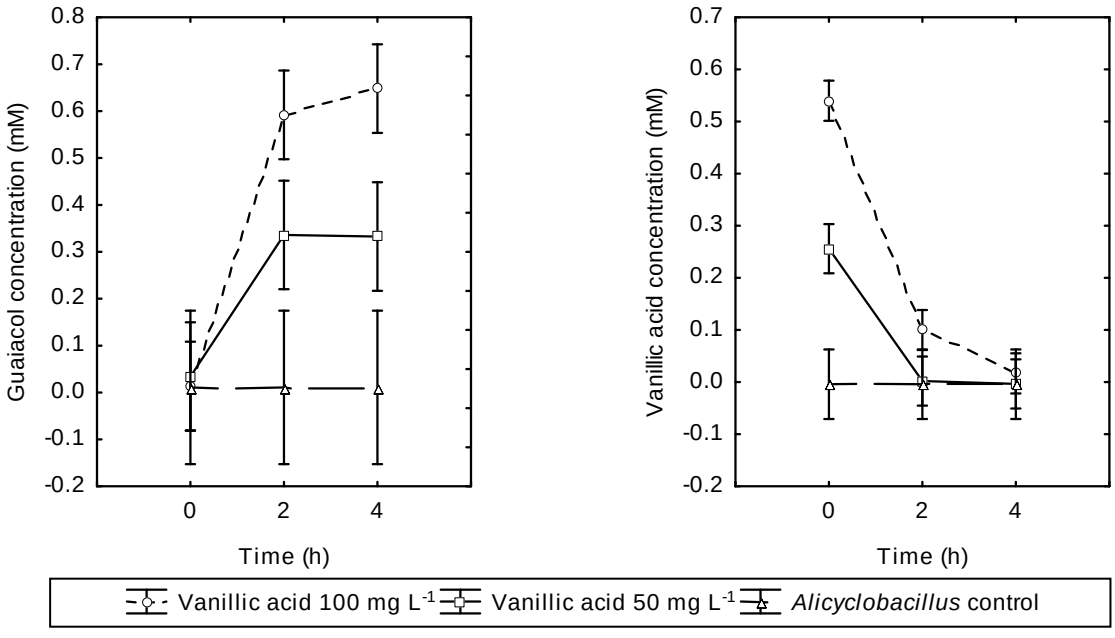

Figure 15 Guaiacol and vanillic acid concentrations in the BAT broth samples supplemented with vanillic acid, measured with HPLC. Vertical bars denote 0.95 confidence intervals.

as the substrates required for guaiacol production in fruit juices are prerequisites for guaiacol spoilage of fruit juice products. In this study vanillin and vanillic acid were confirmed as guaiacol precursors for *A. acidoterrestris* FB2. A metabolic pathway directly from vanillin to guaiacol seemed to be followed by *A. acidoterrestris*, however, the metabolic conversion of vanillic acid to guaiacol by *A. acidoterrestris* was faster than the conversion of vanillin to guaiacol. Previous studies indicated that ferulic acid in fruit juice may be a possible precursor of guaiacol production by *Alicyclobacillus.* In this study, different concentration of ferulic acid was used to determine whether ferulic acid was a precursor of guaiacol production by *A. acidoterrestris*. No guaiacol was, however, produced in any of the ferulic acid samples, indicating that ferulic acid would not influence the spoilage potential of fruit juice.

The precursors of guaiacol present in different types of fruit juice would indicate the spoilage potential of these fruit juices by *Alicyclobacillus* spp. The information gained from the composition of fruit juices can be used to prevent spoilage of the fruit juice products. Furthermore, procedures need to be improved to prevent the contamination of fruit juice products that are highly vulnerable to guaiacol spoilage. Although BAT broth was used as the media in this study to evaluate the pathway of guaiacol production by *A. acidoterrestris*, it is important to evaluate the growth of *Alicyclobacillus* and the spoilage potential of fruit juice containing lower concentrations of vanillin and vanillic acid.

REFERENCES

Álvarez-Rodríquez, M.L., Belloch, C., Villa, M., Uruburu, F., Larriba, G. & Coque, JJ. R. (2003). Degradation of vanillic acid and production of guaiacol by microorganisms isolated from cork samples. FEMS Microbiology Letters, **220**, 49-55.

Anonymous (2006). Product safety – the source for innovative microbiological detection media. DöhlerGroup Press Release. [WWW document]. URL http://www.drinksmediawire.com/upload/pdfs/cdp/DohlerGroup-ENG.pdf. 16 February 2010.

Aziz, N.H., Farag, S.E., Mousa, L.A. & Abo-Zaid, M.A. (1998). Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios,* **93**, 43-54.

Bahçeci, K.S. & Acar, J. (2007). Determination of guaiacol produced by *Alicyclobacillus acidoterrestris* in apple juice by using HPLC and spectrophotometric methods, and mathematical modeling of guaiacol production. *European Food Research and Technology*, **225**, 873-878.

Bahçeci, K.S., Serpen, A., Gökmen, V. & Acar, J. (2005). Study of lipoxygenase and peroxidase as indicator enzymes in green beans: change of enzyme activity, ascorbic acid and chlorophylls during frozen storage. *Journal of Food Engineering*, **66**, 187-192.

Chang, S.S. & Kang, D.H. (2004). *Alicyclobacillus* spp. in the fruit juice industry: history, characteristics, and current isolation/detection procedures. *Critical Reviews in Microbiology*, **30**, 55-74.

Crawford, R.L. & Olson, P.P. (1978). Microbial catabolism of vanillate: decarboxylation to guaiacol. *Applied and Environmental Microbiology*, **36**, 539-543.

Delaqui, P., Stanich, K. & Toivonen, P. (2006). Effect of pH on the inhibition of *Listeria* spp. by vanillin and vanillic acid. *Journal of Food Protection,* **69**, 1724-1727.

Doerge, D.R., Divi, R.L. & Churchwell, M.I. (1997). Identification of the colored guaiacol oxidation product produced by peroxidases. *Analytical Biochemistry*, **250**, 10-17.

Gañan, M., Martínez-Rodríguez, A.J. & Carrascosa, A.V. (2006). Antimicrobial activity of phenolic compounds of wine against *Campylobacter jejuni*. *Food Control*, **20**, 739-742.

Gocmen, D., Elston, A., Williams, T., Parish, M. & Rouseff, R.L. (2005). Identification of medicinal off-flavours generated by *Alicyclobacillus* species in orange juice using GC-olfactometry and GC-MS. *Letters in Applied Microbiology*, **40**, 172-177.

Goodner, K.L., Jella, P. & Rouseff, R. (2000). Determination of vanillin in orange, grapefruit, tangerine, lemon, and lime juices using GC-Olifactometry and GC-MS/MS. *Journal of Agricalture and Food Chemistry*, **48**, 2882-2886.

Goto, K., Nishibori, A., Wasada, Y., Furuhata, K., Fukuyama, M. & Hara, M. (2008). Identification of thermo-acidophilic bacteria isolated from the soil of several Japanese fruit orchards. *Letters in Applied Microbiology*, **46**, 289-294.

Groenewald, W.H., Gouws, P.A., & Witthuhn, R.C. (2009). Isolation, identification and typification of *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius* strains from orchard soil and the fruit processing environment in South Africa. *Food Microbiology*, **26**, 71-76.

Henderson, M.E.K. (1961). The metabolism of aromatic compounds related to lignin by some hyphomycetes and yeastlike fungi of soil. *Journal of General. Microbiology,* **26**, 155-165.

Herrmann, K. (1989). Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *CRC Critical Review in Food Science and Nutrition,* **28**, 315-347.

Huang, Z., Dostal, L. & Rosazza, J.P.N. (1993). Mechanisms of ferulic acid conversions to vanillic acid and guaiacol by *Rhodotorula rubra*. *The Journal of Biological Chemistry*, **268**, 23954-23958.

International Federation of Fruit Juice Producers (IFU). (2007). Method on the detection of taint producing *Alicyclobacillus* in fruit juices. *IFU Method No. 12*. Pp. 1-11. IFU, Paris.

Ishikawa, H., Schubert, W.J. & Nord, E.F. (1963). The degradation by *Polyporus versicolor* and *Fomes fomentarius* of aromatic compounds structurally related to soft wood lignin. *Archives of Biochemistry and. Biophysics,* **100**, 140-149.

Jensen, N. (2000). *Alicyclobacillus* in Australia. *Food Australia*, **52**, 282-285.

Jensen, N. & Whitfield, F.B. (2003). Role of *Alicyclobacillus acidoterrestris* in the development of a disinfectant taint in shelf-stable fruit juice. *Letters in Applied Microbiology*, **36**, 9-14.

Komitopoulou, E., Boziaris, I.S., Davies, E.A., Delves-Broughton, J. & Adams, M.R. (1999). *Alicyclobacillus acidoterrestris* in fruit juices and its control by nisin. *International Journal of Food Science and Technology*, **34**, 81-85.

Mangas, J.J, Rodríguez, R., Moreno, J., Suárez, B. & Blanco, D. (1997). Furanic and phenolic composition of cider brandy. A chemometric study. *Journal of Agricaltural and Food Chemistry*, **45**, 4076-4079.

Martin, A.B., Acree, T.E., Hotchkiss, J.H. & Nagy, S. (1992). Gas chromatography-Olfactometry of orange juice to assess the effects of plastic polymers on aroma character. *Journal of Agricaltural and Food Chemistry*, **40**, 650-654.

Matsubara, H., Goto, K., Matsumura, T., Mochida, K., Iwaki, M., Niwa, M. & Yamasato, K. (2002). *Alicyclobacillus acidiphilus* sp. nov., a novel thermo-acidophilic, ω-alicyclic fatty acid-containing bacterium isolated from acidic beverages. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 1681-1685.

Moon, K.D., Delaquis, P., Toivonen, P., Bach, S. Stanich, K. & Harris, L. (2006). Destruction of Escherichia coli O157:H7 by vanillic acid in unpasteurized juice from six apple cultivars. *Journal of Food Protection,* **69**, 572-547.

Niwa, M. & Kawamoto, A. (2003). Development of a rapid detection method of *A. acidoterrestris*, hazardous bacteria to acidic beverage. *Fruit Processing*, **13**, 102-107.

Pettipher, G.L., Osmundson, M.E. & Murphy, J.M. (1997). Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit juice-containing drinks. *Letters in Applied Microbiology*, **24**, 185-189.

Piacquadio, P., De Stefano, G. & Sciancalepore, V. (2002). Phenolic composition of apple juice treated by laccase immobilized on copper-chelate regenerable carrier. *Italian Journal of Food Science*, **14**, 291-294.

Pyysalo, T., Honkanen, E. & Hirvi, T. (1979). Volitiles of wild strawberries, *Fragaria vesca* L., compared to those of cultivated berries, *Fragaria x ananassa* cv. Senga Sengana. *Journal of Agricaltural and Food Chemistry*, **27**, 19-22.

Rahouti, M., Seigle-Murandi, F., Steiman, R. & Eriksson, K-E. (1989). Metabolism of ferulic acid by *Paecilomyces variotii* and *Pestalotia palmarum*. *Applied and Environmental Microbiology*, **55**, 2391-2398.

Rosazza, J.P.N., Huang, Z., Dostal, L., Volm, T. & Rousseau, B. (1995). Review: Biocatalytic transformations of ferulic acid: an abundant aromatic natural product. *Journal of Industrial Microbiology*, **15**, 457-471.

Sakho, M., Chassagne, D. & Crouzet, J. (1997). African mango glycosidically bound volatile compounds. *Journal of Agricaltural and Food Chemistry*, **45**, 883-888.

Sheu, S.C. & Chen, A.O. (1991). Lipoxygenase as blanching index for frozen vegetable soybeans. *Journal of Food Science*, **56**, 448-451.

Smit, Y. (2009). Growth and guaiacol production of species of *Alicyclobacillus* isolated from the South African fruit processing environment. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Spillman, P.J., Pollnitz, A.P., Liacopoulos, D., Skouroumounis, K. & Sefton, M.A. (1997). Accumulation of vanillin during barrel-aging of white, red, and model wines. *Journal of Agricaltural and Food Chemistry*, **45**, 2584-2589.

Splittstoesser, D.F., Churey, J.J. & Lee, C.Y. (1994). Growth characteristics of aciduric sporeforming bacilli isolated from fruit juices. *Journal of Food Protection*, **57**, 1080-1083.

Sutherland, J.B., Crawford, D.L. & Pometto, A.L. (1983). Metabolism of cinnamic, *p*-coumaric and ferulic acid by *Streptomyces setonii. Canadian. Journal of Microbiology,* **29**, 1253.

Tadasa, K. (1977). Degradation of eugenol by a microorganism. *Agricultural* and Biological Chemistry*,* **41**, 925-929.

Toms, A. & Wood, J.M. (1969). The degradation of trans-ferulic acid by *Pseudomonas acidovorans. Biochemistry,* **9**, 337.

Topakas, E., Kalogeris, E., Kekos, D., Macris, B.J. & Christakopoulos, P. (2003). Bioconversion of ferulic acid into vanillic acid by the thermophilic fungus *Sporotrichum thermophile*. *Lebensmittel-Wissenschaft und -Technologie*, **36**, 561-565.

Walls, I. & Chuyate, R. (1998). *Alicyclobacillus* - historical perspective and preliminary characterization study. *Dairy, Food and Environmental Sanitation*, **18**, 499-503.

Yamazaki, K., Teduka, H. & Shinano, H. (1996). Isolation and identification of *Alicyclobacillus acidoterrestris* from acidic beverages. *Bioscience, Biotechnology, and Biochemistry*, **60**, 543-545.

APPENDIX

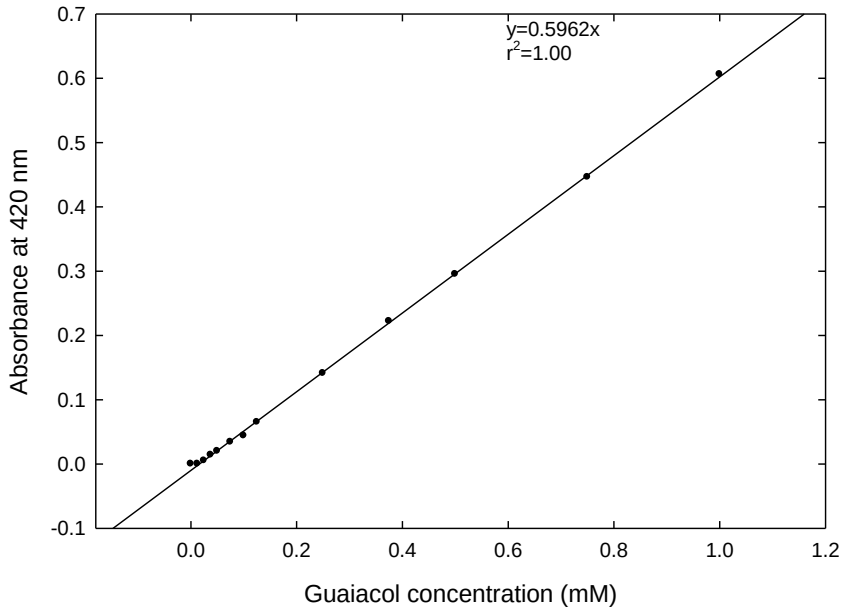

Figure 1A The standard curve of known guaiacol concentrations versus absorbance measured with the PECA. The equation of the regression line as well as the r^2 value is indicated on the graph.

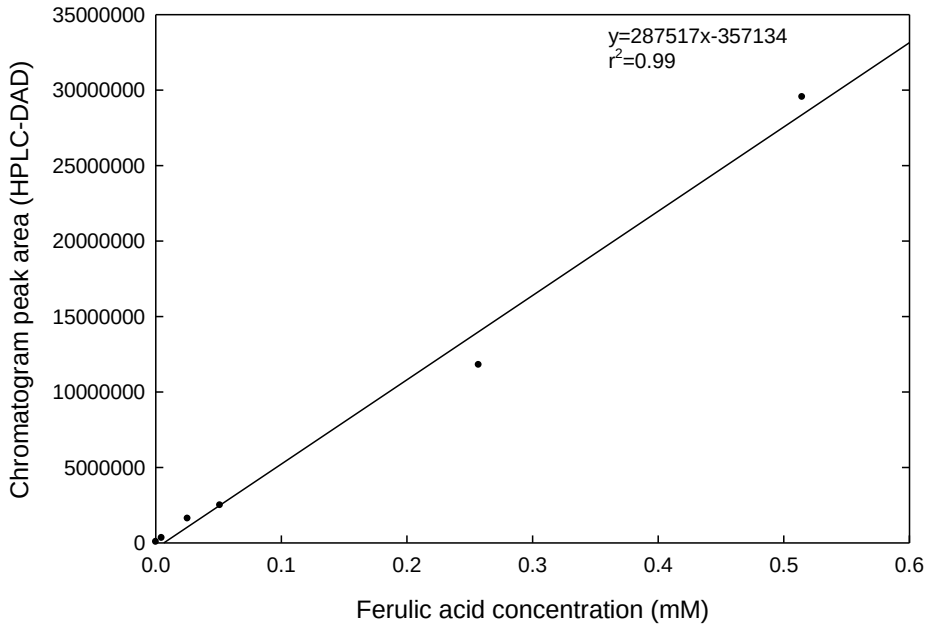

Figure 2A The standard curve for determination of ferulic acid concentration with HPLC-DAD. The equation of the regression line as well as the r^2 value is indicated on the graph.

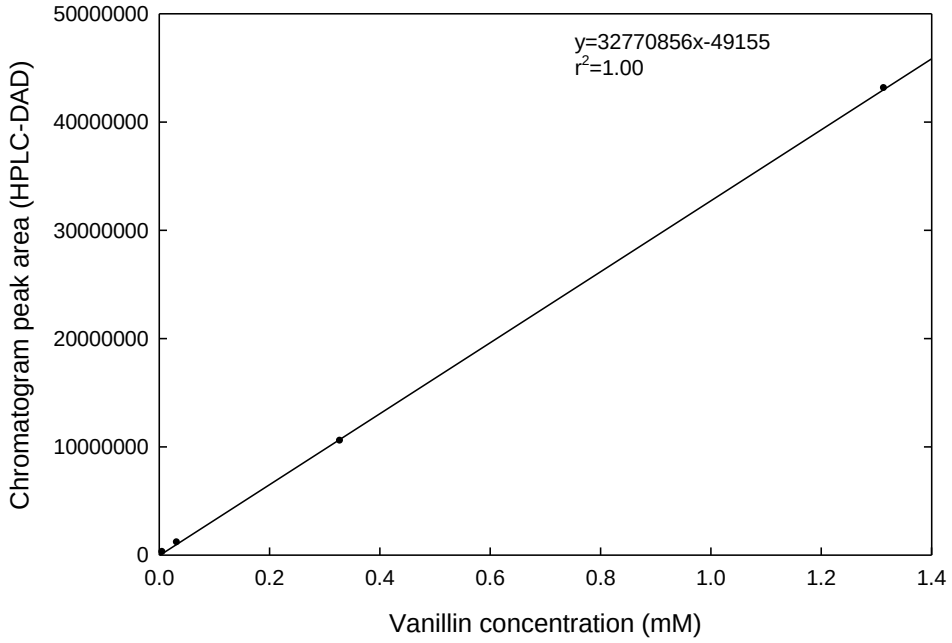

Figure 3A The standard curve for determination of vanillin concentration with HPLC-DAD. The equation of the regression line as well as the r^2 value is indicated on the graph.

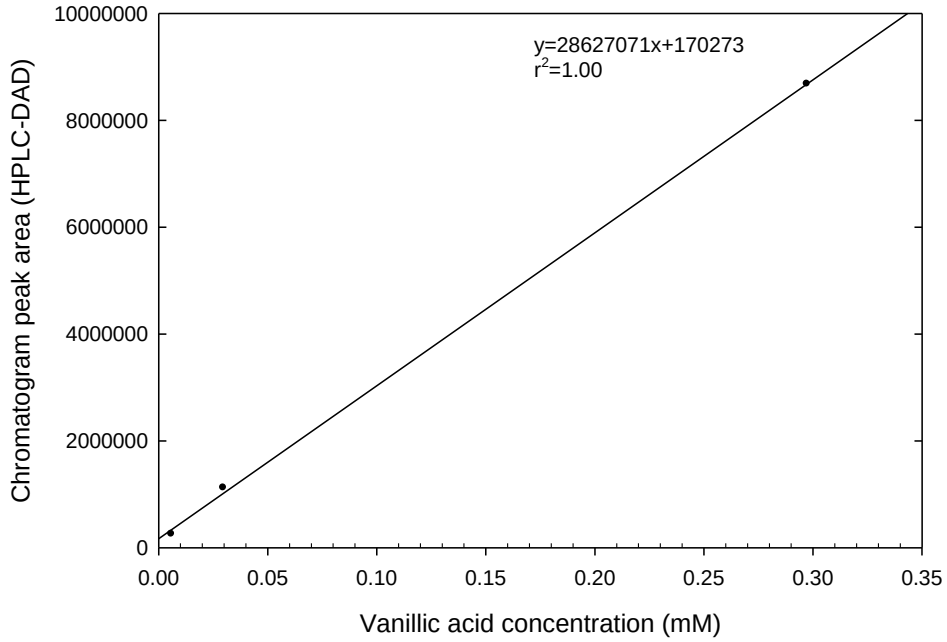

Figure 4A The standard curve for determination of vanillic acid concentration with HPLC-DAD. The equation of the regression line as well as the r^2 value is indicated on the graph.

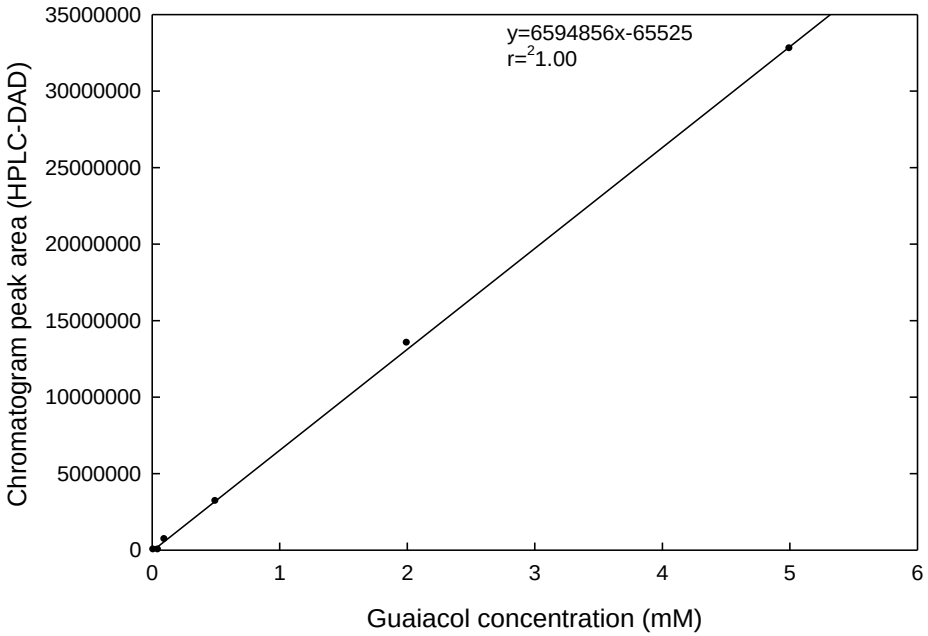

Figure 5A The standard curve for determination of guaiacol concentration with HPLC-DAD. The equation of the regression line as well as the r^2 value is indicated on the graph.

CHAPTER 5

PRODUCTION OF GUAIACOL BY *ALICYCLOBACILLUS ACIDOTERRESTRIS* IN APPLE JUICE FROM THE PRECURSORS VANILLIN AND VANILLIC ACID

ABSTRACT

Alicyclobacillus survive the pasteurisation process and spoil acidic fruit juices through the production of the taint compound, guaiacol. The presence of the precursors for guaiacol production by species of *Alicyclobacillus* may influence the spoilage potential of a specific fruit juice. The aim of this study was to evaluate the spoilage potential of apple juice, supplemented with vanillin and vanillic acid by *Alicyclobacillus acidoterrestris*. Apple juice supplemented with either 10 mg L^{-1} or 100 mg L^{-1} vanillin or vanillic acid was inoculated with *A. acidoterrestris* FB2 and incubated at 45 °C for 7 d. The samples were analysed every 24 h to determine the cell concentration, pH, °Brix, the concentration of supplemented vanillin or vanillic acid using high performance liquid chromatography with UV-diode array detection (HPLC-DAD) and the guaiacol concentration, using both the peroxidase enzyme colourimetric assay (PECA) and HPLC-DAD. The cell concentration of *A. acidoterrestris* FB2 in each sample during the 7 d was above the required vegetative cell concentration of 10^5 cfu mL^{-1} reportedly required for guaiacol production. Guaiacol was produce by *A. acidoterrestris* from vanillin and vanillic acid, confirming that these compounds are precursors of guaiacol production by *A. acidoterrestris* FB2. The HPLC method was sensitive for detecting low concentrations of guaiacol produced, while the PECA did not differentiate between the control samples, containing no guaiacol and the samples with low guaiacol concentrations (6.46 to 14.40 mg L^{-1}). Guaiacol concentrations well above the best estimated threshold (BET) value for taste and odour was produced in apple juice by *A. acidoterrestris* FB2 from supplemented vanillin or vanillic acid at concentrations of 10 mg L^{-1}. Higher concentrations of guaiacol were produce in the samples supplemented with higher concentrations of vanillin and vanillic acid, indicating that the concentration of vanillin and vanillic acid present in apple juice influenced the concentration of taint compound produced and consequently the spoilage potential of the juice.

INTRODUCTION

The genus *Alicyclobacillus* consist of endospore-forming, thermophilic, acidophilic bacteria that can survive the pasteurisation process and spoil fruit juice products (Jensen, 2000; Jensen & Whitfield, 2003; Chang & Kang, 2004). Spoilage by *Alicyclobacillus* spp. is difficult to detect and in several spoilage incidences it was not recognised until consumer complaints were received. In some clarified fruit juices only a light sediment, cloudiness or haze is visible (Walker & Phillips, 2005), with no gas production (Walls & Chuyate, 1998). The only obvious indication of spoilage is the formation of an off-flavour (Walls & Chuyate, 1998; Silva *et al.,* 1999; Pettipher & Osmundson, 2000). Guaiacol, a by-product of the microbial metabolic activity causes a taint that is described as an unpleasant smoky, medicinal or phenolic-like taste with an undesirable odour (Pettipher *et al.,* 1997; Jensen, 2000). The best estimated threshold (BET) value for taste of guaiacol in apple juice range from 0.24 – 2.00 µg L^{-1} and for odour range from 0.50 - 2.32 µg L^{-1} (Pettipher *et al.,* 1997; Orr *et al.,* 2000; Eisele & Semon, 2005; Siegmund & Pöllinger-Zierler, 2006).

Fruit juices that are liable to spoilage by *Alicyclobacillus* include apple, orange, tomato, pineapple, grapefruit, pear, white grape and cranberry juice (Pettipher *et al.,* 1997; Splittstoesser *et al*., 1998; Walls & Chuyate, 1998; Durak *et al.,* 2010). Growth does not occur in concentrates, however, a reduction in the sugar concentration to 16 °Brix under suitable conditions results in optimal growth of *Alicyclobacillus* spp. (Splittstoesser *et al*., 1994).

The production of guaiacol in food products is dependent on the precursors and microbes present in the product. The precursors required for the production of guaiacol can be naturally present in fruit juice and if *Alicyclobacillus* species that have the ability to produce guaiacol are present at the ideal environmental conditions, the taint can be formed (Chang & Kang, 2004). In the suggested pathway for guaiacol production in fruit juice by *Alicyclobacillus*, guaiacol is produced from ferulic acid via vanillin and vanillic acid (Niwa & Kawamoto, 2003; Bahçeci *et al*., 2005). Vanillin and vanillic acid are present in the juice as derivatives of lignin or ferulic acid degradation (Peleg *et al.*, 1992; Chang & Kang, 2004). Piacquadio *et al.* (2002) reported 10.07 (± 1.13) mg L^{-1} vanillic acid in apple juice. In orange, tangerine, lemon, lime and grape fruit juice vanillin concentrations range from 0.20 to 0.60 mg L^{-1} (Goodner *et al.,* 2000).

It has been reported that vanillin and vanillic acid are precursors of guaiacol for *Alicyclobacillus acidoterrestris* and the concentration of these precursors influence the production of guaiacol by *A. acidoterrestris*. Therefore, the availability of vanillin and

vanillic acid can possibly influence the spoilage of fruit juice products. The aim of this study is to evaluate the spoilage potential of apple juice supplemented with vanillin and vanillic acid in the presence of the spoilage microbe *A. acidoterrestris.*

MATERIALS AND METHODS

Bacterial cultures

Alicyclobacillus acidoterrestris FB2 (Department of Food Science Culture Collection, Stellenbosch University), isolated from pear concentrate (Groenewald *et al*., 2009) was used as inuculum in this study. A pure culture of *A. acidoterrestris* FB2 (vegetative cells and endospores) was inoculated into *Bacillus acidoterrestris* (BAT) broth (IFU, 2007). The broth was adjusted to pH 4 using 1 M H_2SO_4 (Merck) and sterilised by autoclaving prior to inoculation. The inoculated media was incubated at 45 °C for 2 d and thereafter, 1 mL of the culture was aseptically removed and inoculated in sterile BAT broth (pH 4) followed by incubation at 45 °C for 5 d. After the 5d incubation period the inoculated broth was centrifuged for 10 min in a Beckman Coulter TJ-25 centrifuge (Beckman Coulter Inc., Fullerton, California, USA) at 5 500 *g,* the supernatant was discarded and the pellet was resuspended in sterile saline solution (SSS) (0.85% (m/v) NaCl (Merck)). The optical density (OD) of the culture was determined with a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA) at 540 nm to determine the approximate cell concentration using a standard curve.

Preparation of apple juice samples

Apple concentrate (70 °Brix) was diluted with sterile dH_2O to 12 °Brix in 1 L Schott bottles. Three mL apple juice supplemented with either 10 mg L^{-1} or 100 mg L^{-1} vanillin or vanillic acid were heat shocked in a water bath for 10 min at 80 °C to activate *Alicyclobacillus* endospores that may have been present in the apple concentrate. The samples were left to cool and after 1 h the samples were pasteurised for 2 min at 90 °C to sterilise the samples (Walker & Philips, 2008). The samples were inoculated with *A. acidoterrestris* FB2 to a final cell concentration of 10^6 cfu mL^{-1}. All the samples were incubated at 45 °C for 7 d and analysed every 24 h to evaluate the concentrations of the precursors and the production of guaiacol. The concentration of 10 mg L^{-1} vanillin or vanillic acid was supplemented in the samples to evaluate the production of guaiacol from the low concentrations of vanillin or vanillic acid possibly present in fruit juice and

100 mg L^{-1} vanillin or vanillic acid was supplemented in the samples to evaluate the effect of increased concentrations of vanillin or vanillic acid available to *A. acidoterrestris* FB2. The vegetative cell concentration, pH and °Brix of each sample was also measured. Guaiacol concentrations were determined using two methods, the peroxidase enzyme colourimetric assay (PECA) and high performance liquid chromatography with UV-diode array detection (HPLC-DAD). The supplemented vanillin and vanillic acid concentrations in the samples were also determined by HPLC analysis. All experiments were performed in triplicate. Three mL apple juice (12 °Brix) inoculated with 10^6 cfu mL^{-1} *A. acidoterrestris* FB2 (without supplement) was used as the *Alicyclobacillus* control sample. The *Alicyclobacillus* control sample was included to indicate that production of guaiacol does not occur without the presence of precursors. Samples of 300 mL apple juice supplemented with either 10 mg L^{-1} or 100 mg L^{-1} vanillic acid or vanillin (without *Alicyclobacillus*) served as the supplement control samples. The supplement control samples were used to indicate that spontaneous production of guaiacol from vanillin and vanillic acid will not occur without the metabolic action of *Alicyclobacillus* species.

Vegetative cell and endospore concentration

The vegetative cell concentration during the 7 d incubation period was determined by serially diluting (10^0 – 10^6) each sample in SSS, followed by enumeration with Potato dextrose agar (PDA) (Merck) (pH 4) using the pour plate technique and incubation at 45 °C for 4 d. The endospore concentration was determined by first subjecting 1 mL of each sample to a heat shock treatment in a water bath at 80 °C for 10 min to eliminate all the viable vegetative cells. Thereafter, the samples were serially diluted (10^0 – 10^6) in SSS, followed by enumeration using PDA (pH 4) and incubation at 45 °C for 4 d. Control plates of PDA (pH 4) incubated at 45 °C for 4 d had no growth. Experiments were conducted in duplicate.

pH and °Brix

Four ml of each sample was used to determine the pH using a HI221 Calibration Check Microprocessor pH meter (Hanna Instruments, Padova, Italy). A drop of the sample was used to determine the °Brix with a handheld refractometer (H-50, ATAGO).

Peroxidase enzyme colourimetric assay (PECA)

The peroxidase enzyme colourimetric assay (Sheu & Chen, 1991; Bahçeci & Acar 2007) was used as a rapid method to determine the guaiacol concentration in all of the samples. One mL of each sample was centrifuged at 2 300 *g* for 10 min (Eppendorf 5415 D centrifuge, Hamburg, Germany), followed by vortexing 300 µL of the supernatant with 2 mL 0.2 M potassium phosphate buffer (4.18 g L^{-1} K_2HPO_4 (Merck) and 23.95 g L^{-1} KH_2PO_4 (Merck), pH 6), 300 µL (5 U) peroxidase enzyme (Merck) and 300 µL 0.5% (m/v) H_2O_2 (Merck). The blank consisted of 2.3 mL 0.2 M potassium phosphate buffer, 300 µL (5 U) peroxidase enzyme and 300 µL 0.5% (m/v) H_2O_2. The colour changes was measured at 420 nm using a Beckman Coulter DU 530 spectrophotometer and the guaiacol concentration was calculated using a standard curve of absorbance at 420 nm versus guaiacol (Sigma-Aldrich, USA) concentration (Fig. 1A, Appendix).

High performance liquid chromatography (HPLC)

Standard solutions

Guaiacol standard solutions were prepared in the range of 0.0125 - 5 mM and vanillin (0.007 - 6.572 mM) and vanillic acid (0.006 - 5.947 mM) standard solutions were prepared in the range of 1 - 1000 mg L^{-1}. The standard solutions were filtered using a 0.8/0.2 µm Supor Membrane Low Protein binding Non-pyrogenic filter (Pall, USA) prior to HPLC analysis.

HPLC analysis

A HPLC system (Spectra Physics) with UV-diode array detection was used to determine the vanillin, vanillic acid and guaiacol concentrations present in each 24 h sample. The samples were diluted 1:1 (v/v) to 6 °Brix prior to HPLC analysis and filtered (0.8/0.2 µm Supor membrane filter) prior to injection in the HPLC system fitted with a silica C_{18} column (Supelco, SA) with dimensions 25 cm x 4.6 mm x 5 µm. The elution program was carried out at 40 °C, the injection volume was set at 2 µL, loop injection at 50 µL, the flow rate was set at 1 mL min^{-1} and the maximum run pressure was 3 000 psi. The mobile phase consisted of 50 % solvent A, a mixture of formic acid (100 %) (Merck) and water (5:95), and 50 % solvent B, consisting of methanol (HPLC grade) (Merck). UV detection was performed at 272 nm, 312 nm and 328 nm and computerised integration was performed using Chromquest v. 3.0 to calculate the peak areas. Identification of vanillin, vanillic acid and guaiacol in the samples were carried out by comparing

retention times and spectral data with those of the appropriate standards. A standard curve of each standard was used to quantify vanillin, vanillic acid and guaiacol in the samples.

Statistical analysis

Repeated measures ANOVA with a mixed model approach were used to analyse the data obtained in this study. Correlation analyses were also done to determine the strength of relationships between the data obtain by the two methods used to quantify guaiacol. Statistical analyses were performed using Statistica 9 and 95 % confidence interval was used as a guideline for indicating significant differences.

RESULTS AND DISCUSSION

Influence of the precursors on the growth of *A. acidoterrestris* FB2

A 10^6 cfu mL^{-1} inoculum of *A. acidoterrestris* was used to ensure that the concentration of cells present in the medium was above the concentrations of 10^4 - 10^5 cfu mL^{-1} that is reportedly required for guaiacol production (Pettipher *et al.,* 1997; Komitopoulou *et al.,* 1999). The vegetative cell concentration in all samples inoculated with *A. acidoterrestris* decreased in the first 24 h, possibly due to the lower pH of the apple juice compared to the BAT broth (pH 4). The vegetative cell concentration in the *Alicyclobacillus* control sample (without precursors)was significantly ($p < 0.05$) higher on day 4 compared to the initial cell concentration, but was similar after 7 d incubation (Fig. 1). The vegetative cell growth of the *Alicyclobacillus* control and the samples supplemented with 100 mg L^{-1} vanillin or 10 mg L^{-1} vanillin followed a similar trend (Fig. 1), except that the vegetative cell concentration of the *Alicyclobacillus* control sample (without precursors)was significant ($p < 0.05$) lower after 3 d incubation than the cell count in the sample supplemented with 10 mg L^{-1} vanillin. The vegetative cell concentration of the samples supplemented with 10 mg L^{-1} or 100 mg L^{-1} vanillic acid was significantly ($p < 0.05$) higher on d 3, 6 and 7 and significantly ($p < 0.05$) lower on day 4 compared to the *Alicyclobacillus* control sample (without precursors)(Fig. 1). The vegetative cell concentration in the 100 mg L^{-1} vanillic acid sample increased on day 2 and 5, while the vegetative cell concentration in the sample supplemented with 10 mg L^{-1} vanillic acid increase significantly ($p < 0.05$) from day 1 to day 3 and on day 5 (Fig. 1). The final cell concentrations of all the samples were above 10^6 cfu mL^{-1},

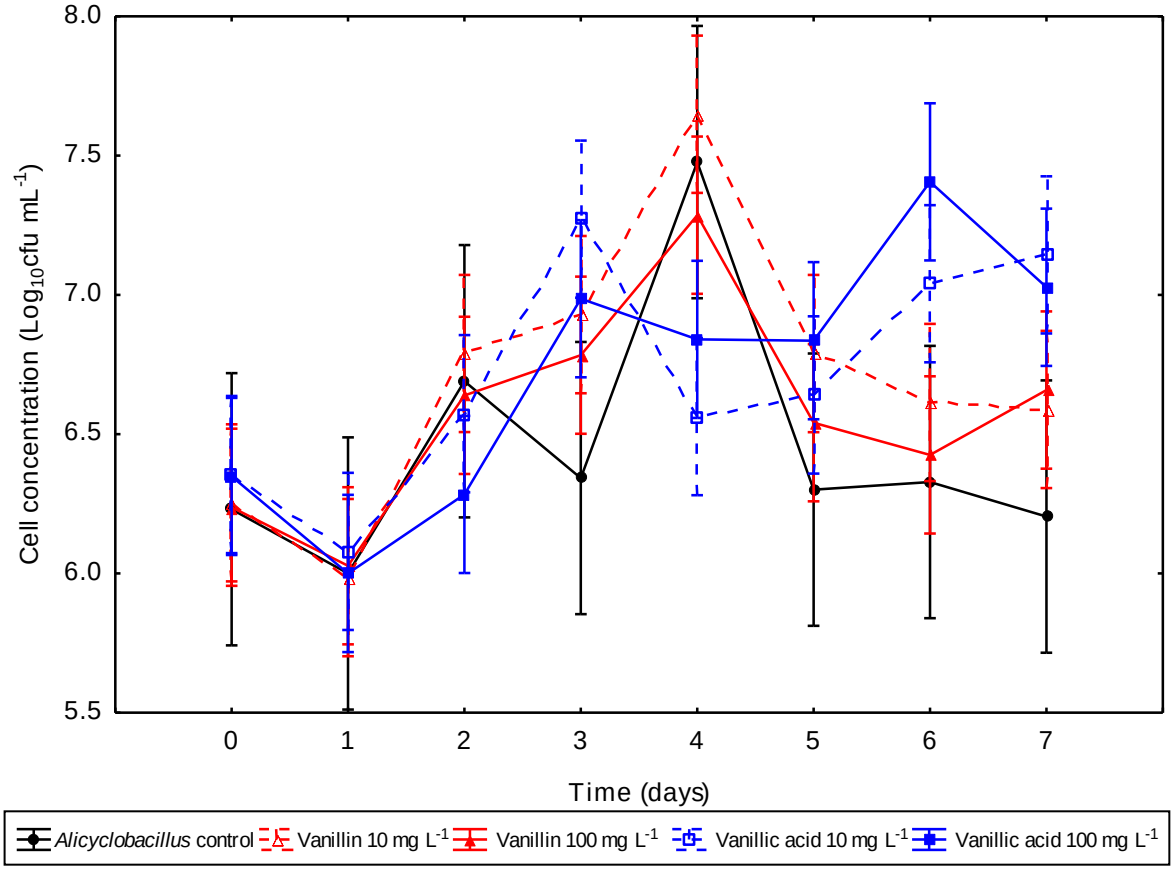

Figure 1 Changes in vegetative cell concentration of *A. acidoterrestris* FB2 at different concentrations of vanillin and vanillic acid (10 mg L^{-1} and 100 mg L^{-1}) in single strength apple juice incubated at 45 °C for 7 d. Vertical bars denote 0.95 confidence intervals.

pasteurised fruit juices. All of the samples, except the *Alicyclobacillus* control sample (without precursors), had a significant ($p < 0.05$) decrease in endospore concentration within the first 24 h (Fig. 2). The endospore concentration of the *Alicyclobacillus* control sample increased significantly ($p < 0.05$) from day 3 to 4, with a significantly ($p < 0.05$) higher endospore count on day 4 and 5 compared to the initial endospore concentration (Fig. 2). The endospore concentration of all the other samples showed similar trends, with a significant ($p < 0.05$) decrease in endospore concentration from day 0 to 1 and a significant increase in endospore concentration from day 2 to 3. The 10 mg L^{-1} vanillic acid sample, however, had a significant ($p < 0.05$) increase in endospore concentration from day 1 to 3 (Fig. 2). The endospore concentration in the 100 mg L^{-1} and 10 mg L^{-1} vanillin samples were significantly ($p < 0.05$) higher on day 3 compared to the *Alicyclobacillus* control sample (without precursors) (Fig. 2). The endospore concentration of the 100 mg L^{-1} vanillic acid sample was significantly ($p < 0.05$) higher from day 3 to 7 compared to the *Alicyclobacillus* control sample, while the endospore concentration in the 10 mg L^{-1} vanillic acid sample was only significantly ($p < 0.05$) higher on day 3 and 6 (Fig. 2). All the samples had a final enodospore concentration above 10^6 cfu mL^{-1}.

Change in pH

The initial pH of the *Alicyclobacillus* control sample (without precursors) and the samples supplemented with vanillin or vanillic acid were all at pH 3.3 (Fig. 3). The slightly lower pH of the samples supplemented with vanillic acid was ascribed to the added vanillic acid (Fig. 3). The *Alicyclobacillus* control sample (without precursors) had an increase and decrease in pH from day 5 to 7, however, the final pH was not significantly ($p \geq 0.05$) higher than the initial pH. The vanillin control sample also had an increase and decrease in pH during the 7 d with a final pH of 3.3 (Fig. 3). Only a slight decrease in pH was observed in the vanillic acid control samples from 3.3 to 3.2.

The 10 mg L^{-1} vanillin sample showed an increase in pH in the first 24 h and the final pH of 3.4 was higher than the initial pH. The final pH was also higher than the pH of the vanillin control sample. The sample supplemented with 100 mg L^{-1} vanillin also had an increase in pH, with a final pH of 3.4 (Fig. 3). The sample supplemented with 10 mg L^{-1} vanillic acid, had an increase in pH until day 4, with a final pH of 3.4. The sample supplemented with 100 mg L^{-1} vanillic acid also showed an increase in pH with a final pH of 3.4 (Fig. 3). Although an increase in pH was observed in the samples

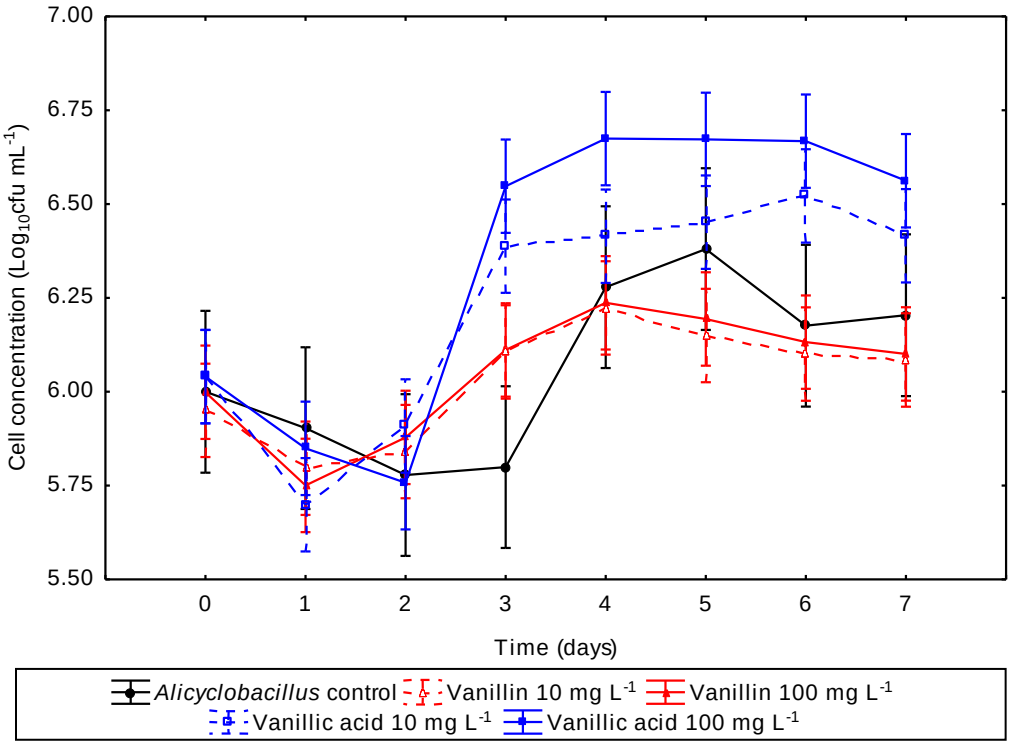

Figure 2 Changes in endospore concentration of *A. acidoterrestris* FB2 at different concentrations of vanillin and vanillic acid (10 mg L^{-1} and 100 mg L^{-1}) in single strength apple juice incubated at 45 °C for 7 d. Vertical bars denote 0.95 confidence intervals.

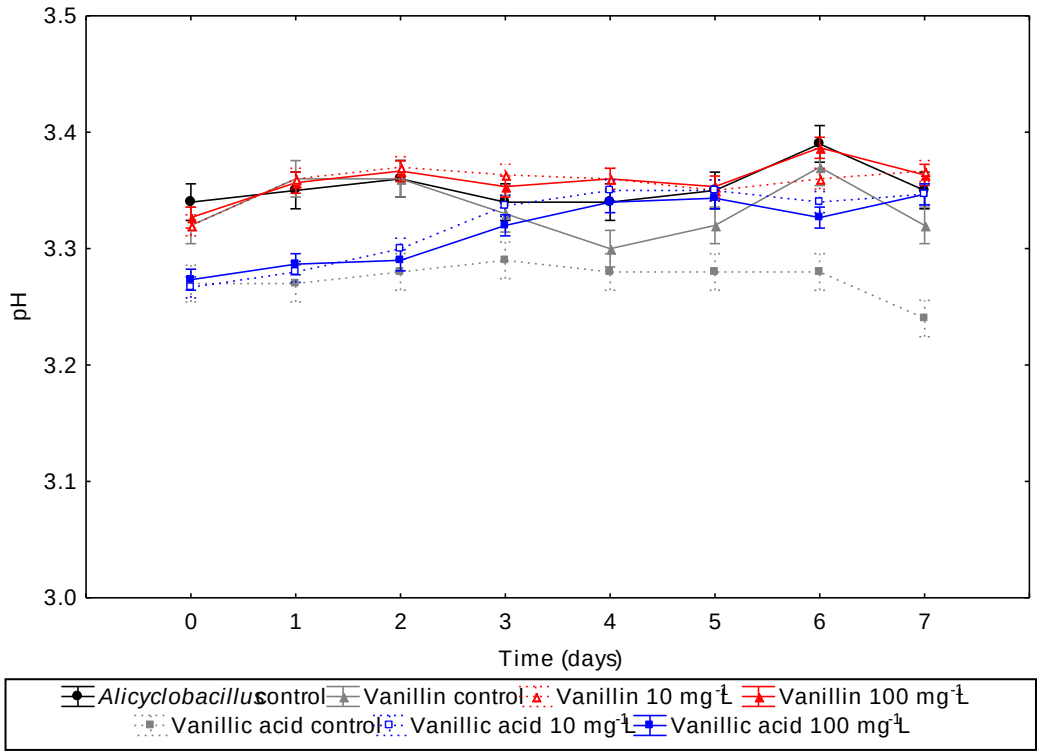

Figure 3 Changes in pH in single strength apple juice supplemented with vanillin and vanillic acid (10 mg L^{-1} and 100 mg L^{-1}) in the presence of *A. acitoterrestris* incubated at 45 °C for 7 d. Vertical bars denote 0.95 confidence intervals.

supplemented with vanillin or vanillic acid and inoculated with *Alicyclobacillus*, the change in pH was less than 0.1. Therefore, the change in pH of fruit juice would not be a clear indication of guaiacol production and spoilage caused by *Alicyclobacillus*.

Guaiacol production measured with PECA

Using the PECA method, no colour change was observed in the *Alicyclobacillus* control or the supplement control samples during the 7 d incubation period. Guaiacol production in the 10 mg L^{-1} vanillin and 10 mg L^{-1} vanillic acid samples were also not detected during the 7 d (Fig. 4) using the PECA method. This may indicate that low concentrations of guaiacol in apple juice could not be detected with the PECA method.

Guaiacol concentration in the sample supplemented with 100 mg L^{-1} vanillic acid increased significantly ($p < 0.05$) in the first 2 d to a maximum concentration of 0.783 mM (97.20 mg L^{-1}) (Fig. 4). The guaiacol concentration measured from day 1 to 7 was significantly ($p < 0.05$) higher than the guaiacol concentration measured in the *Alicyclobacillus* control and supplement controls. In the sample supplemented with 100 mg L^{-1} vanillin the guaiacol concentration increased steadily during the first 24 h and then increase significantly ($p < 0.05$) from day 1 to 6 (Fig 4). The maximum guaiacol concentration was 0.687mM (85.28 mg L^{-1}) on day 7 (Fig. 4), indicating that the PECA was able to detect guaiacol when a higher concentration of precursors was present in the apple juice.

HPLC analysis

The HPLC method was used to quantify the concentration of the precursors, vanillin and vanillic acid, as well as the produced guaiacol in the samples. The standard solutions analysed with the HPLC was used to obtain standard curves for the quantification of vanillin, vanillic acid and guaiacol. The standard curves used for the quantification are given in the appendix (Figs. 2A - 4A, Appendix). Guaiacol was not detected in the *Alicyclobacillus* or the supplement control samples (Fig. 5). *Alicyclobacillus acidoterrestris* was, therefore, responsible for the metabolism of vanillin or vanillic acid and the subsequent production of guaiacol.

Guaiacol was produced in the 10 mg L^{-1} vanillic acid sample, however, the guaiacol concentration did not increase significantly ($p \geq 0.05$) during the 7 d (Fig. 5). A maximum guaiacol concentration of 0.052 mM (6.46 mg L^{-1}) was detected. The production of these low levels of guaiacol could not be detected using the PECA

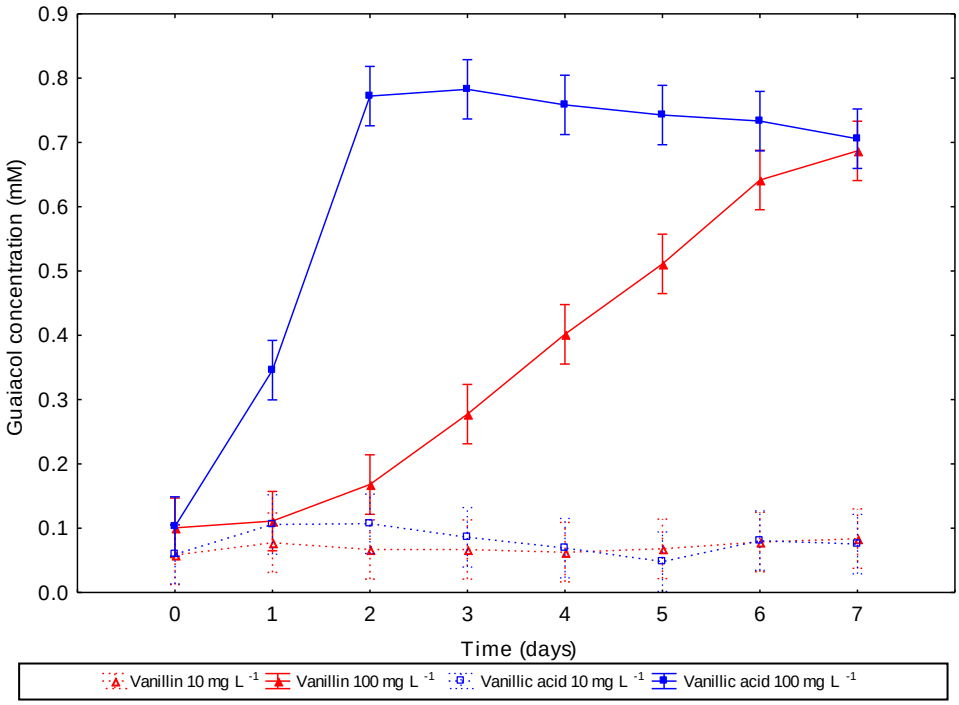

Figure 4 Guaiacol production in the single strength apple juice supplemented with vanillin and vanillic acid (10 mg L^{-1} and 100 mg L^{-1}) in the presence of *A. acidoterrestris* during the 7 d incubation measured with PECA. Vertical bars denote 0.95 confidence intervals.

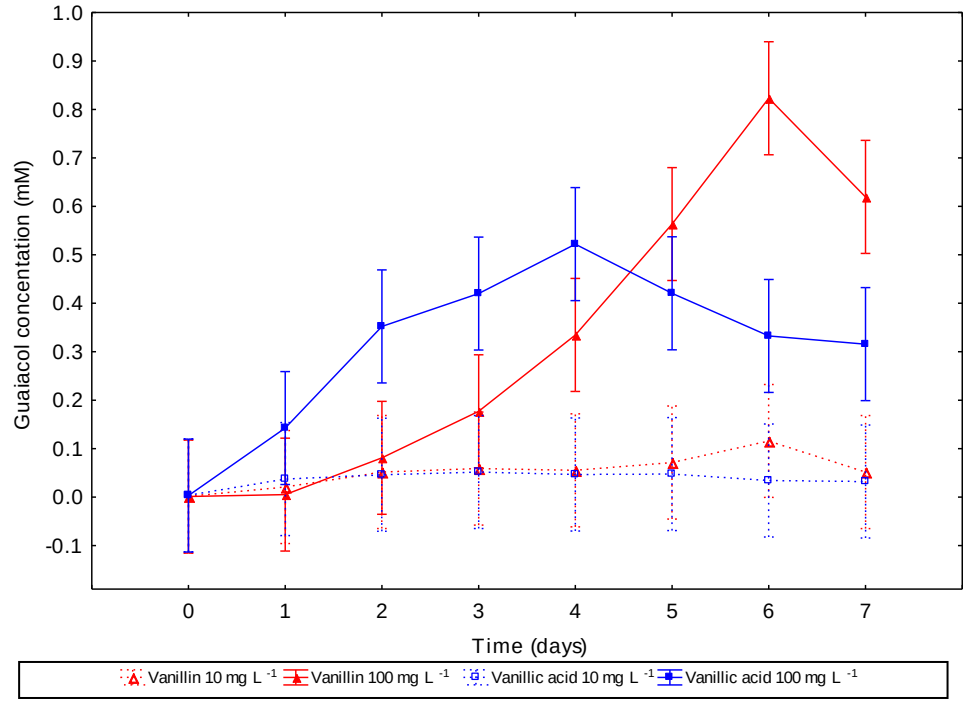

Figure 5 Guaiacol production in the single strength apple juice supplemented with vanillin and vanillic acid (10 mg L^{-1} and 100 mg L^{-1}) in the presence of *A. acidoterrestris* during the 7 d incubation measured with high performance liquid chromatography (HPLC). Vertical bars denote 0.95 confidence intervals.

method. The vanillic acid present in the 10 mg L^{-1} vanillic acid sample was depleted within the first 24 h after inoculation (Fig. 6). The guaiacol concentration in the 100 mg L^{-1} vanillic acid sample increased significantly ($p < 0.05$) in the first 48 h, thereafter the guaiacol concentration increase steadily until day 4 with a maximum guaiacol concentration of 0.522 mM (64.80 mg L^{-1}) (Fig. 5) (PECA, 0.783 mM). The vanillic acid concentration in this sample decreased significantly ($p < 0.05$) until day 2 (Fig. 6) and was not detected from day 2 to 7.

Although the guaiacol concentration in the 10 mg L^{-1} vanillin sample did not increase significantly ($p \geq 0.05$) during the 7 d, low concentrations of guaiacol was produced (Fig. 5). A maximum guaiacol concentration of 0.116 mM (14.40 mg L^{-1}) was detected in the sample on day 6 (Fig. 5). The vanillin concentration in the 10 mg L^{-1} vanillin sample decrease the first 48 h after inoculation and was not detected from day 2 to 7 (Fig. 7). In the 100 mg L^{-1} vanillin sample the guaiacol concentration increased steadily for the first 3 d and a significant ($p < 0.05$) increase was observed from day 3 to 6 (Fig. 5). The maximum guaiacol concentration in this sample was detected as 0.823 mM (102.17 mg L^{-1}) on day 6 (Fig. 5) (PECA, 0.687 mM). The vanillin concentration in this sample decreased during the 7 d and was completely depleted on day 7 (Fig. 7).

Guaiacol concentrations well above the BET values for taste (0.24 – 2.00 µg L^{-1}) and odour (0.50 - 2.32 µg L^{-1}) in apple juice was produced in the 10 mg L^{-1} vanillin and 10 mg L^{-1} vanillic acid samples, indicating that fruit juice with a vanillin or vanillic acid concentration of as low as 10 mg L^{-1} has the potential to spoil if the juice is contaminated with *A. acidoterrestris.* The guaiacol production by *A. acidoterrestris* increased with an increase in vanillin and vanillic acid concentration. This shows that the spoilage potential of a fruit juice will increase with an increase in precursors available for guaiacol production for metabolism by *A. acidoterrestris*.

Although the samples were incubated in sealed containers, a decrease in guaiacol concentration was observed in the samples over time (Fig. 5). Previous research reported the possibility that guaiacol can be reduced to catechol (Pometto *et al*., 1981; Rahouti *et al*., 1989; Álvarez-Rodríguez *et al*., 2003; Topakas *et al*., 2003). However, the reason for a decrease in the guaiacol concentration in apple juice in this study remains unclear.

Comparison of the PECA with HPLC

An interclass correlation was used to indicate the correlation between the data obtained

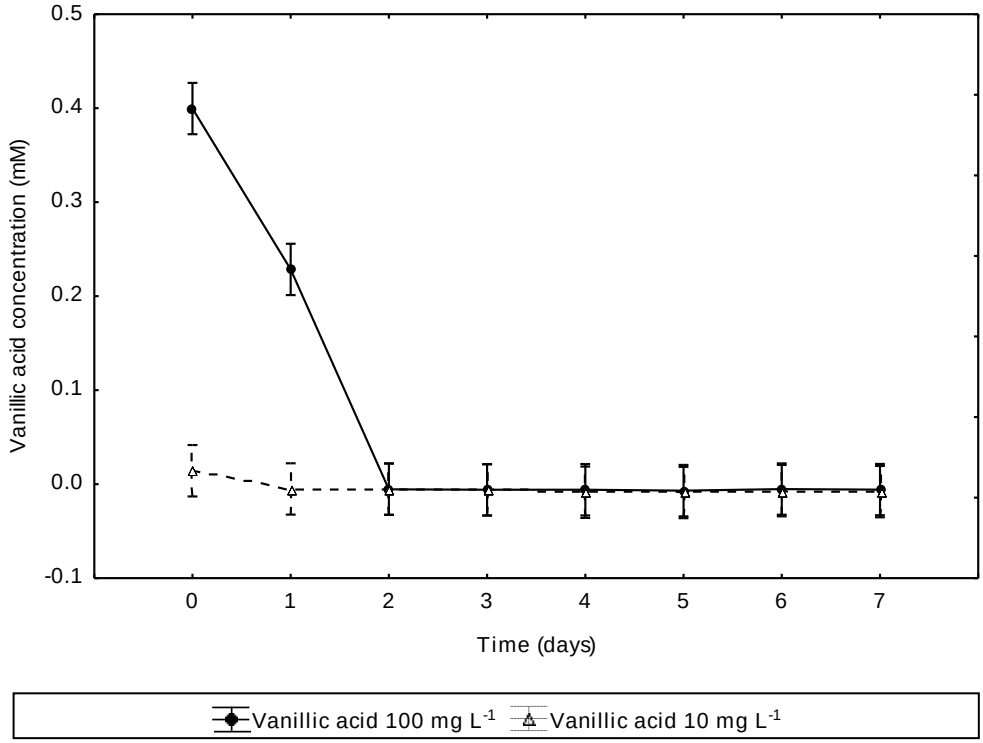

Figure 6 The decrease in vanillic acid concentrations (10 mg L^{-1} and 100 mg L^{-1}) in single strength apple juice in the presence of *A. acidoterrestris* during the 7 d incubation period measured with HPLC. Vertical bars denote 0.95 confidence intervals.

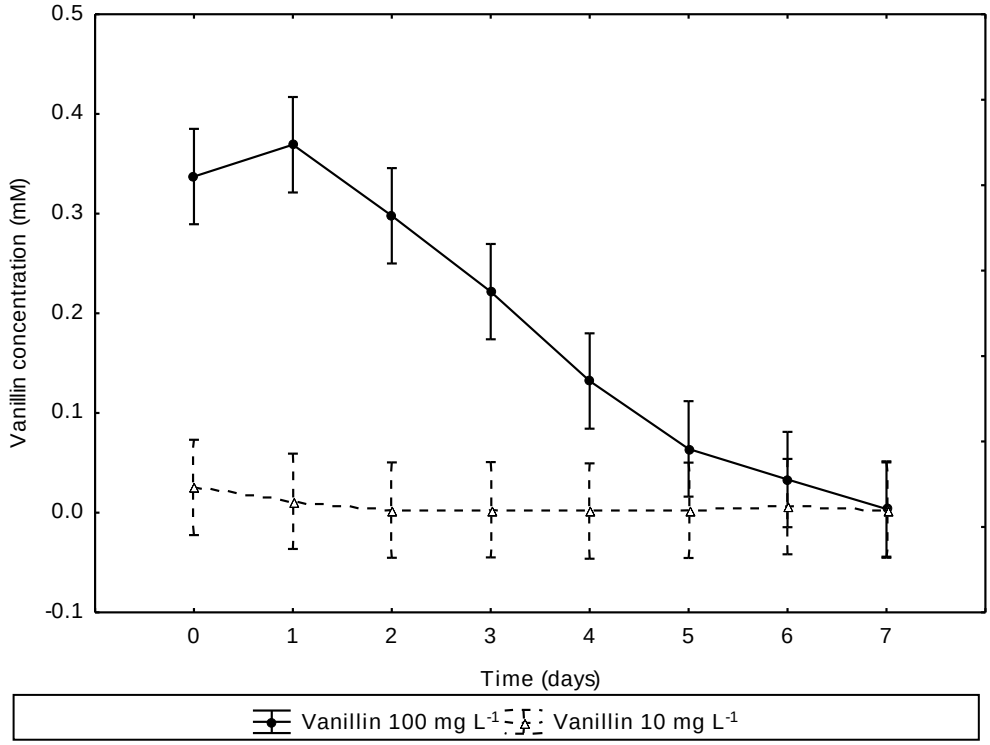

Figure 7 The decrease in vanillin concentrations (10 mg L^{-1} and 100 mg L^{-1}) in single strength apple juice in the presence of *A. acidoterrestris* during the 7 d incubation period measured with HPLC. Vertical bars denote 0.95 confidence intervals.

with the two methods, the PECA and HPLC, used in this study to quantify guaiacol production. High agreement and consistency values of 0.876 and 0.918, respectively were observed between the two methods (Fig. 8), indicating that the trends of the data obtained by the two methods were similar. Most of the samples were, however, below the x = y line (Fig. 8), indicating that higher guaiacol concentrations were measured with the PECA compared to HPLC. This may possibly be due to the colour of the apple juice that influences the PECA measurements, resulting in a higher guaiacol concentration.

CONCLUSION

The precursors, vanillin and vanillic acid and the metabolic activity of *A. acidoterrestris* FB2 are prerequisites for the spoilage of apple juice by guaiacol. Fruit juice with a concentration as low as 10 mg L^{-1} vanillin or vanillic acid have the potential to result in spoilage and an increase in precursors in the fruit juice, increases the spoilage potential of the fruit juice by *A. acidoterrestris*. Therefore, the concentrations of these compounds in commercial fruit juice are important in indicating the spoilage potential. In the development of juice products and different blends of fruit juices, special care must be taken not to concentrate the amount of vanillin and vanillic acid present in the fruit juices

Changes in pH and the guaiacol measured using the PECA method was not adequate to indicate the spoilage of apple juice. Previous studies recommended PECA as a screening tool to indicate the presence of guaiacol, however, the PECA is not sensitive enough for the detection of guaiacol at low concentrations in apple juice samples. The guaiacol production measured with the two methods is very different. The colour of the apple juice may have influenced the guaiacol measurements by the PECA method and the natural compounds in the apple juice may have influence the identification and quantification of the compounds with the HPLC method. Improved sample preparation and separation with HPLC may improve the quantification of the compounds present in fruit juice samples.

REFERENCES

Álvarez-Rodríquez, M.L., Belloch, C., Villa, M., Uruburu, F., Larriba, G. & Coque, JJ. R. (2003). Degradation of vanillic acid and production of guaiacol by microorganisms isolated from cork samples. FEMS Microbiology Letters, **220**, 49-55.

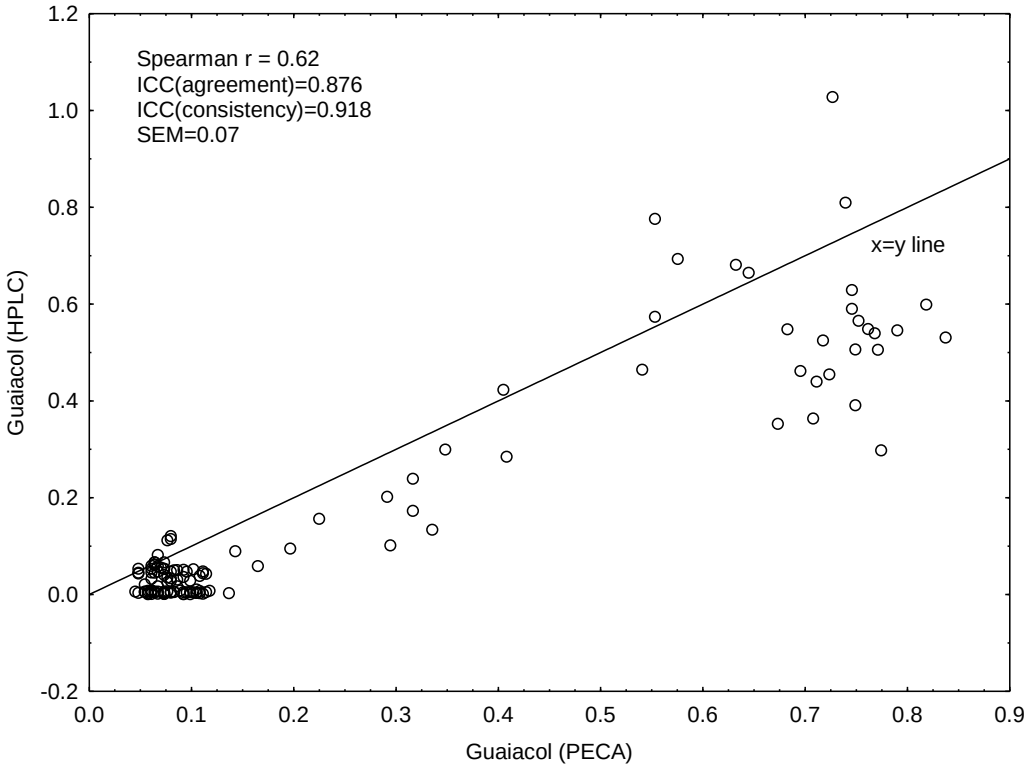

Figure 8 Interclass correlation of the PECA and HPLC methods used to measure the guaiacol concentration.

Bahçeci, K.S., Gökmen, V. & Acar, J. (2005). Formation of guaiacol from vanillin by *Alicyclobacillus acidoterrestris* in apple juice: a model study. *European Food Research and Technology*, **220**, 196-199.

Chang, S.S. & Kang, D.H. (2004). *Alicyclobacillus* spp. in the fruit juice industry: history, characteristics, and current isolation/detection procedures. *Critical Reviews in Microbiology*, **30**, 55-74.

Durak, M.Z., Churey, J.J, Danyluk, M.D. & Worobo, R.W. (2010). Identification and haplotype distribution of *Alicyclobacillus* spp. from different juices and beverages. *International Journal of Food Microbiology,* **142**, 286-291.

Eisele, T.A. & Semon, M.J. (2005). Best estimated aroma and taste detection threshold for guaiacol in water and apple juice. *Journal of Food Science*, **70**, 267-269.

Goodner, K.L., Jella, P. & Rouseff, R. (2000). Determination of vanillin in orange, grapefruit, tangerine, lemon, and lime juices using GC-Olifactometry and GC-MS/MS. *Journal of Agricalture and Food Chemistry*, **48**, 2882-2886.

International Federation of Fruit Juice Producers (IFU). (2007). Method on the detection of taint producing *Alicyclobacillus* in fruit juices. *IFU Method No. 12*. Pp. 1-11. IFU, Paris.

Jensen, N. (2000). *Alicyclobacillus* in Australia. *Food Australia*, **52**, 282-285.

Jensen, N. & Whitfield, F.B. (2003). Role of *Alicyclobacillus acidoterrestris* in the development of a disinfectant taint in shelf-stable fruit juice. *Letters in Applied Microbiology*, **36**, 9-14.

Jensen, N., Varelis, P. & Whitfield, F.B. (2001). Formation of guaiacol in chocolate milk by the psychrotrophic bacterium *Rahnella aquatilis*. *Letters in Applied Microbiology*, **33**, 339-343.

Komitopoulou, E., Boziaris, I.S., Davies, E.A., Delves-Broughton, J. & Adams, M.R. (1999). *Alicyclobacillus acidoterrestris* in fruit juices and its control by nisin. *International Journal of Food Science and Technology*, **34**, 81-85.

Niwa, M. & Kawamoto, A. (2003). Development of a rapid detection method of *A. acidoterrestris*, hazardous bacteria to acidic beverage. *Fruit Processing*, **13**, 102-107.

Orr, R.V., Shewfelt, R.L., Huang, C.J., Tefera, S. & Beuchat, L.R. (2000). Detection of guaiacol produced by *Alicyclobacillus acidoterrestris* in apple juice by sensory and chromatographic analyses, and comparison with spore and vegetative cell populations. *Journal of Food Protection*, **63**, 1517-1522.

Peleg, H., Naim, M., Zehavi, U., Rouseff, R.L. & Nagy, S. (1992). Pathways of 4-vinylguaiacol formation from ferulic acid in model solutions of orange juice. *Journal of Agricultural and Food Chemistry,* **40**, 764-767.

Pettipher, G.L. & Osmundson, M.E. (2000). Methods for the detection, enumeration and identification of *Alicyclobacillus acidoterrestris*. *Food Australia*, **52**, 293-295.

Pettipher, G.L., Osmundson, M.E. & Murphy, J.M. (1997). Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit juice-containing drinks. *Letters in Applied Microbiology*, **24**, 185-189.

Piacquadio, P., De Stefano, G. & Sciancalepore, V. (2002). Phenolic composition of apple juice treated by laccase immobilized on copper-chelate regenerable carrier. *Italian Journal of Food Science*, **14**, 291-294.

Pometto, A.L., Sutherland, J.B. & Crawford, D.L. (1981). *Streptomyces setonii*: catabolism of vanillic acid via guaiacol and catechol. *Canadian Journal of Microbiology,* **27**, 636-638.

Rahouti, M., Seigle-Murandi, F., Steiman, R. & Eriksson, K-E. (1989). Metabolism of ferulic acid by *Paecilomyces variotii* and *Pestalotia palmarum*. *Applied and Environmental Microbiology*, **55**, 2391-2398.

Sheu, S.C. & Chen, A.O. (1991). Lipoxygenase as blanching index for frozen vegetable soybeans. *Journal of Food Science*, **56**, 448-451.

Siegmund, B. & Pöllinger-Zierler, B. (2006). Odor thresholds of microbially induced off-flavor compounds in apple juice. *Journal of Agricultural and Food Chemistry*, **54**, 5984-5989.

Silva, F.M.S., Gibbs, P., Vieira, M.C. & Silva, C.L.M. (1999). Thermal inactivation of *Alicyclobacillus acidoterrestris* spores under different temperature, soluble solids and pH conditions for the design of fruit processes. *International Journal of Food Microbiology,* **51**, 95-103.

Splittstoesser, D.F., Churey, J.J. & Lee, C.Y. (1994). Growth characteristics of aciduric sporeforming bacilli isolated from fruit juices. *Journal of Food Protection*, **57**, 1080-1083.

Splittstoesser, D.F., Lee, C.Y. & Churey, J.J. (1998). Control of *Alicyclobacillus* in the juice industry. *Dairy, Food and Environmental Sanitation*, **18**, 585-587.

Topakas, E., Kalogeris, E., Kekos, D., Macris, B.J. & Christakopoulos, P. (2003). Bioconversion of ferulic acid into vanillic acid by the thermophilic fungus

Sporotrichum thermophile. *Lebensmittel-Wissenschaft und -Technologie*, **36**, 561-565.

Walker, M. & Phillips, C.A. (2005). The effect of intermittent shaking, headspace and temperature on the growth of *Alicyclobacillus acidoterrestris* in stored apple juice. *International Journal of Food Science and Technology*, **40**, 557-562.

Walls, I. & Chuyate, R. (1998). *Alicyclobacillus* - historical perspective and preliminary characterization study. *Dairy, Food and Environmental Sanitation*, **18**, 499-503.

APPENDIX

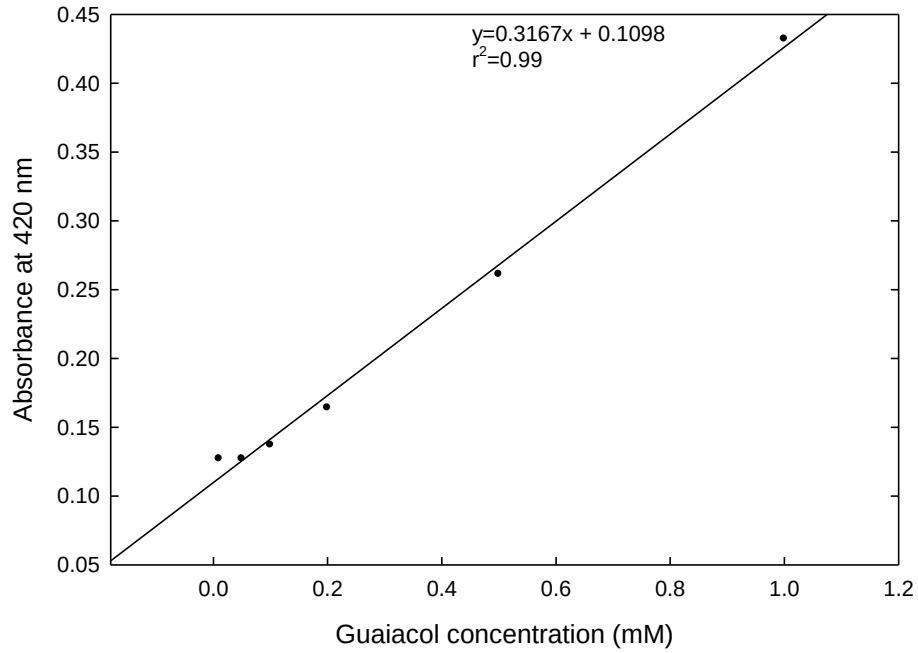

Figure 1A The standard curve for determination of guaiacol concentration in apple juice with the PECA. The equation of the regression line as well as the r^2 value is indicated on the graph.

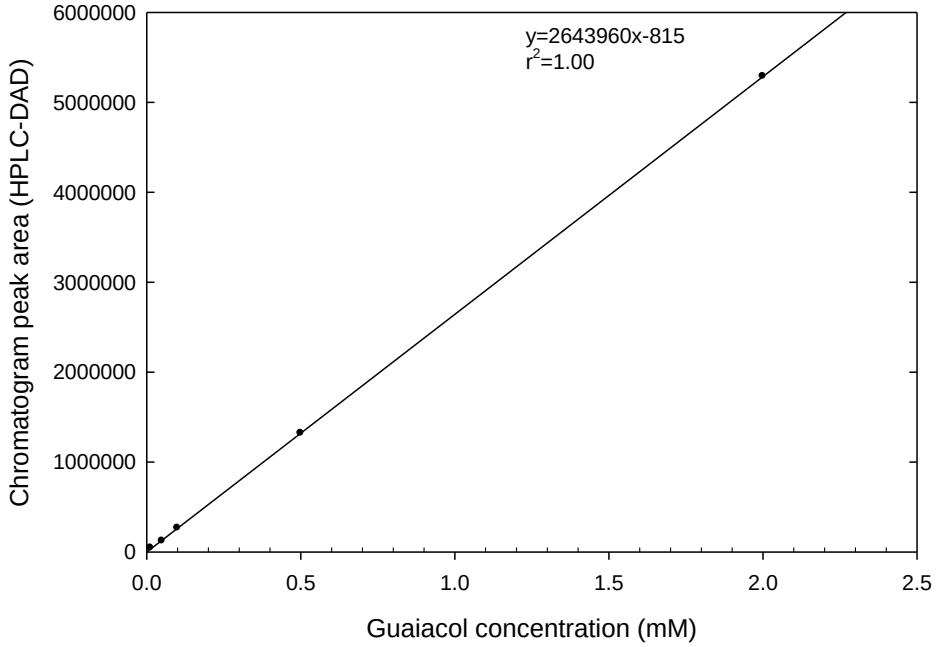

Figure 2A The standard curve for determination of guaiacol concentration by HPLC-DAD. The equation of the regression line as well as the r^2 value is indicated on the graph.

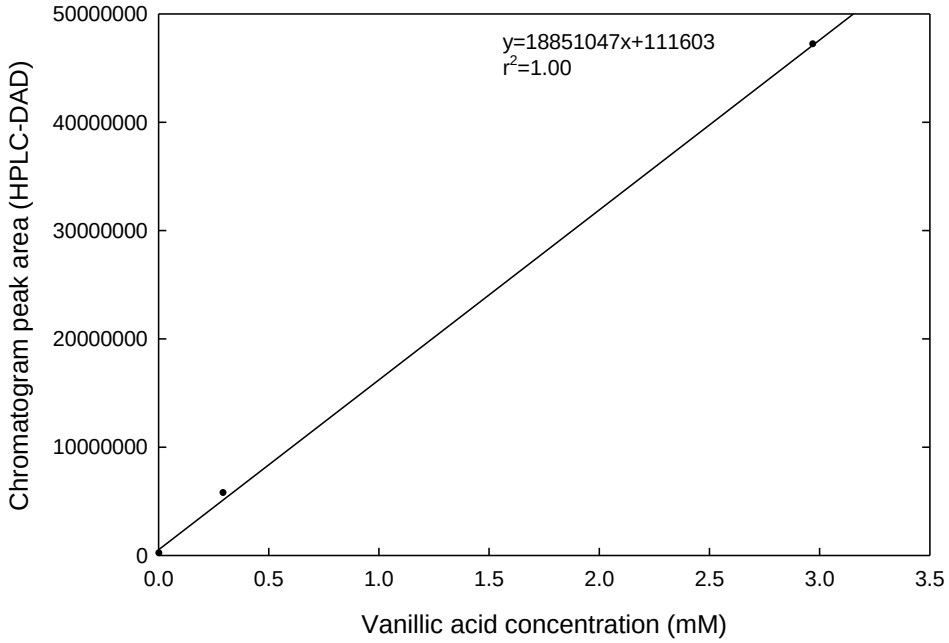

Figure 3A The standard curve for determination of vanillic acid concentration by HPLC-DAD. The equation of the regression line as well as the r^2 value is indicated on the graph.

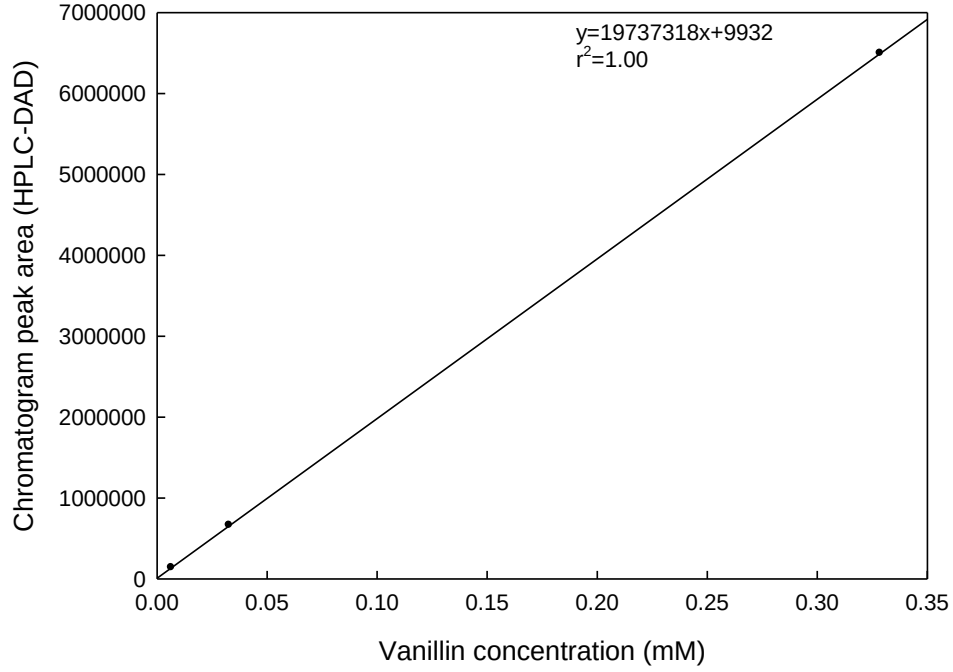

Figure 4A The standard curve for determination of vanillin concentration by HPLC-DAD. The equation of the regression line as well as the r^2 value is indicated on the graph.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The spoilage of fruit juice and fruit juice products by *Alicyclobacillus* is of major concern to the beverage industry due to the production of the taint chemical guaiacol. *Alicyclobacillus* spp. have been acknowledged by the fruit juice industry as the target microbe in the design of pasteurisation processes for acidic products (Silva *et al*., 2000; Vieira *et al*., 2002; Silva & Gibbs, 2004). Although previous research has focused on the development of methods to control *Alicyclobacillus*, it remains challenging to eliminate these bacteria from the fruit processing environment. Numerous factors influence the spoilage of fruit juice with *Alicyclobacillus* spp. and the production of guaiacol (Splittstoesser *et al*., 1994; Chang & Kang, 2004), but little information exists on the metabolic pathway and the substrates that are required for guaiacol production (Chang & Kang, 2004).

A high performance liquid chromatography method with UV-diode array detection (HPLC-DAD) was developed for the simultaneous quantification of guaiacol and its precursors. This method was used to separate and quantify the supplemented precursors in *Bacillus acidoterrestris* (BAT) broth and apple juice, as well as the produced guaiacol. The guaiacol concentrations measured with the HPLC-DAD method was compared to the guaiacol concentrations measured with the peroxidase enzyme colourimetric assay (PECA). *Alicyclobacillus acidoterrestris* FB2 was incubated at 45 °C for 7 d in BAT broth supplemented with ferulic acid, vanillin or vanillic acid, respectively to evaluate the possible precursors of guaiacol production and to establish the pathway of guaiacol production by *A. acidoterrestris*. The spoilage potential of apple juice was evaluated by supplementing apple juice with either 10 mg L^{-1} or 100 mg L^{-1} vanillin or vanillic acid, inoculated with *A. acidoterrestris* FB2 and incubated at 45 °C for 7 d.

Vanillin and vanillic acid was confirmed as precursors of guaiacol production by *A. acidoterrestris*. A pathway of guaiacol production by *A. acidoterrestris* from vanillin and vanillic acid was established. The metabolic conversion of vanillic acid to guaiacol by *A. acidoterrestris* was found to be faster than the conversion of vanillin to guaiacol, indicating that spoilage from vanillic acid can occur in a shorter time period than spoilage from vanillin. Guaiacol was not produced by *A. acidoterrestris* in the samples supplemented with ferulic acid. Therefore it was concluded that under the conditions

used in this study, *A. acidoterrestris* cannot produce guaiacol directly from ferulic acid. Ferulic acid must first be converted to vanillin or vanillic acid before guaiacol can be produced by *A. acidoterrestris.* Therefore, fruit juice with vanillin and vanillic acid pose a risk for spoilage by *A. acidoterrestris.* Furthermore, it was found that *A. acidoterrestris* could not produce guaiacol in the absence of the precursors nor could guaiacol be formed without the metabolic action of *A. acidoterrestris* in medium and in apple juice. As the production of guaiacol increased with an increase in vanillin or vanillic acid concentration, it is evident that the concentration of precursors present in fruit juice will influence the spoilage potential of the juice. Guaiacol concentrations well above the best estimated threshold value for taste (0.24 – 2.00 µg L^{-1}) and odour (0.50 - 2.32 µg L^{-1}) (Pettipher *et al.,* 1997; Orr *et al.,* 2000; Eisele & Semon, 2005; Siegmund & Pöllinger-Zierler, 2006) was produced by *A. acidoterrestris* in apple juice supplemented with 10 mg L^{-1} vanillin or 10 mg L^{-1} vanillic acid. Thus, fruit juice with a low concentration of vanillin or vanillic acid has the potential to spoil if contaminated with *A. acidoterrestris*.

The pH of single strength apple juice supplemented with vanillin and vanillic acid (10 mg L^{-1} and 100 mg L^{-1}) in the presence of *A. acidoterrestris* was evaluated to determine if guaiacol spoilage have an effect on the pH of the fruit juice. An increase of 0.1 in pH was observed in the samples supplemented with the precursors and inoculated with *Alicyclobacillus*. Therefore, the change in pH of fruit juice is not a clear indication of guaiacol production and spoilage caused by *Alicyclobacillus*. The peroxidase enzyme colourimetric assay (PECA) generally used as an absent/present test of guaiacol (Chang & Kang, 2005) was used as a rapid method to detect and quantify guaiacol using a standard curve of known guaiacol concentrations and absorbance at 420 nm. Although the PECA method is an inexpensive, rapid method that is easy to use and does not require expensive and complicated equipment or chemicals, this method is not recommended for the detection and quantification of guaiacol. The high concentrations (1 000 mg L^{-1}) of vanillin and vanillic acid caused a pale yellow discolouration with the PECA, resulting in higher guaiacol measurements. The PECA could also not differentiate between the apple juice samples with low concentrations of guaiacol and the control samples without guaiacol. The significant difference observed between the sensitivity of the PECA method and HPLC-DAD method with regards to guaiacol detection shows that HPLC-DAD should be used for quantification of guaiacol.

Concluding remarks

A metabolic pathway of guaiacol production by *A. acidoterrestris* from vanillin and vanillic acid was established. This study shows that the precursors of guaiacol, vanillin and vanillic acid are prerequisites for the production of guaiacol by *Alicyclobacillus* spp. Furthermore, the concentrations of these precursors present in fruit juice will give an indication of the spoilage potential of a specific fruit juice. It is recommended that the concentration of vanillin and vanillic acid in commercial fruit juices are determined to provide an indication of its spoilage potential. The knowledge of the spoilage potential could assist in the effective use or processing of these products to prevent contamination and guaiacol spoilage. Fruit juice products with high guaiacol spoilage potential could be used in confectionary or baking, as fresh juice with refrigeration requirements or a reduced shelf-life to prevent the production of guaiacol. Care must be taken in the development of juice products not to concentrate or increase the amount of vanillin and vanillic acid present. The removal or minimisation of precursors in fruit juices can prevent or reduce the spoilage of fruit juices.

References

Chang, S.S. & Kang, D.H. (2004). *Alicyclobacillus* spp. in the fruit juice industry: history, characteristics, and current isolation/detection procedures. *Critical Reviews in Microbiology*, **30**, 55-74.

Eisele, T.A. & Semon, M.J. (2005). Best estimated aroma and taste detection threshold for guaiacol in water and apple juice. *Journal of Food Science*, **70**, 267-269.

Orr, R.V., Shewfelt, R.L., Huang, C.J., Tefera, S. & Beuchat, L.R. (2000). Detection of guaiacol produced by *Alicyclobacillus acidoterrestris* in apple juice by sensory and chromatographic analyses, and comparison with spore and vegetative cell populations. *Journal of Food Protection*, **63**, 1517-1522.

Pettipher, G.L., Osmundson, M.E. & Murphy, J.M. (1997). Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit juice-containing drinks. *Letters in Applied Microbiology*, **24**, 185-189.

Siegmund, B. & Pöllinger-Zierler, B. (2006). Odor thresholds of microbially induced off-flavor compounds in apple juice. *Journal of Agricultural and Food Chemistry*, **54**, 5984-5989.

Silva, F.V.M. & Gibbs, P. (2004). Target selection in designing pasteurization processes for shelf-stable high-acid fruit products. *Critical Reviews in Food Science and Nutrition*, **44**, 353-360.

Silva, F.V.M., Gibbs, P. & Silva, C.L.M. (2000). Establishing a new pasteurisation criterion based on *Alicyclobacillus acidoterrestris* spores for shelf-stable high-acidic fruit products. *Fruit Processing*, **4**, 138-141.

Splittstoesser, D.F., Churey, J.J. & Lee, C.Y. (1994). Growth characteristics of aciduric sporeforming bacilli isolated from fruit juices. *Journal of Food Protection*, **57**, 1080-1083.

Vieira, M.C., Teixeira, A.A., Silva, F.M., Gaspar, N. & Silva, C.L.M. (2002). *Alicyclobacillus acidoterrestris* spores as a target for Cupuaçu (*Theobroma grandiflorum*) nectar thermal processing: kinetic parameters and experimental methods. *International Journal of Food Microbiology*, **77**, 71-81.