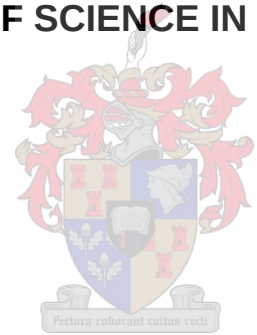
DETECTION AND ISOLATION OF THERMOPHILIC ACIDOPHILIC BACTERIA FROM FRUIT JUICES

WINEEN DUVENAGE

Thesis approved in fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN FOOD SCIENCE



Department of Food Science
Faculty of AgriSciences
Stellenbosch University

Study Leader: Dr. R.C. Witthuhn
Co-study Leader: Prof. P.A. Gouws

April, 2006

DECLARATION

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

_____ _____

Wineen Duvenage Date

ABSTRACT

Fruit juices were until recently considered to only be susceptible to spoilage by yeasts, mycelial fungi and lactic acid bacteria. Spoilage by these organisms was prevented by the acidic pH of fruit juices and the heat-treatment applied during the hot-fill-hold process. Despite these control measures, an increasing number of spoilage cases of fruit juices, fruit juice products and acidic vegetables due to contamination by thermophilic acidophilic bacteria (TAB) have been reported. The genus *Alicyclobacillus*, containing TAB were first classified as *Bacillus*, but were reclassified in 1992. Species of *Alicyclobacillus* are Gram-positive, rod-shaped, endospore-forming bacteria. The unique characteristic of these organisms is the presence of ω-alicyclic fatty acids, such as ω-cyclohexane and ω-cycloheptane, as the major components of the cellular membrane. This organism has been shown to survive pasteurisation conditions of 95˚C for 2 min and grows within a pH range of 2.5 to 6.0 and temperatures between 25˚ and 60˚C. The genus currently consists of 11 species, with *A. acidoterrestris*, *A. acidocaldarius* and *A. pomorum* being the only species associated with the spoilage of fruit juices and fruit juice products.

The aim of this study was to evaluate culture-dependent and culture-independent approaches for the detection and isolation of *Alicyclobacillus* spp. from pasteurised South African fruit juices and concentrates. The culture-dependent approach was evaluated by comparing five different growth media, for growth and recovery of *A. acidoterrestris*, *A. acidocaldarius* and *A. pomorum* at different incubation temperatures, from sterile saline solution (SSS) (0.85% (m/v) NaCl), diluted and undiluted fruit juice concentrates. The five media evaluated included potato dextrose agar (PDA), orange serum agar (OSA), K-agar, yeast extract (YSG)-agar and *Bacillus acidocaldarius* medium (BAM). The culture-independent approach was used to identify the micro-organisms present in fruit juices and concentrates from different South African manufacturers before and after pasteurisation, using polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) and DNA sequencing.

Spread plates of PDA at pH 3.7 and incubation temperature of 50˚C for 3 days was found to be the best isolation media for species of *Alicyclobacillus* from fruit juice and fruit juice concentrate. With the inclusion of a heat shock treatment at 80˚C for 10 min the growth media of preference for spores of *Alicyclobacillus* from fruit juice concentrates was OSA at pH 5.5 and an incubation temperature of 50˚C for 3 days.

The culture-dependent approach could detect cells or endospores at a minimum concentration of 10^4 cfu.ml^{-1} in SSS and diluted fruit juices.

PCR-based DGGE analysis was more sensitive and detected cells of *Alicyclobacillus* spp. from fruit juices and concentrates at a minimum concentration of 10^3 cfu.ml^{-1}. *Alicyclobacillus acidoterrestris* was found to be present in South African apple juice, pear juice, white grape juice and aloe vera juice. White grape juice was also found to contain *A. pomorum*. Other organisms present in the orange, apple, mango and pear juices were two uncultured bacteria that were identified as members of the genus *Bacillus*, and one uncultured bacterium closely related to *Alcaligenus faecalis*. This study confirmed the presence of TAB in pasteurised South African fruit juices and concentrates and emphasises the need for the rapid and accurate detection of TAB in food products.

UITTREKSEL

In die verlede is aanvaar dat vrugtesap slegs bederf word deur giste, skimmels en melksuurbakterieë. Bederf deur hierdie organismes is uitgeskakel deur die natuurlike lae pH van vrugtesap tesame met die hitte-behandeling wat toegepas word tydens die warm-vul proses. Ten spyte van hierdie beheermaatreëls wat tydens vervaardiging van die vrugtesap toegepas word, is 'n toenemende hoeveelheid gevalle aangemeld waar vrugtesap, vrugtesap produkte en groentes bederf is as gevolg van kontaminasie deur termofiliese asidofiliese bakterieë (TAB). Die genus *Alicyclobacillus*, waarin lede van TAB voorkom, is eers geklassifieer in die genus *Bacillus*, maar in 1992 is dit hergeklassifiseer. Spesies van *Alicyclobacillus* is Gram-positiewe, staaf-vormige, endospoor vormende bakterieë, wat ook nie-patogenies is. Die unieke eienskap van hierdie organisme is die teenwoordigheid van ω-alisikliese vetsure, soos ω-sikloheksaan en ω-sikloheptaan, as die hoof komponente van die selmembraan. Hierdie organisme kan pasteurisasie temperature van 95˚C vir 2 min oorleef en groei binne 'n pH reeks van 2.5 tot 6.0 en by temperature tussen 25˚ en 60˚C. Die genus bestaan uit 11 spesies, met *A. acidoterrestris*, *A. acidocaldarius* en *A. pomorum* die enigste spesies wat tans met bederf van vrugtesappe en vrugtesap produkte geassosieër word.

Die doel van hierdie studie was om kultuur-afhanklike en kultuur-onafhanklike benaderings vir die deteksie en isolasie van spesies van *Alicyclobacillus* vanuit gepasteuriseerde Suid-Afrikaanse vrugtesap en konsentrate te evalueer. Die kultuur-afhanklike benadering is geëvalueer deur die deteksie en isolasie van *A. acidoterrestris*, *A. acidocaldarius* en *A. pomorum* vanuit fisiologiese sout oplossing (FSO) (0.85% (m/v) NaCl) asook verdunde en onverdunde vrugtesap konsentraat, by verskillende inkubasie temperature te vergelyk. Die media wat het ingesluit aartappel dekstrose agar (PDA), lemoen serum agar (OSA), K-agar, gis ekstrak (YSG)-agar and *Bacillus acidocaldarius* medium (BAM). Die kultuur-onafhanklike benadering is geëvalueer deur die mikro-organismes teenwoordig in die vrugtesap en konsentraat van verskillende Suid-Afrikaanse vervaardigers voor en na pasteurisasie te identifiseer, deur gebruik te maak van polimerase kettingreaksie (PKR)-gebaseerde denaturerende gradiënt jelelektroforese (DGGE) en DNA volgorde bepaling.

Spreiplate van PDA met 'n pH van 3.7 en geïnkubeer by 50˚C vir 3 dae is die mees geskikte media vir die deteksie van *Alicyclobacillus* spesies vanuit vrugtesap en vrugtesap konsentraat. OSA is die beste medium vir die deteksie van endospore

van *A. acidoterrestris* en die insluiting van ‘n hitte-behandeling teen 80˚C vir 10 min het die deteksie meer selektief gemaak. Die kultuur-afhanklike metode kon selle of endospore van *Alicyclobacillus* spp. bo ‘n konsentrasie van 10^4 kolonie vormende eenhede per ml (kve.ml^{-1}) vanuit FSO en vrugtesap waarneem.

Die PKR-gebaseerde DGGE analise was meer sensitief en het selle van spesies van *Alicyclobacillus* tot so laag as 10^3 kve.ml^{-1} waargeneem. Daar is gevind dat *A. acidoterrestris* teenwoordig was in Suid-Afrikaanse appelsap, peersap, wit druiwesap en aloe vera sap. Wit druiwesap was ook met *A. pomorum* gekontamineer. Ander organismes teenwoordig in die vrugtesap was ongekweekte bakterieë, geïdentifiseer as lede van die genus *Bacillus*, en ongekweekte bakterieë verwant aan *Alcaligenus faecalis*. Hierdie studie het die teenwoordigheid van TAB in gepasteuriseerde Suid-Afrikaanse vrugtesap en konsentrate bevestig en beklemtoon die behoefte vir die vinnige en akkurate opsporing van TAB in voedselprodukte.

dedicated to my parents

with deep gratitude for their love and support

and for making every opportunity possible

ACKNOWLEDGEMENTS

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

Dr. R.C. Witthuhn, Study-leader and Chairman of the Department of Food Science, for investing so much of her time to develop my potential and for her expert guidance, enthusiasm and support during the course of my study, as well as her valuable criticism during the preparation of this thesis;

Prof. P.A. Gouws, Co-study leader and Associate Professor - Food Microbiology at the Department of Biotechnology, University of Western Cape, for his expert guidance, enthusiasm and support during the course of my study and valuable advice in preparation of this thesis;

The National Research Foundation (Scarce Skills Bursary), *Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF*, Stellenbosch University (Merit Bursaries 2004 and 2005), Ernst and Ethel Eriksen Trust (2005) and the South African Association for Food Science and Technology (Brian Koeppen Memorial Scholarship, 2005) for financial support;

Mrs. C. Lamprecht and L. Mouton for technical assistance and Mr. E. Brooks for his help and support;

Maricel Keyser for her skilled practical assistance in the molecular laboratory;

Michelle Cameron for her invaluable advice and help throughout the completion of this study and this thesis;

My fellow post-graduate students and friends for their support, friendship and numerous coffee breaks;

My parents, sister and brother for their unconditional love, continual inspiration and support;

and

My Heavenly Father for giving me the strength and guidance to successfully complete my studies.

CONTENTS

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

CHAPTER 1

INTRODUCTION

The demand for natural and healthy beverages has recently increased due to a greater number of health-conscious consumers. Therefore, the demand for phenol-rich beverages, such as fruit juices, have also increased as phenol-rich beverages exert a strong antioxidant activity and may play a role in maintaining health and preventing cardiovascular diseases (Serafini *et al*., 2000; Ruel *et al*., 2005).

The spoilage of fruit juices has been limited by applying a heat treatment to the fruit juices during manufacturing and by the natural low pH of the juices (Walls & Chuyate, 1998; Jensen, 1999). Spoilage of fruit juices and fruit juice products by thermophilic acidophilic bacteria (TAB) has become a major concern as spoilage cases of acidic vegetables, fruit juices and fruit juice products by *Alicyclobacillus* spp. has been reported (Cerny *et al*., 1984; Splittstoesser *et al*., 1994; Baumgart *et al*., 1997; Pettipher *et al*., 1997; Walls & Chuyate, 1998; Jensen, 1999; Komitopoulou, 1999; Chang & Kang, 2004; Gouws *et al*., 2005; Walker & Philips, 2005). *Alicyclobacillus* is a Gram-positive, endospore forming bacteria and has the ability to survive extreme conditions such as high temperatures and low pH (Splittstoesser *et al*., 1994; Jensen, 1999). The distinctive characteristic of *Alicyclobacillus* spp. is the presence of ω-alicyclic fatty acids as the major components of the cellular membrane (Silva & Gibbs, 2001; Goto *et al*., 2002). *Alicyclobacillus* spp. has been shown to grow at temperatures between 20˚ and 60˚C (Yamazaki *et al*., 1996; Jensen, 1999; Chang & Kang, 2004) and the endospores can survive a pasteurisation temperature of 95˚C for over 2 min in apple juice (Komitopoulou *et al*., 1999). This microbial species can also grow at a pH range of between 2.5 and 6.0 (Deinhard *et al*., 1987; Yamazaki *et al*., 1996; Jensen, 1999; Silva *et al*., 1999; Chang & Kang, 2004). The genus *Alicyclobacillus* currently consists of 11 species, of which *A. acidoterrestris*, *A. acidocaldarius* and *A. pomorum* have been isolated from spoilt fruit juices and are currently the only species associated with fruit juice spoilage (Cerny *et al*., 1984; Splittstoesser *et al*., 1994; Goto *et al*., 2003; Jensen & Whitfield, 2003; Gouws *et al*., 2005).

Species of *Alicyclobacillus* causes a flat-sour type spoilage, mainly attributed to the formation of the offensive-smelling compound guaiacol. Other taint chemicals, such as the halophenols 2,6-dichlorophenol (2,6-DCP) and 2,6-dibromophenol

(2,6-DBP) can also be produced in fruit juice by *A. acidoterrestris* (Yamazaki *et al*., 1996; Komitopoulou *et al*., 1999; Jensen & Whitfield, 2003). Spoilage of apple, pear, orange, peach, mango and white grape juice, as well as fruit juice blends, fruit juice containing drinks and tomato products have been reported, while no growth of *A. acidoterrestris* has been observed in red-grape juice (Splittstoesser *et al*., 1994; Splittstoesser *et al*., 1998; Jensen, 1999).

The need has arisen for an accurate, sensitive and standardised technique for the isolation and detection of *Alicyclobacillus* spp. from fruit juices and fruit juice products (Chang & Kang, 2004). Media plating and membrane filtration are the two main isolation methods currently used (Chang & Kang, 2004). Popular enumeration methods include spread plating on potato dextrose agar (PDA), orange serum agar (OSA), K-agar, yeast-starch-glucose (YSG)-agar and *Bacillus acidocaldarius* medium (BAM) with a pH adjusted to between 3.5 and 5.5, followed by incubation at temperatures ranging between 37˚ and 55˚C for 3 to 7 days (Farrand *et al*., 1983; Deinhard *et al*., 1987; Splittstoesser *et al*., 1994; McIntyre *et al*., 1995; Pettipher *et al*., 1997; Splittstoesser *et al*., 1998; Eiora *et al*., 1999; Komitopoulou *et al*., 1999; Silva *et al*., 1999; Silva *et al*., 2000; Walls & Chuyate, 2000; Goto *et al*., 2002; Chang & Kang, 2004; Gouws *et al*., 2005). For greater sensitivity, either membrane filtration or a heat shock treatment at 80˚C for 10 min is included in the enumeration procedures (Splittstoesser *et al*., 1994; Pettipher, 2000).

The aim of this study was to evaluate culture-dependent and culture-independent approaches for the detection and isolation of *Alicyclobacillus* spp. from pasteurised South African fruit juices. The effectiveness of the heat-treatment applied during manufacturing of the fruit juices was evaluated and the microbial community present in different fruit juices throughout the manufacturing process was determined.

References

Baumgart, J., Husemann, M. & Schmidt, C. (1997). *Alicyclobacillus acidoterrestris*: occurrence, significance and detection in beverages and beverage base. *Flussiges Obst*, **64**, 178.

Cerny, G., Hennlich, W. & Poralla, K. (1984). Fruchtsaftverderb durch *Bacillen*: isolierung und charakterisierung des verderbserrengers. *Zeitschrift feur Lebensmittel-Untersuchung und -Forsuchung*, **179**, 224-227.

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

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

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

Farrand, S.G., Linton, J.D., Stephenson, R.J. & MacCarthy, W.V. (1983). The use of response surface analysis to study growth of *Bacillus acidocaldarius* throughout the growth range of temperature and pH. *Archives of Microbiology*, **135**, 272-275.

Goto, K., Tanimoto, Y., Tamura, T., Mochida, K., Arai, D., Asahara, M., Suzuki, M., Tanaka, H. & Inagaki, K. (2002). Identification of thermo-acidophilic bacteria and a new *Alicyclobacillus* genomic species isolated from *acidic* environments in Japan. *Extremophiles*, **6**, 333-340.

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

Gouws, P.A., Gie, L., Pretorius, A. & Dhansay, N. (2005). Isolation and identification of *Alicyclobacillus acidocaldarius* by 16S rDNA from mango juice and concentrate. *International Journal of Food Science and Technology*, **40**, 789-792.

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

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.

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.

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

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

Pettipher, G.L. (2000). *Alicyclobacillus* spp., their detection and control in fruit juice. *Soft Drinks International*, 31-32.

Ruel, G., Pomerleau, S., Couture, P., Lamarche, B. & Couillard, C. (2005). Changes in plasma antioxidant capacity and oxidized low-density lipoprotein levels in men after short-term cranberry juice consumption. *Metabolism*, **54**, 856-861.

Serafini, M., Laranjinha, J.A.N., Almeida, L.M. & Maiani, G. (2000). Inhibition of human LDL lipid peroxidation by phenol-rich beverages and their impact on plasma total antioxidant capacity in humans. *Journal of Nutritional Biochemistry*, **11**, 585-590.

Silva, F.V.M., 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.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*, **10**, 138-141.

Silva, F.V.M. & Gibbs, P. (2001). *Alicyclobacillus acidoterrestris* spores in fruit products and design of pasteurization processes. *Trends in Food Science & Technology*, **12**, 68-74.

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.

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.

Walls, I. & Chuyate, R. (2000). Isolation of *Alicyclobacillus acidoterrestris* from fruit juices. *Journal of AOAC International*, **83**, 1115-1120.

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

Background

Foods with a pH lower than 4 are considered as high in acid and are generally regarded as not being susceptible to spoilage by a variety of micro-organisms (Jay, 1998a). At this low pH, spoilage is mostly caused by acid-tolerant yeasts and mycelial fungi, while bacterial spores will not germinate and grow under these acidic conditions. The acid or acidified foods with a pH below 4.6 are not subjected to a heat-treatment at temperatures sufficient to destroy bacterial spores (Walls & Chuyate, 1998). During the heat-treatment of foods, pathogens and most non-spore-forming micro-organisms are killed, but a heat process sufficient to destroy all the microbial spores will have a detrimental effect on the organoleptic quality of the product.

Traditionally, fruit juices are considered to be susceptible to spoilage only by yeasts, mycelial fungi and lactic acid bacteria. The low pH is considered sufficient to prevent the growth of almost all bacterial spore-formers. Spores of *Clostridium botulinum* can not germinate or produce the lethal botulinum toxin in an environment with a pH below 4.6 (Chang & Kang, 2004). Another common thermophilic spoilage organism, *Bacillus stearothermophilus*, can not grow and cause the characteristic flat sour type spoilage below a pH of 5.3 (Chang & Kang, 2004). Other organisms of concern are *C. pasteurianum* and *B. coagulans,* being the only spore-formers able to grow at pH 3.8 (Jay, 1998b). This has allowed the fruit beverage industry to successfully apply a hot-fill-hold process to pasteurise the products. This pasteurisation process holds the product at temperatures between 88˚ and 96˚C for 2 min and is sufficient to destroy heat-liable spoilage organisms such as yeasts, lactic acid bacteria and some mycelial fungi. The products are then commercially sterile for the duration of the specified shelf-life, until the container is opened (Blocher & Busta, 1983).

Recently, an increasing number of spoilage cases of acid foods, such as fruit juices have been reported (Jensen, 1999). Cerny *et al.* (1984) reported the first spoilage case of commercially available pasteurised fruit juice and found shelf-stable, aseptically packaged apple juice to have an off-flavour. Following this report, a

number of cases were reported from all over the world and almost all of these spoilage incidents were caused by the spore-forming, thermo-acidophilic bacteria from the genus *Alicyclobacillus*.

Historical Perspective

The researchers Uchino & Doi (1967) initiated the interest in *Alicyclobacillus* after reporting the isolation of an aerobic, acidophilic, spore-forming bacterium from thermal waters from hot springs in Japan. Preliminary morphological and cultural studies showed the isolates to be closer related to *B. coagulans* than to other thermophiles. The bacteria grew over a pH range of 2.3 to 5.0 and over a temperature range of 45˚ to 71˚C. In 1971 this bacterial species was also isolated from hot springs in the USA (Darland & Brock, 1971). It was recommended that a new species of *Bacillus* should be created to accommodate these isolates as no other species of *Bacillus* possessed ω-alicyclic fatty acids and hopanoids in the bacterial membrane (Walls & Chuyate, 1998). It was named *B. acidocaldarius* and described as thermo-acidophilic, aerobic spore-formers containing ω-alicyclic fatty acids as the major membrane component.

In the years to follow, acidophilic spore-formers were isolated from various other environmental sources, including garden soil, forest soil, apple juice and water (Pontius *et al*., 1998), emphasising the fact that they can survive non-thermal conditions in the form of endospores (Hippchen *et al*., 1981). These bacteria are adapted to different climate zones around the world and can even be isolated from commercial juice products, showing their ability to survive from the environment into the retail market (Wisse & Parish, 1998; Eiroa *et al*., 1999).

The environmental isolates found in the years to follow the discovery of Uchino & Doi (1967) were shown to differ from *B. acidocaldarius*, regarding their growth requirements and biochemical characteristics (Walls & Chuyate, 1998). The optimum growth pH of these isolates was found to be between 2.0 and 5.0, while the optimum temperature for growth ranged from 22˚ to 62˚C. The fatty acid composition of the cellular membrane was analysed and also found to contain ω-alicyclic fatty acids. Sub-terminal or terminal endospores were seen to be contained in slightly swelled sporangia. These findings lead to the conclusion that a new species, different from *B. acidocaldarius* was isolated (Deinhard *et al*., 1987). Another bacteria with the same growth requirements as *B. acidocaldarius*, but with ω-cycloheptane (instead of

ω-cyclohexane) membrane fatty acids were later isolated (Poralla & König, 1983). Deinhard *et al.* (1987) described the two new species and named them *B. acidoterrestris* and *B. cycloheptanicus*.

The three species *B. acidocaldarius, B. acidoterrestris* and *B. cycloheptanicus* were sufficiently different from other *Bacillus* spp. to warrant reclassification into a new genus (Walls & Chuyate, 1998). The genus *Alicyclobacillus* was described in 1992 based on 16S ribosomal RNA (rRNA) gene sequence analyses, as well as the unique fatty acid profile of the bacterial membrane (Wisotzkey *et al*., 1992; Goto *et al*., 2002a).

Characteristics

There are currently eleven species recognised within the genus *Alicyclobacillus* (Table 1). Species of *Alicyclobacillus* are rod-shaped, non-pathogenic and Gram-positive (Fig. 1). They are referred to as thermophilic acidophilic bacteria (TAB) due to their ability to survive acidic conditions and elevated temperatures. The cell size of species of *Alicyclobacillus* ranges from 2.9 to 4.3 μm in length and 0.6 to 0.8 μm in width (Jensen, 1999). The colonies are described as circular, creamy white, flat, translucent to opaque and 3 to 5 mm in diameter (Walls & Chuyate, 1998; Chang & Kang, 2004). The endospores are located terminally or sub-terminally and the sporangium is not necessarily swollen. Isolates from *A. acidoterrestris* sporulate rapidly, usually within 24 h in liquid and solid media, as well as in fruit juice (Splittstoesser *et al*., 1994).

The unique and distinctive characteristic of *Alicyclobacillus* spp. is the presence of ω-alicyclic fatty acids as the major components of the cellular membrane (Silva & Gibbs, 2001; Goto *et al*., 2002a). The main membrane component in the species *A. acidocaldarius, A. acidoterrestris, A. hesperidium* and *A. acidiphilus* is ω-cyclohexane fatty acids (Fig. 2) (Deinhard *et al*., 1987; Jensen, 1999; Albuquerque *et al*., 2000; Matsubara *et al*., 2002; Chang & Kang, 2004). The fatty acid ω-cycloheptane are mainly present in the membranes of *A. cycloheptanicus* and *A. herbarius* (Poralla & König, 1983; Goto *et al*., 2002a; Chang & Kang, 2004). These fatty acids are believed to play an important role in the acid- and heat-resistance of *Alicyclobacillus* spp. (Wisotzkey *et al*., 1992). When they are tightly packed in a cyclohexane ring structure it may serve as a protective coating for the

cell membrane, contributing to the survival of the cells under extreme conditions (Chang & Kang, 2004).

Growth temperature varies between the different species of *Alicyclobacillus* and might be influenced by the pH of the growth medium (Farrand *et al*., 1983). The temperatures supporting growth is reported to be between 20˚ and 70˚C, with optimum temperatures between 42˚ and 60˚C (Yamazaki *et al*., 1996; Jensen, 1999; Chang & Kang, 2004). Growth at extreme temperatures of 12˚ to 80˚C has also been reported, but at these temperatures growth initiation is extremely slow, usually taking several weeks (Deinhard *et al*., 1987). The optimum growth temperature for *A. acidoterrestris* is between 35˚ and 55˚C, while *A. acidocaldarius* has a higher optimum growth temperature range of between 45˚ and 70˚C (Simbahan *et al*., 2004).

Pathogenicity was of concern in food products contaminated with *Alicyclobacillus* destined for human consumption. A study on the pathogenicity of *A. acidoterrestris* in fruit juice was conducted by Walls & Chuyate (2000) during which they injected spores into mice and fed inoculated juice to guinea pigs. No illnesses or deaths were reported and they concluded that *A. acidoterrestris* was not pathogenic at the levels tested. The risk of secondary growth of other pathogens such as *Clostridium botulinum* is not of concern, as growth of *A. acidoterrestris* does not affect the pH of the juice (Brown, 2000). Juice spoilage by *Alicyclobacillus* spp. has a major economical impact on the fruit juice industry, but there is no health risk involved in consuming fruit juice containing this bacterium or its spores.

An interesting and unusual characteristic of this genus is that when they are grown in an unbuffered, liquid medium containing amino acids they will produce ammonia from the amino acids, increasing the pH to inhibitory levels (Jensen, 1999). Growth is initiated at a of pH 6, but does not continue due to the increase in the pH of the growth medium (Splittstoesser *et al*., 1998).

Walker & Phillips (2005) found that the presence of headspace in the packaging affects the growth of *A. acidoterrestris* in fruit juice, but samples having 25, 50 and 75% headspace showed no significant differences in cell numbers. The presence or absence of headspace thus affected the growth of *A. acidoterrestris*, and not the percentage of headspace. Intermittent shaking was also found to increase growth of *A. acidoterrestris* in fruit juice at a suboptimal growth rate at 30˚C (Walker & Philips, 2005).

Thermal Resistance

The heat resistance of bacterial vegetative cells and spores is measured as the decimal reduction time (*D*-value). The *D*-value is the time required to destroy 90% of the bacteria at a given temperature and is equal to the minutes required for the survival curve to traverse one log cycle at a given temperature (Jay, 1998b). Varying *D*-values have been reported and all show that *Alicyclobacillus acidoterrestris* spores survive the industrial pasteurisation temperatures applied during the hot-fill-hold processes used in the production of commercial fruit juices. The *D*-values reported are $D_{87.9}$ 11 min, $D_{91.1}$ 3.8 min and D_{95} 1.0 min (Walls & Chuyate, 1998; Chang & Kang, 2004).

The reason *Alicyclobacillus* spp. survive pasteurisation and hot-fill-hold processes are still unclear (Splittstoesser *et al*., 1994). Environmental factors, such as pH, soluble solid content and temperature conditions influence the heat resistance of the spores (Chang & Kang, 2004). Splittstoeser *et al.* (1998) obtained comparable D-values of grape and apple juice to illustrate the influence of the soluble solids content on the heat resistance of the spores (Table 2) and reported that a soluble solid content of 18˚Brix or higher has an inhibitory effect on *A. acidoterrestris*. An orange juice drink, fruit drink and fruit nectar was also evaluated by Baumgart *et al.* (1997) (Table 2). From these results it is clear that it is difficult to destroy the spores in concentrate, compared to destroying the spores in single strength juice. Thus the higher the soluble solids content of the juice, the higher the resistance of the spores to heat treatments.

The heat resistance of members of the genus *Alicyclobacillus* is influenced by the pH at temperatures around 88˚ to 91˚C (Jensen, 1999). At these temperatures a pH increase of just over half a unit caused the *D*-values to double. Pontius *et al.* (1998) found that the type of organic acid present did not have a significant influence on the heat resistance of *A. acidoterrestris* spores, while the temperature and pH did have a major effect on the heat sensitivity. The resistance of the spores increased with an increase in the pH to an optimum just higher than the optimum pH for growth, after which it decreased rapidly as the pH of the medium decreased (Chang & Kang, 2004). The heat sensitivity of the spores was most affected by temperatures between 85˚ and 97˚C and was less affected by the pH value (Silva *et al.*, 1999). A non-linear decrease of the *D*-values was observed when the temperature was increased, while a linear decrease of the *D*-value was observed with a decrease in

the soluble solid content and pH (Silva *et al.,* 1999). The *z*-value refers to the degrees Fahrenheit needed for the thermal destruction curve to be reduced by one log cycle (Jay, 1998b). By monitoring the *z*-values during the reduction of the media pH, the spores of *A. acidoterrestris* were not significantly influenced by reducing the pH of the heating medium (Murakami *et al.*, 1998). The *z*-values remained constant over the pH range of 3.0 to 8.0, showing that the heat resistance of the spores are not influenced by the pH of the medium in which the vegetative cells or spores are suspended during heating.

Sulphur dioxide and sorbic acid are the preservatives used in fruit juice to decrease the heat resistance of mycelial fungi ascospores, but were found to have no effect on the heat resistance of *Alicyclobacillus* spores at concentrations as high as 100 $mg.l^{-1}$ (Splittstoesser *et al*., 1998). It was later found that sorbic acid, benzoic acid, or a combination of these two prevented spoilage of the fruit juice drink by *Alicyclobacillus* spp. (Pettipher & Osmundsen, 1999). Growth was also inhibited in unpreserved juices through carbonation.

A number of different factors that may contribute to the thermal resistance of these bacterial spores include the presence of heat stable proteins and enzymes, dehydration, dipicolinic acid (DPA) content and mineralisation (De Rosa *et al*., 1971; Chang & Kang, 2004). Lipids with a high ω-cyclohexane fatty acid content are stabilised by a high acyl chain density at the free fatty acid side in the centre of the membrane, which can aid in heat resistance (Kannenberg *et al*., 1984; Moore *et al*., 1997). Furthermore, the density of these chains also influences the permeability of the membrane. In addision to the reason for the thermal stability of *Alicyclobacillus* spp., enzymes stable at these high temperature and low pH conditions have also generated interest, as these enzymes could have many potential industrial application (Matzke *et al*., 1997; Füll & Poralla, 1999; Matzke *et al*., 2000; Eckert *et al*., 2002).

Demineralisation and remineralisation play a role in the heat sensitivity of bacterial spores, with a decrease in the heat sensitivity of demineralised spores (Alderton *et al*., 1964). The heat resistance of demineralised bacterial spores can be increased by reminiralisation with divalent cations such as Ca^{2+} or Mn^{2+} (Chang & Kang, 2004). However, in a study done by Silva & Gibbs (2001) on the heat sensitivity of *Alicyclobacillus* spores, the addition of divalent cations, such as Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+} and Sr^{2+} in the medium used for sporulation, did not affect the heat resistance of the spores in fruit products. Contradictory to these findings, two other

studies reported that different pH values caused fast demineralisation of the spores, as the binding characteristics of *A. acidoterrestris* spores to these two cations was showed to be stronger under low pH conditions, making the spores more sensitive to heat (Alderton *et al*., 1964; Bender & Marquis, 1985). The small change in calcium-dipicolinic acid (Ca-DPA) concentration and the ability to strongly bind divalent cations are thought to be related to the specific heat resistance of *A. acidoterrestris* spores (Yamazaki *et al*., 1997; Chang & Kang, 2004).

Spoilage and taint production

Interest in species of *Alicyclobacillus* increased when a thermophilic, acidophilic *Bacillus* spp. was identified as the spoilage organism in a large-scale spoilage incidence of shelf-stable apple juice in Germany in 1982 (Cerny *et al*., 1984). A number of spoilage cases of fruit juice or fruit-based products by *A. acidoterrestris* were reported in the 1990s from Europe, USA, the United Kingdom and Japan (Walls & Chuyate, 1998; Jensen, 1999). During the past decade, *A. acidoterrestris* has become a major cause of spoilage in pasteurised fruit juices (Walls & Chuyate, 1998). Cases were previously thought to be sporadic, but the National Food Processors Association (NFPA) undertook a survey in 1998 to estimate the impact of spoilage caused by thermo-acidophilic spore-formers (Walls & Chuyate, 1998). Just over half of the manufacturers who responded to the survey (35% of the 60% who responded) reported incidence of spoilage of juice products, consistent with the growth of thermo-acidophilic bacteria (Walls & Chuyate, 1998). Spoilage seemed to occur in the warmer seasons and apple juice was most commonly spoilt. The pH of the spoilt products ranged from 3.2 to 4.1 and spoilage was mainly due to an off-flavour, with or without cloudiness.

Alicyclobacillus acidoterrestris, *A. pomorum* and *A. acidocaldarius* are the only species of the genus currently associated with spoilage of food-products (Jensen, 1999; Silva & Gibbs, 2001; Goto *et al*., 2003; Gouws *et al*., 2005), with fruit juices and fruit based products the most susceptible (Komitopoulou *et al*., 1999). Spoilage has been reported in fruit juices that include apple, pear, orange, peach, mango and white grape juice, as well as in fruit juice blends, fruit juice containing drinks and tomato products, such as tomato juice and canned tomatoes (Splittstoesser *et al*., 1994; Jensen, 1999). No growth was, however, observed in red-grape juice (Splittstoesser *et al*., 1998) and it is believed that certain phenolic compounds in red-

grape juice inhibit growth. Alcohol levels of more than 6% have been shown to have an inhibitory effect on the growth of *A. acidoterrestris*. Therefore, table wines will not be spoilt, but some ciders might be at risk.

Alicyclobacillus acidoterrestris causes a flat-sour type of spoilage, mainly attributed to the formation of guaiacol, an offensive-smelling compound. Taint in fruit juice products caused as a result of guaiacol production can easily be formed since substrates required for guaiacol production can be present in the fruit juice (Chang & Kang, 2004).

Other taint chemicals, such as the halophenols 2,6-dichlorophenol (2,6-DCP) (Fig. 3A) and 2,6-dibromophenol (2,6-DBP) (Fig. 3B) can also be produced in fruit juice by *A. acidoterrestris* (Yamazaki *et al*., 1996; Komitopoulou *et al*., 1999; Jensen & Whitfield, 2003). However, it has not clearly been shown that the halophenols are from microbial origin (Jensen & Whitfield, 2003). Gocmen *et al*. (2005) published results where aroma compounds from orange juice contaminated with *A. acidoterrestris* was examined using gas chromatography-olfactory (GC-O) and confirmed with GC-mass spectroscopy (GC-MS). The medicinal off-odours were found to be from guaiacol and at least one halogenated phenol.

Guaiacol

Guaiacol (2-methoxyphenol) (Fig. 4) is frequently used as a synthetic flavouring in foods (Furia & Bellanca, 1975), producing a sweet, burnt aroma and smoky taste (Wasserman, 1966). It has also been described as having an odour comparable to the smell of smoky bacon (Pettipher & Osmundsen, 1999). In roasted products, guaiacol is formed by the thermal decomposition of phenolic precursors (Chang & Kang, 2004) and is responsible for the characteristic odour of Arabica coffee (De Maria *et al*., 1994) and barley malt (Topakas *et al*., 2003). In most cases, the sensory odour caused is undesirable (Whitfield, 1998).

The odour threshold of guaiacol in fruit juice was found to be approximately 2 parts per billion (ppb) (Pettipher *et al*., 1997; Orr *et al*., 2000). This is much lower than the sensory threshold of 0.02 $mg.l^{-1}$ reported for guaiacol in dry white wine (Chang & Kang, 2004). Guaiacol is present in much higher concentrations in the fruit juice and it is more volatile than the halophenols, and is, therefore, seen as the major off odour in the spoilage of fruit juices by *A. acidoterrestris* (Jensen, 2000).

Guaiacol is produced from vanillin by *A. acidoterrestris* (Jensen, 2000). Vanillic acid, which is formed after the oxidation of vanillin and is decarboxylised to guaiacol, can also be naturally derived from lignin or be present as a result of contamination of fruit juices. The synthetic pathway for the production of guaiacol from lignin is presented in Fig. 4. Ferulic acid, a major component of lignin, is first converted to vanillin or 4-vinyl-guaiacol by decarboxylation of the ferulic acid. The 4-vinyl-guaiacol is oxidised to vanillin, which is then further oxidised to vanillic acid, which forms the taint chemical guaiacol after a decarboxylation step (Crawford & Olson, 1978; Pometto *et al.*, 1981; Huang *et al.*, 1993). The amino acid tyrosine may also be a possible precursor for guaiacol (Jensen, 1999). Apple juice naturally contains tyrosine up to concentrations of 4.1 $\mu l.ml^{-1}$, while higher concentrations of up to 13.5 $\mu l.ml^{-1}$ are found in orange juice. This reaction has not been thoroughly investigated and guaiacol production as a result of lignin degradation is most widely accepted.

Vanillic acid has been reported to be converted to guaiacol by several strains of *B. megaterium* (Crawford & Olsen, 1978; Topakas *et al*., 2003), *Streptomyces setonii* (Pometto *et al*., 1981), unidentified species of *Streptomyces* and from ferulic acid by *Rhodotorula rubra* (Huang *et al*., 1993). Vanillic acid was identified as the immediate precursor of guaiacol in each of these pathways (Jensen *et al*., 2001).

Factors affecting guaiacol production in *Alicyclobacillus* spp. are the concentration of the species present, the storage temperature and the heat shock treatment applied (Pettipher *et al*., 1997). Guaiacol was detected in both apple and orange juice when *A. acidoterrestris* was present at a concentration of 10^5 $cfu.ml^{-1}$ after 4 days. Results from growth experiments indicated that low numbers of *A. acidoterrestris* present in fruit juice increased to numbers where guaiacol is produced, indicating their potential to spoil fruit juice even though they are initially present in low numbers. Significant sensory differences and differences in guaiacol content in fruit juice and chocolate milk samples, incubated at different temperatures, were reported (Jensen *et al*., 2001). It is believed that guaiacol production increases as the temperature of incubation increases. Active vegetative cells present are responsible for the production of guaiacol, therefore, heat shock treatments of samples leads to the activation and growth of spores (Splittstoesser *et al*., 1998; Jensen, 2000; Chang & Kang, 2004) and it was found that 80˚C for 10 min resulted in the highest guaiacol concentration (Walls & Chuyate, 2000). By accelerating the formation of guaiacol, the detection of this taint chemical can be simplified if this is to

be used as an indication of the presence of *A. acidoterrestris* in food products (Chang & Kang, 2004).

Halophenols

The halophenols 2,6-DCP and 2,6-DBP have been linked to taint production in fruit juices caused by *A. acidoterrestris* (Fig. 3A and 3B) (Baumgart *et al*., 1997; Borlinghaus & Engel, 1997; Jensen, 1999). Similar to guaiacol, 2,6-DCP and 2,6-DBP also produce taints described as 'medicinal' or 'disinfectant' (Whitfield, 1998).

The contamination pathway of these two compounds can be divided into two groups, chemical contamination and microbial synthesis (van Pée, 1996; Flodin & Whitfield, 1999). Chemical contamination occurs when fruit juice comes into contact with weak halogen solutions present in the sanitisers used to wash the raw fruit or processing lines (van Pée, 1996). Thus, if they are not completely removed from contact areas 2,6-DCP and 2,6-DBP can be formed. Alternatively, these spoilage compounds can be formed through bacterial synthesis (Flodin & Whitfield, 1999). Phenolic precursors, hydrogen peroxide and halide ions play a vital role in taint formation or halogenation. The presence of these three compounds in fruit juice, together with the bacterial enzyme haloperoxidase, may result in taint formation in fruit juices. The possibility that halogenation can occur as a result of the presence of *A. acidoterrestris* is strengthened by evidence that strains of this species contain enzyme systems for the conversion of chemical compounds to 2,6-DCP and 2,6-DBP (Borlinghaus & Engel, 1997; Jensen & Whitfield, 2003).

The prevention of 2,6-DCP and 2,6-DBP formation, causing taint in fruit juice products, is challenging because of the low sensory thresholds reported. The threshold of 2,6-DBP is in the parts per trillion (ppt), with the taste threshold in water at 0.5 ppt (Borlinghaus & Engel, 1997). Although the exact threshold of the halophenols in fruit juices is still uncertain, reported numbers are estimated to be 0.5 parts per billion (ppb) for 2,6-DCP and 30 ppt for 2,6-DBP (Jensen, 1999).

A factor affecting the halophenol production in *Alicyclobacillus* spp. is the type of medium containing the bacteria, since taint formation was detected in fruit juice, but not in acidified water (Jensen, 1999). A further factor affecting halophenol production is the headspace of the packaging of the fruit juice (Jensen & Whitfield, 2003). A larger headspace results in an increase in the growth rate of

A. acidoterrestris and thus an increased rate of halophenol production (Jensen & Whitfield, 2003).

Sources of contamination

Members from the genus *Alicyclobacillus* were first isolated from soil samples taken from hot springs in the Tohoku district in Japan (Uchino & Doi, 1967). Hippchen *et al*., (1981) isolated a further 23 strains of thermo-acidophilic bacilli, containing ω-cyclohexane fatty acids from soil, showing that they need neither hot nor acidic conditions for survival. It might be that these bacteria depend on the presence of certain minerals in the soil for survival (Hippchen *et al*., 1981). Species of *Alicyclobacillus* have also been isolated from a wide variety of other habitats and substrates including geothermal sites, sediments of thermal aquifers, small rivers and creeks, and submarine hot springs (Splittstoesser *et al*., 1998; Goto *et al*., 2002a). The occurrence of *Alicyclobacillus* in these different habitats indicates that it is wide-spread (Wisse & Parish, 1998; Eiora *et al*., 1999).

Soil is considered to be the main repository for spores of *A. acidoterrestris* and it is believed that fruit in contact with the soil during harvesting become contaminated (Splittstoesser *et al*., 1998). Fruit juice contamination results from unwashed or poorly washed raw fruit that is processed, as well as contaminated water used during the production of fruit juices (Pontius *et al*., 1997).

Detection, isolation and enumeration

In the years following the discovery of *Alicyclobacillus* spp. it was established as a major spoilage organism of fruit juice and various methods for the detection, isolation, identification, confirmation and quantification of *Alicyclobacillus* were developed (Yamazaki *et al*., 1996; Borlinghaus & Engel, 1997; Pettipher *et al*., 1997; Wisse & Parish, 1998; Pettipher, 2000; Walls & Chuyate, 2000; Pacheco, 2002). Currently, no standard method exists for the isolation and identification of *Alicyclobacillus* spp. from fruit juice (Chang & Kang, 2004).

Media plating and membrane filtration are the two main isolation methods used (Chang & Kang, 2004). Membrane filtration is commonly used to collect micro-organisms from both liquid and gas samples. A primary advantage of membrane filtration is that large sample volumes can be tested (Chang & Kang, 2004).

Splittstoesser *et al.* (1994) applied this technique to the detection of *Alicyclobacillus* in fruit juice. Beverages was filtered through membranes with a pore size of 0.45 μm and plated onto potato dextrose agar (PDA), pH 3.5 and incubated for 5 to 7 days at 43˚C. Pettipher (2000) used membrane filtration in combination with a heat treatment at 80˚C for 10 min to detect *A. acidoterrestris* at low contamination levels in cans of juice products, where the filter was incubated on orange serum agar (OSA) for increased sensitivity (Table 3). New species of *Alicyclobacillus*, namely *A. hesperidum* and *A. herbarius* was isolated from soil suspensions and dried hibiscus flowers using a membrane filter with a pore size of 0.45 μm and a diameter of 47 mm (Table 3) (Albuquerque *et al*., 2000; Goto *et al*., 2002b).

Counts of 15 to 200 cfu.20 ml^{-1} fruit juice was obtained when membrane filtration was used in combination with a heat treatment and 1 to 79 cfu.20 ml^{-1} fruit juice when only membrane filtration was used to isolate *Alicyclobacillus* spp. from fruit juice, clearly showing membrane filtration to increase the detection sensitivity for the isolation of *Alicyclobacillus*. Counts of 5 cfu.ml^{-1} for *Alicyclobacillus* spp. was obtained when spread plating was used with or without a heat treatment for the analysis of fruit juice (Pettipher *et al*., 1997). Currently, the fruit juice industry is widely incorporating membrane filtration as part of their routine quality control procedures. The method has not been standardised, as different materials, membranes and procedures are currently used. Incorporation of membrane filters in the fruit juice production process is recommended to remove spores of *Alicyclobacillus* spp., but this can only be used for clear juices, as the pore size of the membrane will have to be small enough (between 0.45 and 0.6 μm) to filter out the bacterial spores without blocking the membrane (Vieira *et al*., 2002).

Spread plating on either OSA or PDA with a pH between 3.5 and 5.5, followed by incubation at temperatures ranging between 37˚ and 55˚C for 3 to 5 d are popular enumeration methods (Pettipher *et al*., 1997; Chang & Kang, 2004). For greater sensitivity, either membrane filtration or a heat shock treatment at 80˚C for 10 min is included in the enumeration procedures (Splittstoesser *et al*., 1994; Pettipher, 2000). Using membrane filtration, the filter itself can be incubated on the isolation media for greater sensitivity (Pettipher, 2000).

The different pre-treatments of contaminated samples are listed in Table 3 and may include freezing of the sample, dilution, centrifugation, incubation at various temperatures or heat-treatments before enumeration (Borlinghaus & Engel, 1997; Eiroa *et al*., 1999; Goto *et al*., 2001). Heat treatments activate the spores, which

leads to an increased viable count, especially if mainly spores are present in the sample (Chang & Kang, 2004). Enumeration is influenced by the plating technique, with spread plates giving higher counts than pour plates (Pettipher *et al*., 1997). The isolation media is another factor to consider and OSA, PDA, *Bacillus acidocaldarius* medium (BAM), K-agar and yeast extract agar (YSG-agar) are most commonly used for the isolation of *Alicyclobacillus* spp. (Pettipher *et al*., 1997; Walls & Chuyate, 2000; Chang & Kang, 2004). The pH of the isolation media influences recovery of *Alicyclobacillus* spp. and an acidification step to a pH of 3.7 is recommended to isolate this bacterium (Walls & Chuyate, 1998; Chang & Kang, 2004). Incubation temperatures can range from 30˚ to 60˚C (Splittstoesser *et al*., 1994; Pontius *et al*., 1998; Komitopoulou *et al*., 1999; Chang & Kang, 2004). Higher temperatures, such as 40˚ to 45˚C favoured the growth of *Alicyclobacillus* spp., while inhibiting the growth of many non-thermophilic organisms (Pettipher *et al*., 1997). Incubation at 50˚ to 53˚C further inhibited the growth of heat resistant moulds, such as *Byssochlamys* without decreasing the recovery of *Alicyclobacillus* spp. (Splittstoesser *et al*., 1998).

YSG-agar was the first medium used for the enumeration of *Alicyclobacillus* (then known as a thermo-acidophilic *Bacillus* species) (Uchino & Doi, 1967). Since Darland & Brock (1971) researched *Bacillus acidocaldarius*, later renamed *Alicyclobacillus acidocaldarius* (Wisotzkey *et al*., 1992) and developed BAM, most researchers used this media for the enumeration of *Alicyclobacillus*. Generally, the use of BAM for the isolation of *A. acidoterrestris* is limited to temperatures between 25˚ and 60˚C and pH values ranging from 2.5 to 5.5 (Deinhard *et al*., 1987; Yamazaki *et al*., 1996).

Isolates of *Alicyclobacillus* does not grow on brain heart infusion agar, veal infusion agar, trypticase soy agar, standard plate count agar and nutrient agars, even when the pH is adjusted to 3.5 (Splittstoesser *et al.*, 1998). The inability of *Alicyclobacillus* spp. to grow on these media may be due to the presence of inhibitory substances such as peptones. Jensen (1999) found that *A. acidoterrestris* will grow on most media, including nutrient agar, if the pH is adjusted to below 5.8 and the media is incubated aerobically. K-agar have been used for isolation of *Alicyclobacillus* strains, adjusted to a pH of 3.7 (Walls & Chuyate, 1998). Growth of *Alicyclobacillus* on K-agar was compared to growth of *Alicyclobacillus* on semi-synthetic medium with a pH of 4, OSA with a pH of 3.5 and minimal salts medium with a pH of 4 and the highest enumeration results was obtained on K-agar with incubation at 43˚C for 5 to 7 days (Walls & Chuyate, 2000). Malt extract agar (MEA)

adjusted to pH 4 and incubated for 3 to 4 d at 37˚C, have also been found to support the growth of *Alicyclobacillus* (Yamazaki *et al*., 2000), as does thermo acidurance agar (TA) (pH 4) and PDA at pH 3.5 and 5.6 (Splittstoesser *et al*., 1998; Jensen, 1999).

Identification

The different *A. acidoterrestris* strains have been identified by characterisation of the biochemical profile, using Gram stains and API 50 CH test strips (Deinhard *et al*., 1987; Yamazaki *et al*., 1996; Walls & Chuyate, 1998; Silva *et al*., 1999; 2001). Deinhard *et al*. (1987) tested 13 strains of *A. acidoterrestris* and all strains formed acid from glycerol, erythritol, L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, mannitol, esculin and cellobiose.

Tests currently being used in the fruit juice industry to identify *Alicyclobacillus* spp. include the presence or absence detection method which consists of pre-incubating the juice for 48 h at 44˚C before streaking it out on OSA and then incubating the plates for 48 h at 44˚C before examining the colonies (Pettipher *et al.*, 1997). Another method is the microscopic method (Pettipher & Osmundsen, 1999). This test uses a direct epifluorescent filter technique, which is a combination of membrane filtration with a nucleopore polycarbonate membrane with a pore size of 0.6 μm, a fluorescent dye such as acridine orange and epifluorescence microscopy. With this technique it is possible to see the rod shaped bacteria if they are present in the sample tested. Off-odour production is another approach used for the detection of *Alicyclobacillus* spp. in fruit juice. This approach is done by olfactory evaluation and if the distinct disinfectant-like taint is produced by colonies on the isolation media, it is regarded as a presumptive positive (Pettipher & Osmundsen, 1999).

Rapid detection methods for *Alicyclobacillus* spp. include polymerase chain reaction (PCR) (Yamazaki *et al*., 1996; Yamazaki *et al*., 1997; Gouws *et al*., 2005), a 24 h detection technique using reverse transcription polymerase chain reaction (RT-PCR) (Yamazaki *et al*., 1996) and real-time PCR (RT-PCR), that targets the squalene-hopene cyclase-encoding (*shc*) gene and rapidly detects less than 100 cells of *A. acidocaldarius* and *A. acidoterrestris* in fruit juice (Connor *et al*., 2004; Luo *et al*., 2004).

Control

Good hygiene alone is not sufficient to control the occurrence of *Alicyclobacillus acidoterrestris* in fruit juice (Pettipher, 2000). The only viable control measure at present is the thorough washing of the raw material before it is processed (Brown, 2000). Orr & Beuchat (2000) tested the efficiency of different disinfectants against spores of *A. acidoterrestris*. Spores treated with 8% trisodium phosphate or 80 parts per million (ppm) Tsunami were not significantly reduced. Spores were also suspended in 200 ppm chlorine, 500 ppm acidified sodium chlorite and 0.2% (v/v) hydrogen peroxide for 10 min at 23˚C. These treatments led to significant reductions in viable spore counts. Further reductions of up to 5 logs were achieved when spores were treated with 1000 ppm chlorine or 4% (v/v) hydrogen peroxide. Treatment of aqueous solutions of *A. acidoterrestris* showed a greater reduction in spore counts with a higher concentration (ppm) of chlorine dioxide. Using 80 ppm or 120 ppm free chlorine dioxide for 5 min both reduced spore counts of *A. acidoterrestris* in aqueous solutions to less than 0.7 log cfu.ml^{-1} (Lee *et al*., 2004).

Chemical disinfectants are less effective against spores of *A. acidoterrestris* on the surface of fruits and treatment with 500 ppm chlorine and 1200 ppm acidified sodium chlorite for 1 min on *A. acidoterrestris* spores on the surface of apples only led to reductions of less than 1 log. A 2% (v/v) solution of hydrogen peroxide failed to kill the spores remaining on the apple surfaces after treatment. Chlorine dioxide at different concentrations is effective against spores of *A. acidoterrestris* both in aqueous solutions and on the surface of apples (Lee *et al*., 2004). Applying 40 ppm free chlorine dioxide to the surface of apples for 1, 2, 3 and 4 min reduced the number of *A. acidoterrestris* spores by 1.5, 3.2, 4.5 and >4.8 log. No synergistic effect was observed when the chlorine dioxide treatment was used in conjunction with a heat treatment.

Heat treatment alone has been shown to be inefficient to eliminate *A. acidoterrestris* from fruit juice without altering the organoleptic qualities or vitamin content of fruit juice (Splittstoesser *et al*., 1994; Jensen, 1999; Chang & Kang, 2004). Silva *et al*. (2000) designed a pasteurisation process for cupuaçu pulp using *A. acidoterrestris* as reference micro-organism and recommended that it be done for other acidic fruit products, as the heat resistance of the microbial targets normally used for fruit products are much less than that of the spores of *A. acidoterrestris* (Silva & Gibbs, 2000). The use of high pressure alone is also not sufficient to reduce

the number of viable spores of *A. acidoterrestris* in apple juice, but when it is combined with a heat treatment, the effectivity increases as the temperature of the heat treatment increases (Lee *et al*., 2002). The higher pressure ensures that temperatures are kept low enough as not to alter the taste of the fruit juice. Treatment of 6000 $kg.cm^{-2}$ for 10 min at 47˚C has been reported to eliminate bacterial spores in fruit juice (Farr, 1990). Treatment of apple juice under pressure decreased viable spores to undetectable levels, and it was reported that the amount of pressure used was not as important as the period of time it was applied (Lee *et al*., 2002).

Heat stable bacteriocins produced by lactic acid bacteria may play a role in controlling *Alicyclobacillus* in fruit juice (Oh *et al*., 1999). *Alicyclobacillus acidoterrestris* is sensitive to the bacteriosin nisin, decreasing the *D*-value by up to 40% when added during heating, indicating that the use of nisin is a potential way of controlling this organism in fruit juice (Komitipoulou *et al*., 1999). The bacteriocin from *Lactococcus* sp. CU216 was found to have an inhibitory effect against strains of *Alicyclobacillus*, leading to the rapid inactivation of all *Alicyclobacillus* strains tested when added to spores and vegetative cells (Oh *et al*., 1999). An enterocin from *Enterococcus faecalis* was also found to be active against *Alicyclobacillus* spp. (Grande *et al*., 2005). Enterocin AS-48 inhibited vegetative cells of *A. acidoterrestris* in orange and apple juices stored at 37˚C and no growth was seen after two weeks of incubation. In commercial fruit juices 2.5 $\mu g.ml^{-1}$ of the enterocin eliminated viable cells after 15 min of incubation and no viable cells were detected in the fruit juice during incubation at 37˚, 15˚ and 4˚C for 90 days. The enterocin prevented spoilage of apple, peach and grapefruit juices by *A. acidoterrestris* for 60 days at 37˚C. It seems the enterocin causes cell damage, bacterial lyses and disruption of the endospore structure (Grande *et al*., 2005).

The beverage industry uses calcium lactate to fortify fruit juice and the effect of concentrations equivalent to 0% and 5% dietary reference intake of calcium lactate on spoilage and pathogenic organisms in orange juice with a pH of 3.6 and 4 was investigated (Yeh *et al*., 2004). *Alicyclobacillus acidoterrestris* was inhibited in all fruit juices stored at 4˚C, but was able to grow in the orange juice with a higher pH stored at higher temperatures. The use of temperature and pH may be a possible control measure for *Alicyclobacillus* in fruit juice.

The use of antimicrobial release films in fruit juice packaging has been investigated as a possible control measure (Buonocore *et al.*, 2004). It was reported

that the active ingredients released by lysozyme and nisin were effective in inhibiting microbial growth, but release kinetics and active films must be investigated further if it is to be used by the fruit juice industry.

Conclusions

Increasing amounts of thermo-acidophilic spore-forming bacteria have been isolated from spoiled beverages since 1982 and the presence of *Alicyclobacillus acidoterrestris* has specifically been linked to spoilage incidents of pasteurised fruit juices and fruit juice products (Cerny *et al*., 1984; Wisotzkey *et al*., 1992; Splittstoesser *et al*., 1994; Yamazaki *et al*., 1996; Pettipher *et al*., 1997). The current isolation and identification methods for *Alicyclobacillus* spp. differ regarding the isolation media used, the time and temperature of the heat-shock treatment applied and the time and temperature of incubation (Chang & Kang, 2004). Currently, there exists confusion about which media is most appropriate for the isolation of *Alicyclobacillus* spp. from fruit juice and fruit juice products (Darland & Brock, 1971; Deinhard *et al*., 1987; Wisotzkey *et al*., 1992; Yamazaki *et al*., 1996; Pettipher *et al*., 1997; Palop *et al*., 2000; Walls & Chuyate, 2000), and therefore comparison of the different isolation media for the isolation of *Alicyclobacillus* spp. from fruit juice and fruit juice products is necessary to aid in the development of a standard method. Culture-dependent methods are not always a true indication of the bacteria population in juice and are more time consuming than culture-independent methods. PCR-based DGGE is a highly specific, culture-independent molecular technique which could be a useful tool for the detection of thermo-acidophilic organisms in fruit juice.

References

Alderton, G., Thompson, P.A. & Snell, N. (1964). Heat adaptation and ion exchange in *Bacillus megaterium* spores. *Science*, **143**, 141-143.

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.

Baumgart, J., Husemann, M. & Schmidt, C. (1997). *Alicyclobacillus acidoterrestris*: occurrence, significance and detection in beverages and beverage base. *Flussiges Obst*, **64**, 178.

Bender, G.R. & Marquis, R.E. (1985). Spore heat resistance and specific mineralization. *Applied and Environmental Microbiology*, **50** (6), 1414-1421.

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

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

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

Buonocore, G.G., Sinigaglia, M., Corbo, M.R., Bevilacqua, A., La Notte, E. & Del Nobile, M.A. (2004). Controlled release of antimicrobial compounds from highly swellable polymers. *Journal of Food Protection*, **67**, 1190-1195.

Cerny, G., Hennlich, W. & Poralla, K. (1984). Fruchtsaftverderb durch *Bacillen*: isolierung und charakterisierung des verderbserrengers. *Zeitschrift feur Lebensmittel-Untersuchung und -Forsuchung*, **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.

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

Connor, C.J., Hongliang, L., McSpadden, B.B. & Wang, H.H. (2004). Development of a real-time PCR-based system targeting the 16S rRNA gene sequence for rapid detection of *Alicyclobacillus* spp. in juice products. *International Journal of Food Microbiology*, **99**, 229-235.

Crawford, R.L. & Olsen, 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., an acidophilic thermophilic spore-forming bacterium. *Journal of Genetic Microbiology*, **67**, 9-15.

De Maria, C.A.B., Trugo, L.C., Moreira, R.F.A. & Werneck, 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. (1971). Cyclohexane fatty acids from a thermophilic bacterium. *Chemical Communications*, 1334.

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

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

Eckert, K., Zielinski, F., Leggio, L.L. & Schneider, E. (2002). Gene cloning, sequencing and characterization of a family 9 endoglucanase (CelA) with an unusual pattern of activity from the thermoacidophile *Alicyclobacillus acidocaldarius* ATCC27009. *Applied Microbiology and Biotechnology*, **60**, 428-436.

Farr, D. (1990). High pressure technology in the food industry. *Trends in Food Science and Technology*, **1**, 14-16.

Farrand, S.G., Linton, J.D., Stephenson, R.J. & MacCarthy, W.V. (1983). The use of response surface analysis to study growth of *Bacillus acidocaldarius* throughout the growth range of temperature and pH. *Archives of Microbiology*, **135**, 272-275.

Flodin, C. & Whitfield, F.B. (1999). 4-Hydroxybenzoic acid: a likely precursor of 2,4,6-tribromophenol in *Ulva Lactuca*. *Phytochemistry*, **51**, 249-255.

Füll, C. & Poralla, K. (1999). Conserved Tyr residues determine functions of *Alicyclobacillus acidocaldarius* squalene – hopene cyclase. *FEMS Microbiology Letters*, **183**, 221-224.

Furia, T.E. & Bellanca, N. (1975). Fenaroli's Handbook of Flavor Ingredients, Vol. 2. Pp. , Cleveland, OH: CRC 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-olfactory and GC-MS. *Letters in Applied Microbiology*, **40**, 172-177.

Goto, K., Tanimoto, Y., Tamura, T., Mochida, K., Arai, D., Asahara, M., Suzuki, M., Tanaka, H. & Inagaki, K. (2002a). Identification of thermo-acidophilic bacteria and a new *Alicyclobacillus* genomic species isolated from *acidic* environments in Japan. *Extremophiles*, **6**, 333-340.

Goto, K., Matsubara, H., Mochida, K., Matsumura, T., Hara, Y., Niwa, M. & Yamasato, K. (2002b). *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., Moshida, K., Asahara, M., Suzuki, M., Kasai, H. & Yokota, A. (2003). *Alicyclobacillus pomorum* sp. nov., a novel thermo-acidophillic, endospore-forming bacterium that does not possess omega-alicyclic fatty acids, and emended description of the genus *Alicyclobacillus*. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 1537-1544.

Gouws, P.A., Gie, L., Pretorius, A. & Dhansay, N. (2005). Isolation and identification of *Alicyclobacillus acidocaldarius* by 16S rDNA from mango juice and concentrate. *International Journal of Food Science and Technology*, **40**, 789-792.

Grande, M.J., Lucas, R., Abriouel, H., Ben Omar, N., Maqueda, M., Martínez-Bueno, M., Martínez-Cañamero, M., Valdivia, E. & Gálvez, A. (2005). Control of *Alicyclobacillus acidoterrestris* in fruit juices by enterocin AS-48. *International Journal of Food Microbiology* (in press)

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

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.

Jay, J.M. (1998a). Intrinsic and extrinsic parameters of foods that affect microbial growth. In: *Modern Food Microbiology*, 5th ed. Pp. 38-44. New York: Chapman & Hah.

Jay, J.M. (1998b). High-temperature food preservation and characteristics of thermophilic microorganisms. In*: Modern Food Microbiology*, 5th ed. Pp. 354-355. New York: Chapman & Hah.

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.

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.

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.

Kannenberg, E., Blume, E. & 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 tolrans* 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.

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.

Lee, S-Y., Dougherty, R.H. & Kang, D-H. (2002). Inhibitory effects of high pressure and heat on *Alicyclobacillus acidoterrestris* spores in apple juice. *Applied and Environmental Microbiology*, **68**, 4158-4161.

Lee, S-Y., Gray, P.M., Dougherty, R.H. & Kang, D-H. (2004). The use of chlorine dioxide to control *Alicyclobacillus acidoterrestris* spores in aqueous suspensions and on apples. *International Journal of Food Microbiology*, **92**, 121-127.

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

Matzke, J., Schwermann, B. & Bakker, E.P. (1997). Acidostable and acidophilic proteins: the example of the α-amylase from *Alicyclobacillus acidocaldarius*. *Comparative Biochemistry and Physiology*, **118A**, 475-479.

Matzke, J., Herrmann, A., Schneider, E. & Bakker, E.P. (2000). Gene cloning, nucleotide sequence and biochemical properties of a cytoplasmic cyclomaltodextrinase (neopullulanase) from *Alicyclobacillus acidocaldarius*, reclassification of a group of enzymes. *FEMS Microbiology Letters*, **183**, 51-61.

Moore, B.S., Walker, K., Tornus, I., Handa, S., Poralla, K. & Floss, H.G. (1997). Biosynthetic studies of ω-cycloheptyl fatty acids in *Alicyclobacillus cycloheptanicus.* Formation of cycloheptanecarboxylic acid from phenylacetic acid. *Journal of Organic Chemistry*, **62**, 2173-2185.

Murakami, M., Tedzuka, H., & Yamazaki, K. (1998). Thermal resistance of *Alicyclobacillus acidoterrestris* spores in different buffers and pH. *Food Microbiology*, **15**, 577.

Oh, S., Churey, J.J. & Worobo, R.W. (1999). Inhibitory activity of *Alicyclobacillus* strains by bacteriocin of *Lactococcus* sp. CU216. *The IFT Annual Meeting*, 37D-32.

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.

Pacheco, C.P. (2002). Sensibility and specificity of methods for *Alicyclobacillus* detection and quantification: a collaborative study. *Fruit Processing*, 478-482.

Palop, A., Alvarez, I., Razo, J. & Condon, S. (2000). Heat resistance of *Alicyclobacillus acidocaldarius* in water, various buffers and orange juice. *Journal of Food Protection*, **61**, 1377-1380.

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

Pettipher, G.L. (2000). *Alicyclobacillus* spp., their detection and control in fruit juice. *Soft Drinks International*, 31-32.

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.

Pinhatti, M.E.M.C., Variane, S., Eguchi, S.Y. & Manfio, G.P. (1997). Detection of acidotermophilic bacilli in industrialized fruit juices. *Fruit Processing*, **7**, 350-353.

Previdi, M.P., Quintavalla, S., Lusardi, C. & Vicini, E. (1997). Heat resistance of *Alicyclobacillus* spores in fruit juices. *Industrial Conserve*, **72**, 353-358.

Silva, F.V.M., 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.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*, **10**, 138-141.

Silva, F.V.M. & Gibbs, P. (2001). *Alicyclobacillus acidoterrestris* spores in fruit products and design of pasteurization processes. *Trends in Food Science & Technology*, **12**, 68-74.

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.

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 termophile*. *Lebensmittel-Wissenschaft und -Technologie*, **36**, 561-565.

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-thermophillic bacteria from thermal waters. *Agricultural Biology and Chemistry*, **31**, 817-822.

van Pée, K.H. (1996). Biosynthesis of halogenated metabolites by bacteria. *Annual Reviews in Microbiology*, **50**, 375-399.

Vieira, M., Teixeira, A.A., Silva, F.M., Gaspar, N. & Silva, C.L.M. (2002). *Alicyclobacillus acidoterrestris* spores as a target for Cupuacu (*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.

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

Walls, I. & Chuyate, R. (2000). Isolation of *Alicyclobacillus acidoterrestris* from fruit juices. *Journal of AOAC International*, **83**, 1115-1120.

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

Wisotzkey, J.D., Jurtshuk, P., Jr, Fox, G.E., Deinhart, 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.

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

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

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., Kawai, N., Inoue, N. & Shinano, H. (1997). Influence of sporulation medium and divalent ions on the heat resistance of *Alicyclobacillus acidoterrestris* spores. *Letters in Applied Microbiology*, **25**, 153-156.

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.

Yeh, J-Y., Ellis, H. & Chen, J. (2004). Influence of calcium lactate on the fate of spoilage and pathogenic microorganisms in orange juice. *Journal of Food Protection*, **67**, 1429-1433.

CHAPTER 3

EVALUATION OF DIFFERENT GROWTH MEDIA AND INCUBATION TEMPERATURES FOR THE ISOLATION OF SPECIES OF *ALICYCLOBACILLUS*

Abstract

Species of *Alicyclobacillus* are acid-tolerant and heat-resistant bacteria that causes spoilage of fruit juices stored at room temperature. These endospore-formers can survive pasteurisation of 95°C for 2 min and grow at a pH range of 2.5 to 6.0. The aim of this study was to evaluate five different isolation media, namely potato dextrose agar (PDA), orange serum agar (OSA), K-agar, yeast-starch-glucose (YSG)-agar and *Bacillus acidocaldarius* medium (BAM) for the isolation of *Alicyclobacillus* spp. from sterile saline solution (SSS) and diluted and undiluted fruit juice concentrates. Different incubation temperatures for the five media were also evaluated. Spread plates of PDA at pH 3.7 recovered 2.85 x 10^6 cfu.ml^{-1} of an initial inoculum of 3.85 x 10^6 cfu.ml^{-1} vegetative cells of *A. acidoterrestris* from single strength pear juice concentrate, after 5 days incubation at 50°C (74% recovery). Recovery of *A. pomorum* was found to be the highest on PDA at pH 3.7 (100% recovery) from a 2.6 x 10^5 cfu.ml^{-1} initial inoculum. The recovery of endospores from single strength pear juice was evaluated after the samples were heat shocked at 80°C for 10 min before plating. Recovery was highest on spread plates of OSA at pH 5.5, incubated for 5 days at 50°C, with recovery of 2.81 x 10^6 cfu.ml^{-1} from an initial inoculum of 2.91 x 10^6 cfu.ml^{-1} (97% recovery). Recovery of vegetative cells from pear juice concentrate proved more difficult and was highest on PDA at pH 3.7, with an average recovery of 8.8 x 10^4 cfu.ml^{-1} from an initial inoculum of 3.85 x 10^6 cfu.ml^{-1} (2.3% recovery). The detection limit was determined as the lowest dilution of a known concentration of *Alicyclobacillus* spp. in SSS that showed growth on PDA plates. It was found that the detection limit for *A. acidoterrestris* on PDA at pH 3.7 was 10^4 cfu.ml^{-1} and for *A. acidocaldarius* it was 10^5 cfu.ml^{-1}.

Introduction

Alicyclobacillus acidoterrestris is an acid-tolerant and heat-resistant bacterium that can spoil heat-treated fruit juices by the formation of taint chemicals such as guaiacol and halophenols (Jensen & Whitfield, 2003). Species of *Alicyclobacillus* was

originally isolated from water samples from hot springs in the Tohoku district in Japan (Uchino & Doi, 1967). This endospore-former can survive pasteurisation at 95°C for 2 min in apple juice (Komitipoulou *et al*., 1999), grows at a pH range of 2.5 to 6.0, at temperatures between 25° and 60°C, forms a light sediment and produces no gas in fruit juices (Walls & Chuyate, 1998; Jensen, 1999).

As *Alicyclobacillus* spp. are recognised as spoilage microbes in fruit juices, there is a need for an accurate and standard detection method from fruit juice and fruit juice products (Chang & Kang, 2004). Current isolation procedures consist of membrane filtration (Splittstoesser *et al*., 1994; Pettipher, 2000) and plating on different growth media (Uchino & Doi, 1967; Darland & Brock, 1971; Deinhard *et al*., 1987; Wisotzkey *et al*., 1992; Yamazaki *et al*., 1996; Pettipher *et al*., 1997). Current identification methods include presence/absence testing (Pettipher *et al*., 1997), microscopic analysis, off-odour detection by olfactory analysis (Pettipher & Osmundsen, 2000) and molecular detection using the polymerase chain reaction (PCR) (Murakami *et al*., 1998; Connor *et al*., 2004; Gouws *et al*., 2005).

Yeast-starch-glucose agar (YSG-agar) was the first media used for the isolation of *Alicyclobacillus* spp. (Uchino & Doi, 1967) and Matsubara *et al*. (2002) isolated the new species, *A. acidiphilus* from fruit juice using this medium. Goto *et al*. (2003) combined the use of YSG-agar with membrane filtration to isolate the new species *A. herbarius* from dried hibiscus flowers. YSG-agar plates at pH 3.7, incubated at 55˚C, have also been used to detect the presence of thermophilic acidophilic bacteria (TAB) in mango juice purée (Gouws *et al*., 2005).

Darland & Brock (1971) developed *Bacillus acidocaldarius* medium (BAM) for the isolation of *B. acidocaldarius*, later renamed *A. acidocaldarius* (Wisotzkey *et al*., 1992). Since the development of BAM, it has been extensively used for the isolation of *Alicyclobacillus* spp. (Farrand *et al*., 1983; Deinhard *et al*., 1984; Silva *et al*., 1999; Silva *et al*., 2000) at incubation temperatures between 25˚ and 60˚C and media pH values from 2.5 to 5.5 (Deinhard *et al*., 1987; Yamazaki *et al*., 1996).

Orange serum agar (OSA) was originally developed for the isolation of bacteria associated with spoilage of citrus products. It has also been used for isolation of *Alicyclobacillus* spp. at incubation temperatures ranging from 43˚ to 45˚C and at a pH of 5.5 (Pettipher *et al*., 1997; Walls & Chuyate, 1998; Eiora *et al*., 1999; Pettipher & Osmundsen, 2000; Chang & Kang, 2004).

Walls & Chuyate (1998) first described and used K-agar for the isolation of *Alicyclobacillus* spp., incubated at 35˚C at a pH of 3.7, 4.5 and 5.0. K-agar has been

compared to PDA and OSA and was found to give higher enumeration results for *Alicyclobacillus* spp. after incubation at 43˚C for 5 to 7 days (Orr & Beuchat, 2000; Walls & Chuyate, 2000). The method described by Walls & Chuyate (2000) using K-agar is widely used in the United States of America (USA) to test fruit juices for the presence of *A. acidoterrestris*, however many contaminated products are still not detected, stressing the call for a more sensitive method for the isolation of *Alicyclobacillus* spp. from fruit juices and fruit juice products (Chang & Kang, 2004).

Various researchers have used PDA for the isolation of *Alicyclobacillus* spp. (Splittstoesser *et al*., 1994; McIntyre *et al*., 1995; Pettipher *et al*., 1997; Splittstoesser *et al*., 1998; Komitopoulou *et al*., 1999;). McIntyre *et al.* (1995) reported the detection of *Alicyclobacillus* on PDA after 24 h of incubation at 36˚C and increased growth was observed when the incubation temperature was raised to 50˚ and 55˚C. Splittstoesser *et al*. (1998) recommended PDA as a good selective media for *Alicyclobacillus* spp. at a pH of 3.5, and incubation temperatures of 43˚ or 53˚C. In the USA, PDA was also used by the National Food Processors Association (NFPA) in a survey done to evaluate the extent of spoilage of fruit juice products by acidophilic endospore-forming bacteria (Walls & Chuyate, 1998).

A comparison of the various media used for the isolation of *Alicyclobacillus* spp. is necessary, since uncertainty exists about which media is most effective for the isolation of *Alicyclobacillus* spp. from fruit juices and fruit juice products (Wisotzkey *et al*., 1992; Yamazaki *et al*., 1996; Pettipher *et al*., 1997; Walls & Chuyate, 2000). The aim of this study was to evaluate growth and recovery of *A. acidoterrestris*, *A. acidocaldarius* and *A. pomorum* on PDA, OSA, YSG-agar, BAM, and K-agar from sterile saline solution (SSS), and pasteurised diluted and undiluted fruit juice concentrate, using different incubation temperatures and pH values.

Materials and methods

Strains

Isolates of *A. acidoterrestris* SA01 and *A. acidocaldarius* PM02 was obtained from the Food Microbiology Research Group Culture Collection (FMRGCC) at the University of the Western Cape. The isolates were cultivated on PDA (Oxoid) at pH 3.7 and incubated at 55˚C for 3 days. Isolates of *A. acidoterrestris* K13, *A. acidoterrestris* K47 and *A. pomorum* K20 were obtained from fruit juice

manufacturers in the Western Cape, South Africa and previously identified using 16S ribosomal RNA gene sequence analysis (data not shown).

All isolates were grown in YSG-broth (2 $g.l^{-1}$ yeast extract (Merck), 1 $g.l^{-1}$ D-glucose (Merck), 2 $g.l^{-1}$ soluble starch (Merck)), acidified with 2 N H_2SO_4 to pH 3.7. After 3 days of growth at 50˚C, the cells were centrifuged at 5 000 g for 6 min with a Beckman Coulter TJ-25 Centrifuge (Beckman Coulter, South Africa). The pellet was resuspended in 9 ml sterile saline solution (SSS) (0.85% (m/v) NaCl (Merck)) and the optical density (OD) was measured at 540 nm using a Beckman Coulter DU 530 Life Science UV/Vis Spectrophotometer (Beckman Coulter, South Africa). The data from a standard curve was used to determine the cell concentration used for the inoculum of the SSS and the fruit juices.

Recovery of *Alicyclobacillus* spp. on potato dextrose agar and determination of the detection limit

A detection limit for vegetative cells of *A. acidoterrestris* SA01 and *A. acidocaldarius* PM02 was determined from cells diluted in SSS and plated on PDA (Merck), pH 3.7 (pH adjusted using 1M HCl) (Pettipher *et al.,* 1997; Splittstoesser *et al.,* 1998; Orr & Beuchat, 2000). Cells were grown in YSG-broth at pH 3.7 and growth was limited to 3 days, to ensure vegetative cells are still present. Cells were centrifuged at 5 000 g for 6 min. The pellet was resuspended in 9 ml SSS and dilutions (1:1, 1:2, 1:3, 1:4, 1:5, 1:7, 1:9, 1:11, 1:13 and 1:15) were made from a concentration of 1.24 x 10^6 $cfu.ml^{-1}$ for *A. acidoterrestris* cells and from a concentration of 5.33 x 10^7 $cfu.ml^{-1}$ for *A. acidocaldarius*. The optical density (OD) was measured at 540 nm, using a Beckman Coulter DU 530 Life Science UV/Vis Spectrophotometer (Beckman Coulter, South Africa). A serial dilution (10^{-1} to 10^{-6}) was made of each dilution in SSS and spread plated in duplicate, on PDA, pH 3.7. To avoid clamping of the cells, samples were vortexed briskly while preparing the dilution series. The plates were incubated at 50˚C for 5 days and counted on days 3 and 5. The experiment was done in duplicate.

Recovery of *Alicyclobacillus acidoterrestris* from fruit juice and fruit juice concentrate on five different media

Cells of *A. acidoterrestris* SA01 were grown for 3 days at 50˚C in YSG-broth, pH 3.7 and 3.85 x 10^6 $cfu.ml^{-1}$ was inoculated into diluted (1:4) and undiluted pear juice

concentrate (68.5˚Brix) and a dilution series (10^{-1} to 10^{-6}) was made. A concentration of 3.0 x 10^6 cfu.ml^{-1} cells were also inoculated in SSS and serially diluted, serving as the control. Duplicate spread plates of the dilution series of vegetative cells of *A. acidoterrestris* were made on PDA, pH 3.7 (Pettipher *et al.,* 1997; Splittstoesser *et al.,* 1998; Orr & Beuchat, 2000); OSA (Oxoid), pH 5.5 (Pettipher *et al.,* 1997; Walls & Chuyate, 1998; Jensen, 1999); YSG-agar (2 g.l^{-1} yeast extract (Merck), 1 g.l^{-1} glucose (Merck) and 2 g.l^{-1} soluble starch (Merck), 14 g.l^{-1} bacteriological agar (Merck)), pH 3.7 (Silva & Gibbs, 2001); K-agar (2.5 g.l^{-1} yeast extract (Merck), 5 g.l^{-1} peptone (Merck), 1 g.l^{-1} glucose (Merck), 1 g.l^{-1} Tween 80 (Merck), 14 g.l^{-1} bacteriological agar (Merck)), pH 3.7 (Walls & Chuyate, 1998; Wisse & Parish, 1998; Orr & Beuchat, 2000) and BAM (5 g.l^{-1} yeast extract (Merck), 5 g.l^{-1} proteose peptone (Merck), 5 g.l^{-1} glucose (Merck), 4 g.l^{-1} dipotassium phosphate (Merck), 20 g.l^{-1} bacteriological agar (Merck)), pH 5.0, as was used in the standard International Federation of Fruit Juice Producers (IFU) procedure (Wisotzkey *et al.,* 1992; Silva *et al.,* 1999). The plates were incubated at 50˚C for 5 days and counted on days 3 and 5.

Effect of incubation temperature and media pH

The effect of two different incubation temperatures (43˚ and 50˚C) was evaluated for the isolation of *A. acidoterrestris* SA01 from fruit juice. Cells were grown in YSG-broth, pH 3.7 at 50˚C for 5 to 7 days, to ensure spores are present. A concentration of 2.91 x 10^6 cfu.ml^{-1} was inoculated into pear juice. A dilution series (10^{-1} to 10^{-6}) of the inoculated pear juice was prepared in SSS and each dilution spread plated, in duplicate, on PDA (pH 3.7 and 5.6) and OSA (pH 3.7 and 5.5). The experiment was done in triplicate. One set of plates were incubated at 43˚C, while the other set was incubated at 50˚C for 5 days. The same dilution series was submitted to a heat shock treatment at 80˚C for 10 min to activate endospores and plated on PDA (pH 3.7 and 5.6) and OSA (pH 3.7 and 5.5) and incubated at 43˚ and 50˚C for 5 days. The plates were counted on days 3 and 5.

Recovery of *Alicyclobacillus* spp. from contaminated white grape juice concentrate

Three spoilt white grape juice concentrates, contaminated with unknown concentrations of either *A. acidoterrestris* K13, *A. acidoterrestris* K47 or *A. pomorum*

K20. A dilution series (10^{-1} to 10^{-6}) of the three samples were made in SSS and were duplicate spread plated on PDA, pH 3.7 and OSA, pH 5.5. Each dilution series was also heat treated at 80˚C for 10 min to activate endospores and to eliminate vegetative cells and the serial dilution was spread plated on PDA and OSA. All the plates were incubated at 50˚C for 5 days and counted on days 3 and 5.

Recovery of *A. pomorum* on different isolation media

Alicyclobacillus pomorum (K20) cells were grown in YSG-broth to a concentration of 4.1 x 10^5 cfu.ml^{-1} and inoculated into single strength white grape juice concentrate. Duplicate spread plates of the dilution series (10^{-1} to 10^{-6}) of vegetative cells of *A. pomorum* were made on PDA, pH 3.7; OSA, pH 5.5; YSG-agar, pH 3.7; K-agar, pH 3.7 and BAM, pH 5.0. The plates were incubated at 50˚C for 5 days and counted on days 3 and 5.

Statistical analysis

A repeated measures analysis of variance (ANOVA) was done using Satistica™ 7.1 for Windows™, the source of variance being the recovery obtained for *Alicyclobacillus* spp. on different isolation media at different pH values and incubation temperatures. Mean values were considered significantly different at $p < 0.05$. Where interactions were non-significant, main effects were interpreted directly and if the samples differed significantly, a Bonferroni- and a Bootstrap multiple comparisons procedure was used to determine which samples differed.

Results and discussion

Recovery of *Alicyclobacillus* spp. on potato dextrose agar and determination of the detection limit

The main objective was to assess the recovery of *Alicyclobacillus* spp. on PDA to give an indication of the efficiency and sensitivity of this media as an isolation media and to determine the lowest number of *Alicyclobacillus* cells that can be detected from SSS on PDA. A 100% recovery of an initial inoculum of 1.24 x 10^6 cfu.ml^{-1} of *A. acidoterrestris* cells was obtained on PDA at pH 3.7 for seven of the 10 dilutions (Fig. 1). The detection limit of this culture-dependent method for the recovery of

A. acidoterrestris cells from SSS was 10^4 cfu.ml^{-1}, as no cells were recovered from dilutions containing cells at concentrations less than 10^4 cfu.ml^{-1}. These results are in accordance with results obtained by Pettipher *et al*., (1997) who reported counts of 6.0 x 10^4 cfu.ml^{-1} on spread plates of PDA for the isolation of *A. acidoterrestris* on from inoculated fruit juice.

Recovery of vegetative cells of *A. acidocaldarius* was also high on PDA at pH 3.7 (Fig. 2), with 100% recovery for 5 of the 10 dilutions from an initial inoculum of 5.33 x 10^7 cfu.ml^{-1}. The detection limit of this method for the recovery of *A. acidocaldarius* cells from SSS was 10^5 cfu.ml^{-1}, as no cells were recovered from dilutions containing cells at concentrations less than 10^5 cfu.ml^{-1}. The higher detection limit obtained for *A. acidocaldarius* on PDA compared to that for *A. acidoterrestris* might indicate that PDA is a better isolation media for *A. acidoterrestris* than for *A. acidocaldarius*.

The incubation temperature used for the cultivation of the cells was increased from 43˚ to 50˚C (data not shown) as this temperature is within the optimum growth range of both microbes. *Alicyclobacillus acidoterrestris* has an optimum growth temperature of between 35˚ and 55˚C, while *A. acidocaldarius* has a higher optimum growth temperature of between 45˚ and 70˚C (Cerny *et al*., 1984; Deinhard *et al*., 1987). The higher inoculum concentration of *A. acidocaldarius* after 3 days of growth in YSG-broth might be because the incubation temperature of 50˚C is closer to the optimum growth temperature of *A. acidocaldarius*. No heat treatment was needed to activate the endospores as the cells were only grown for 3 days and was therefore still in the vegetative state.

These results indicate that PDA, pH 3.7, used at an incubation temperature of 50˚C for a minimum of 3 days incubation, is a suitable isolation medium to use for growth and enumeration of vegetative cells of *A. acidoterrestris* from SSS at concentrations above 10^4 cfu.ml^{-1} and *A. acidocaldarius* from SSS at concentrations above 10^5 cfu.ml^{-1}.

Recovery of *Alicyclobacillus acidoterrestris* from fruit juice and fruit juice concentrate on five different media

The number of microbial cells inoculated and recovered from SSS on each of the different media (control) and the amount inoculated and recovered from diluted pear juice concentrate are represented in Figure 3. The highest recovery of vegetative

cells of *A. acidoterrestris* was obtained on PDA, pH 3.7, incubated at 50˚C for 5 days. After inoculation of 3.85 x 10^6 cfu.ml^{-1} in diluted pear juice concentrate, recovery on PDA was 2.85 x 10^6 cfu.ml^{-1} (74% recovery) and on OSA it was 2.52 x 10^6 cfu.ml^{-1} (65% recovery). Statistical analysis of the data for PDA and OSA resulted in a p-value of 0.64, showing either of the media can be used as there is not a significant difference between isolation of *A. acidoterrestris* from single strength pear juice concentrate on PDA or OSA. No recovery of *A. acidoterrestris* from inoculated pear juice was obtained on K-agar, BAM and YSG-agar (Fig. 3).

Recovery of *A. acidoterrestris* from undiluted pear juice concentrate was also assessed on the five different media (Fig. 4). Vegetative cells at an average concentration of 3.85 x 10^6 cfu.ml^{-1} was inoculated into pear juice concentrate and recovery was the highest on PDA, pH 3.7, with an average recovery of 8.80 x 10^4 cfu.ml^{-1} (2.3% recovery) and on OSA with an average recovery of 3.81 x 10^3 cfu.ml^{-1} (0.1% recovery) (Fig. 4). No growth was obtained on K-agar, BAM or YSG-agar.

As expected, recovery of vegetative cells of *A. acidoterrestris* was lower from fruit juice and fruit juice concentrate than from SSS, because of other compounds present in the fruit juice and concentrate that might interfere with the isolation procedure. During this study it was found that the isolation of *Alicyclobacillus* endospores was more difficult from concentrates, with a higher soluble solid content, than from single strength juice. This is in accordance with Baumgart *et al*. (1997) who reported that the soluble solid content of fruit juice influences the heat resistance of the endospores and proved that it was more difficult to destroy endospores in concentrates than in single strength juice. These findings make it more difficult to identify a standard isolation media as recovery varies, depending on

whether the sample is from single strength juice or from concentrate and on the different pre-treatments, such as dilution, filtration or heat shocking, that the samples are subjected to before analysis. The soluble solid content of the sample to be tested is therefore an important factor to consider when developing a detection method for the isolation of *Alicyclobacillus* spp. from fruit juice and fruit juice products.

Although recovery was low, the growth media of preference for the isolation of *Alicyclobacillus* spp. cells from fruit juice concentrate was PDA at pH 3.7 at an incubation temperature of 50˚C for 5 days, compared to OSA, BAM, YSG and K-agar ($p = 0.002$) (Fig. 4). Research showed BAM and K-agar to give better recovery when

membrane filtration was included (Silva *et al*., 1999; Chang & Kang, 2004) and recovery on these media might also be influenced by different incubation temperatures and media pH values. The main focus of this research however, was to find a quick, simple and sensitive method for the isolation of *A. acidoterrestris* from fruit juice and fruit juice concentrate for the fruit juice industry. PDA and OSA were identified as the media conforming the best to these requirements.

Effect of incubation temperature and media pH

Various incubation temperatures for the isolation of *Alicyclobacillus* spp. are suggested in the literature (Splittstoesser *et al*., 1994; Pettipher *et al*., 1997; Walls & Chuyate, 1998; Jensen, 1999; Chang & Kang, 2004). In this study, two incubation temperatures (43˚ and 50˚C) were evaluated for the recovery of *A. acidoterrestris* from fruit juices and the results are depicted in Figure 5.

Recovery before a heat shock treatment on PDA, pH 3.7 and 5.6 and OSA, pH 3.7 and 5.5 was included as a control and showed vegetative cells to still be present after 7 days of growth at 50˚C (Fig. 5). Results also indicated that the heat shock treatment did not have a significant effect on recovery of *A. acidoterrestris* SA01 endospores on PDA, while recovery was significantly different before and after a heat shock treatment on OSA ($p = 0.040$).

Recovery of *A. acidoterrestris* endospores was highest on OSA at pH 5.5 and incubation at 50˚C for 3 days, after a heat shock treatment of the samples to activate endospores. The media pH significantly influenced recovery at 50˚C after a heat shock treatment, with recovery of 2.81×10^6 cfu.ml^{-1} from an initial inoculum of 2.91×10^6 cfu.ml^{-1} (97% recovery), compared to recovery at pH 3.7 (76% recovery) ($p = 0.006$). Recovery on OSA was higher after a heat shock treatment at 50˚C for both pH 3.7 and 5.5 than recovery after a heat shock treatment at 43˚C (70 % and 73% recovery, respectively). Statistical analysis showed that the difference was only significant at an incubation temperature of 50˚C ($p = 0.001$), indicating that recovery of endospores of *A. acidoterrestris* was significantly higher on plates of OSA, pH 5.5 incubated at 50˚C than at 43˚C, with inclusion of a heat shock treatment (Fig. 5).

The highest recovery on PDA for endospores of *A. acidoterrestris* from pear juice was obtained at a media pH of 3.7 and an incubation temperature of 50˚C (83% recovery) after a heat shock treatment (Fig. 5). Recovery of *A. acidoterrestris* endospores on PDA at pH of 3.7 was higher at 50˚C than at pH 5.6 (83% and 77%

recovery, respectively), but recovery did not differ significantly (p = 0.35). Recovery at 43˚C did also not differ significantly at the two pH values (p = 0.19).

Differences between recovery on PDA and OSA showed to be significant at the two incubation temperatures used (p = 0.04), but the pH did not have a significant influence on recovery of *Alicyclobacillus* endospores on the two media (p = 0.25). With the inclusion of all three variables, namely the media, incubation temperature and media pH in the analysis, it was found that recovery of endospores of *Alicyclobacillus* from fruit juice was significantly influenced by the interactions of these factors (p = 0.003), but mostly by the incubation temperature (p = 0.007). The highest recovery obtained on OSA, pH 5.5 (97% recovery) differed significantly from the highest recovery obtained on PDA, pH 3.7 (83% recovery) (p = 0.021), both at an incubation temperature of 50˚C for 3 days.

Fruit juice concentrate has a high soluble solid content, therefore *A. acidoterrestris* is mostly present in the form of endospores in fruit juice concentrate. A heat shock treatment is thus essential to activate the endospores when testing products that encourage endospore-forming, such as fruit juice concentrate. These results lead to the recommendation that the most effective isolation media to test for the presence of *A. acidoterrestris* endospores should include a heat shock treatment at 80˚C for 10 min, followed by plating the samples on OSA at pH 5.5 and incubating at 50˚C for 3 days.

Recovery of *Alicyclobacillus* spp. from contaminated white grape juice concentrate

The dilution series made of each of the three contaminated white grape juice concentrate samples were spread plated on PDA, pH 3.7 and OSA, pH 5.5 as these two media were found to give the highest enumeration results of *Alicyclobacillus* cells from fruit juice and fruit juice concentrate. The white grape juice samples were analysed for the presence of *Alicyclobacillus* spp. and the results are depicted in Figure 6. Recovery of *A. acidoterrestris* K13 and *A. acidoterrestris* K47 from white grape juice concentrate was the highest on PDA, pH 3.7 where 8.0×10^5 cfu.ml^{-1} and 4.52×10^5 cfu.ml^{-1} were recovered from the two samples, respectively (Fig. 6). Recovery of *A. pomorum* K20 from white grape juice concentrate was also the highest on PDA, pH 3.7 where 2.64×10^5 cfu.ml^{-1} cells were recovered.

The amount of organism recovered on PDA was included as the control in Figure 6 (the first column of each group of three), to enable comparison of the highest value obtained for the recovery of the *Alicyclobacillus* spp., as the original level of contamination was unknown. On OSA, pH 5.5, recovery was higher after the heat shock treatment than before. Recovery of 1.75 x 10^5 cfu.ml^{-1} (22% recovery) for *A. acidoterrestris* K13 and 1.17 x 10^4 cfu.ml^{-1} (2.6% recovery) for *A. acidoterrestris* K47, was still lower than when PDA was used. Recovery for *A. pomorum* K20 on OSA was 9.0 x 10^3 cfu.ml^{-1} (3.4% recovery).

The highest enumeration results for the recovery of *A. acidoterrestris* and *A. pomorum* from white grape juice concentrate was obtained on PDA at pH 3.7, with recovery being significantly lower on OSA than on PDA for *A. acidoterrestris* K20 ($p = 0.006$) and *A. acidoterrestris* K47 ($p = 0.008$). Recovery before a heat shock treatment on PDA was significantly different from recovery on OSA for all three samples ($p = 0.000$). From these results it could be seen that the best media for recovery of *A. acidoterrestris* (K13 and K47) and *A. pomorum* (K20) was PDA, pH 3.7 at an incubation temperature of 50˚C for 3 days (Fig. 6).

Recovery of *Alicyclobacillus pomorum* on different isolation media

The growth of *A. pomorum* (K20) from fruit juice on different isolation media has, to date not been assessed. The recovery of this species on PDA, OSA, K-agar, YSG-agar and BAM was evaluated, as this species has also been found to be present in fruit juice and may lead to spoilage of the fruit juice. The highest recovery was obtained on PDA, pH 3.7, where 2.6 x 10^5 cfu.ml^{-1} was recovered from an initial inoculum of 2.65 x 10^5 cfu.ml^{-1} (98% recovery) and the same results were obtained with or without a heat shock treatment at 80˚C for 10 min (Fig. 7).

Recovery on OSA was 6.9 x 10^4 cfu.ml^{-1} (30% recovery) and was significantly better before the samples were subjected to a heat treatment ($p = 0.015$). No recovery was obtained on BAM, either before or after a heat shock treatment, while there was growth on K-agar and YSG before a heat shock treatment, however, at very low concentrations of 1 x 10^3 cfu.ml^{-1} and 8 x 10^3 cfu.ml^{-1}, respectively. Recovery on YSG after a heat shock treatment was also very low, at 1 x 10^3 cfu.ml^{-1}. These results indicate that PDA, pH 3.7 is the best isolation media to use for the isolation of *A. pomorum* from pasteurised white grape juice

concentrate, as recovery on this media differed significantly from recovery on the other four media ($p = 0.000$).

Conclusions

Microbiological enumeration procedures are known to give an underestimation of the true microbial community, as all the micro-organisms are not recovered (Swanson *et al.,* 1992). From the results of this study it can be concluded that PDA, pH 3.7 and OSA, pH 5.5 at an incubation temperature of 50˚C for 3 days, are the best isolation and identification media to use for the detection and isolation of *Alicyclobacillus* spp. from various pasteurised fruit juices and fruit juice concentrates. The inclusion of a heat shock treatment at 80˚C for 10 min is beneficial if endospores are mainly present in the product being tested and the isolation media used is OSA, pH 5.5 at 50˚C. Results showed the growth characteristics of different species of *Alicyclobacillus* to differ and that different species can occur in fruit juice and fruit juice concentrates, either alone or simultaneously, and lead to spoilage. This emphasises the fact that research should not just focus on *A. acidoterrestris*, as other species have also been isolated from spoilt fruit juice. This culture-dependent isolation method is not sensitive enough to use on its own as an indication of contamination of fruit juice and fruit juice concentrates and the combination of this method with a culture-independent method, such as PCR-based DGGE analysis is recommended.

References

Baumgart, J., Husemann, M. & Schmidt, C. (1997). *Alicyclobacillus acidoterrestris*: occurrence, significance and detection in beverages and beverage base. *Flussiges Obst*, **64**, 178.

Cerny, G., Hennlich, W. & Poralla, K. (1984). Fruchtsaftverderb durch *Bacillen*: isolierung und charakterisierung des verderbserrengers. *Zeitschrift feur Lebensmittel-Untersuchung und -Forsuchung*, **179**, 224-227.

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

Connor, C.J., Hongliang, L., McSpadden, B.B. & Wang, H.H. (2004). Development of a real-time PCR-based system targeting the 16S rRNA gene sequence for rapid detection of *Alicyclobacillus* spp. in juice products. *International Journal of Food Microbiology*, **99**, 229-235.

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

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

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

Farrand, S.G., Linton, J.D., Stephenson, R.J. & MacCarthy, W.V. (1983). The use of response surface analysis to study growth of *Bacillus acidocaldarius* throughout the growth range of temperature and pH. *Archives of Microbiology*, **135**, 272-275.

Goto, K., Moshida, 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 omega-alicyclic fatty acids, and emended description of the genus *Alicyclobacillus. International Journal of Systematic and Evolutionary Microbiology*, **53**, 1537-1544.

Gouws, P.A., Gie, L., Pretorius, A. & Dhansay, N. (2005). Isolation and identification of *Alicyclobacillus acidocaldarius* by 16S rDNA from mango juice and concentrate. *International Journal of Food Science and Technology*, **40**, 789-792.

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

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.

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.

Matsubara, H., Goto, K., Matsumura, T., Mochida, K., Iwaki, M., Niwa M. & Yamasoato, 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.

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

Murakami, M., Tedzuka, H. & Yamazaki, K. (1998). Thermal resistance of *Alicyclobacillus acidoterrestris* spores in different buffers and pH. *Food Microbiology*, **15**, 577.

Orr, R.V. & Beuchat, L.R. (2000). Efficacy 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.

Pettipher, G.L. (2000). *Alicyclobacillus* spp., their detection and control in fruit juice. *Soft Drinks International*, 31-32.

Pettipher, G.L., Osmundsen, 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.

Pettipher, G.L. & Osmundsen, M.E. (2000). Methods for the detection, enumeration and identification of *Alicyclobacillus acidoterrestris. Food Australia,* **57**, 293.

Silva, F.V.M., 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.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*, **10**, 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.

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.

Swanson, K.M.J., Busta, F.F., Peterson, E.H. & Johnson, M.G. (1992). Colony count methods. In: *Compendium of Methods for the Microbiological Examination of Foods* (edited by C. Vanderzant & D. F. Splittstoesser), Pp. 75-95. Ann Arbour: Edwards Brothers.

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

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

Walls, I. & Chuyate, R. (2000). Isolation of *Alicyclobacillus acidoterrestris* from fruit juices. *Journal of AOAC International*, **83**, 1115-1120.

Wisotzkey, J.D., Jurtshuk, P., Fox, G.E., Deinhart, 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**, 504-409.

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

CHAPTER 4

PCR-BASED DGGE IDENTIFICATION OF THERMOPHILIC ACIDOPHILIC BACTERIA (TAB) IN PASTEURISED SOUTH AFRICAN FRUIT JUICES AND CONCENTRATES

Abstract

Contamination of pasteurised fruit juice and fruit juice products by thermophilic acidophilic bacteria (TAB) has become a major concern to producers, as the presence of these bacteria might lead to spoilage and economic losses. The aim of this study was to identify the micro-organisms present in fruit juices and concentrates from different South African manufacturers before and after pasteurisation, using polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) and DNA sequencing. The sequence data obtained for the prominent DGGE bands were compared to 16S ribosomal RNA (rRNA) gene sequences in GenBank using the BLASTn search option. *Alicyclobacillus acidoterrestris* was found to be present in South African apple, pear, white grape and aloe vera juices. White grape juice was also found to contain *Alicyclobacillus pomorum*. Other organisms present in the orange, apple, mango and pear juices were two uncultured bacteria that were identified as members of the genus *Bacillus*, and one uncultured bacteria closely related to *Alcaligenus faecalis*. The results confirmed the presence of TAB in pasteurised South African fruit juices and concentrates and emphasises the need for the rapid and accurate detection of TAB in South African food products.

Introduction

Pasteurisation temperatures of between 85˚ and 95˚C and the acidic nature of fruit juices (with a pH below 4.6) has until recently been considered sufficient to prevent spoilage of fruit juices and fruit juice products by bacteria and fungi (Splittstoesser *et al*., 1994; Walls & Chuyate, 1998; Silva *et al*., 1999). Cerny *et al.* (1984) reported the first spoilage case of commercially available pasteurised fruit juice and found shelf-stable, aseptically packaged apple juice to have an off-flavour, attributed to flavour compounds formed by thermophilic acidophilic bacteria (TAB). Spoilage cases of a number of acidic vegetables, wines, fruit juices and fruit juice products due to

contamination by *Alicyclobacillus acidoterrestris* has also been reported (Splittstoesser *et al*., 1994; Baumgart *et al*., 1997; Pontius *et al*., 1998; Komitopoulou *et al*., 1999; Chang & Kang, 2004; Gouws *et al*., 2005; Walker & Philips, 2005).

Members of the genus *Alicyclobacillus* are rod-shaped, Gram-positive, soil-borne micro-organisms (Deinhard *et al*., 1987; Walls & Chuyate, 1998). These species have been shown to survive pasteurisation conditions of 95°C for 2 min and it grows at a pH range of between 2.5 to 6.0 and at temperatures between 25° to 60°C (Deinhard *et al*., 1987; Jensen, 1999; Silva *et al*., 1999). The unique characteristic of *Alicyclobacillus* spp. is the presence of ω-alicyclic fatty acids, such as ω-cyclohexane or ω-cycloheptane, as the major components of the cellular membrane (Silva & Gibbs, 2001; Goto *et al*., 2002).

Contamination of fruit juices by *Alicyclobacillus* spp. results in an off-flavour, a light sediment and no gas production (Splittstoesser *et al*., 1994; Yamazaki *et al*., 1996). Spoilage by TAB, which can occur at any point during the process, may therefore alter the taste, colour and/or odour of the products (Jensen & Whitfield, 2003; Chang & Kang, 2004; Walker & Phillips, 2005). *Alicyclobacillus acidoterrestris* causes a sour type spoilage and produces the halophenols 2,6-dichlorophenol (2,6-DCP) and 2,6-dibromophenol (2,6-DBP) that has a medicinal and disinfectant-like smell. The flavour compound guaiacol can also be produced in contaminated juices, leading to a smoky, phenolic-like aroma (Jensen, 1999; Jensen & Whitfield, 2003).

Molecular techniques, such as PCR-based DGGE analysis are useful for the identification of the microbial communities and the detection and identification of micro-organisms present in food products (Muyzer & Smalla, 1998; Gonzalez *et al*., 2003; Cocolin *et al*., 2004; Ercolini, 2004). These culture-independent techniques may give a better representation of the microbial content compared to culture-dependent approaches, such as growth on selective culture media. The preparation of the media is labour intensive and all micro-organisms can not be isolated and cultured on growth media (Ampe *et al*., 1999; Cocolin *et al*., 2000; Lee *et al*., 2005). The aim of this study was to identify the different micro-organisms present in South African fruit juices and concentrates before and after pasteurisation, using PCR-based DGGE analysis.

Materials and methods

Isolates, fruit juices and concentrates

Pure isolates of *A. acidoterrestris* SA01 and *A. acidocaldarius* PM02 were obtained from the Food Microbiology Research Group Culture Collection (FMRGCC) at the University of the Western Cape, which served as the reference strains during the PCR-based DGGE analysis of the fruit juice samples. The pure isolates were cultivated on potato dextrose agar (PDA) (Oxoid) at pH 3.7 and incubated at 55˚C for 3 days.

Four orange, six apple, five pear, three white grape and one aloe vera juice samples were obtained from manufacturers in the Western Cape, South Africa. Four mango juice samples were obtained from manufacturers in the Limpopo Province, South Africa. The fruit juice samples were taken directly after maceration the fruit, after evaporation of the clear, unpasteurised juices and of the final product after pasteurisation.

DNA extraction and PCR-based DGGE analysis

The DNA was extracted and purified from the pure isolates and the fruit juice samples using the Wizard Genomic DNA Isolation Kit (Promega). For the isolates, colonies from the PDA plates were suspended in 2 ml of sterile saline solution (SSS) (0.85% (m/v) NaCl) and used for DNA isolation. For the samples from the fruit juice or fruit juice concentrate, 2 ml was used for DNA isolation.

The amplification of the V3 variable region of the 16S ribosomal RNA (rRNA) gene was performed using an Eppendorf Mastercycler Personal (Merck) using the primers R534 (5'-ATT ACC GCG GCT GCT GG-3') and F341 (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGC AGC AG -3') (GC-clamp underlined) (Muyzer *et al*., 1993), yielding 200 base pair (bp) fragments. Each PCR reaction was performed in a total of 50 µl, containing 5 µl of the template DNA, 1 µl of each primer (0.5 µM), 1 U *Taq* polymerase (ABGene, Southern Cross Biotechnology), 2 µl deoxyribonucleoside triphosphate (dNTPs) (200 µM) (Promega), 5 µl of the 10 x PCR buffer (ABGene) and 3 µl $MgCl_2$ (2.5 mM) (ABGene, Southern Cross Biotechnology).

All the PCR amplifications were initiated at 94˚C for 5 min. The samples were heated to 94˚C for 30 s for denaturation, followed by annealing at 54˚C for 1 min, while primer extension was performed at 72˚C for 1 min. These three steps were repeated for 30 cycles. A final elongation step at 72˚C for 5 min was included and the samples were cooled to 4˚C. PCR products were separated on a 1.2% (m/v) agarose gel (Whitehead Scientific), containing ethidium bromide and visualised under UV light (Vilber Lourmat).

The 200 bp PCR fragments were separated using the Bio-Rad DCode™ Universal Mutation Detection System (BioRad Laboratories). DGGE analysis was done by directly applying the PCR products onto 8% (m/v) polyacrylamide gels with a gradient of between 30 and 70%, created by 0 to 100% denaturant, with the 100% denaturant consisting of 7 M urea (Merck) and 40% (v/v) formamide (Saarchem). Electrophoresis was performed at a constant voltage of 130 V for 5 h at 60°C, followed by visualisation under UV light (Vilber Lourmat).

DNA sequencing

The DGGE bands were punched from the gels and directly re-amplified using the primers R534 and F341 (without the GC-clamp) (Muyzer *et al*., 1993). Extracted DNA from fruit juices, containing only one DGGE band and therefore only one species, was also amplified using the primers R1512 (5'- GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT -3') and F8 (5'- CAC GGA TCC AGA CTT TGA TYM TGG CTC AG -3') (Felske *et al*., 1997) producing 1500 bp fragments. The PCR amplification conditions and reaction volumes were the same as previously described.

All the PCR products were purified using the Sigma Spin Post-Reaction Purification Columns (Sigma Aldrich) as specified by the manufacturer. The PCR fragments were sequenced using the ABI PRISM 377 DNA Sequencer (Perkin Elmer) at the DNA Sequencing Facility at Stellenbosch University. The sequences obtained were compared to 16S rRNA gene sequences in the GenBank database using the BLASTn search option to determine the closest known relatives (Altschul *et al*., 1997).

DGGE detection limit

The lowest number of cells of *A. acidoterrestris* SA01 in fruit juice concentrates that could be detected using PCR-based DGGE was determined. Cells were grown to a concentration of 3.6 x 10^6 colony forming units per ml (cfu.ml^{-1}) in yeast-starch-glucose broth (YSG-broth) (2 g.l^{-1} yeast extract (Merck), 1 g.l^{-1} D-glucose (Merck) and 2 g.l^{-1} soluble starch (Merck)) (Uchino & Doi, 1967; Goto *et al*., 2002) with the pH adjusted to 3.7 using 2 N H_2SO_4. The mixture was incubated at 50˚C for 3 days. The optical density (OD) was measured (540 nm) with a Beckman Coulter DU 530 Life Science UV/Vis Spectrophotometer (Beckman Coulter) to determine the concentration of the cells. A dilution series (10^{-1} to 10^{-6}) was made of the inoculated fruit juice, DNA was extracted from 2 ml of each dilution and subjected to PCR amplification using the primers F341 and R534 (Muyzer *et al*., 1993). These fragments were separated using DGGE as described.

Results and discussion

PCR-based DGGE detection limit of *Alicyclobacillus acidoterrestris*

The lowest number of cells of *A. acidoterrestris* SA01 present in fruit juice concentrate that could be detected using PCR-based DGGE was at a concentration of 1.9 x 10^3 cfu.ml^{-1}. From inoculated single strength fruit juice, the lowest number of cells of *A. acidoterrestris* that could be detected was 2.3 x 10^3 cfu.ml^{-1}. Repeated detection limit determinations confirmed these results.

The detection of low concentrations of *A. acidoterrestris* is necessary as a concentration of only 10^3 to 10^4 cfu.ml^{-1} of *A. acidoterrestris* cells can lead to the formation of taint chemicals (Pettipher *et al*., 1997; Jensen & Whitfield, 2003). PCR-based DGGE analysis can therefore detect TAB at lower concentrations than required for spoilage. This method is also more sensitive than the culture-dependent method, with a detection limit of 10^4 cfu.ml^{-1} (Pettipher *et al*., 1997; Gouws *et al*., 2005). The taste threshold of guaiacol is reportedly 2 parts per billion (ppb), while the halophenols 2,6-DCP and 2,6-DBP have a taste threshold of 0.5 ng.l^{-1} and 30 ng.l^{-1}, respectively (Jensen & Whitfield, 2003). These taste thresholds are reached within 6 days at room temperature, or shorter times when the temperature of incubation is higher, therefore rapid detection methods are of great value to prevent the spoilage of fruit juices (Gouws *et al*., 2005). Furthermore, this

culture-independent method offers a rapid detection of spoilage organisms in fruit juices, with results obtained within 2 days, compared to the 3 to 7 days needed for the incubation of selective media (Splittstoesser *et al*., 1994; Pettipher *et al*., 1997; Walls & Chuyate, 1998; Jensen, 1999).

DGGE analysis

White grape juice

Four pasteurised, contaminated white grape juice concentrate samples were analysed using PCR-based DGGE and the fingerprints are represented in Figure 1. Bands a, b and d present in all 3 samples after pasteurisation and corresponded to the position of the band representing *A. acidoterrestris* SA01. The profiles indicated that the white grape juice was spoilt by *A. acidoterrestris*, surviving the pasteurisation temperature of 95˚C for 30 s. This was confirmed by isolating the bands, followed by DNA sequence analysis. The spoilage organism represented by the DGGE band b (96% homology, 770 out of 801 bases) and band d (94% homology, 575 out of 608 bases) was identified as *A. acidoterrestris* (GenBank Accession number AB042058). This thermophilic bacteria has previously been isolated from spoilt fruit juices, including white grape juice (Cerny *et al*., 1984; Splittstoesser *et al*., 1994; Yamazaki *et al*., 1996). It is a soil-borne organism, which has also been isolated from water. Contamination usually occurs due to soil entering the processing line on the surface of the fruits (Uchino & Doi, 1967; Darland & Brock, 1971; Cerny *et al*., 1984; Deinhard *et al*., 1987).

Band c was at a different position on the gel than the other bands, which indicated that a different species was present in this sample. The spoilage organism represented by band c (Fig. 1) was identified as *Alicyclobacillus pomorum* (98% homology, 765 out of 778 bases) (GenBank Accession number AB089840). *Alicyclobacillus pomorum* has previously been isolated from a spoilt mixed fruit juice (Goto *et al*., 2003). It does not possess the characteristic ω-alicyclic fatty acids in the cell membrane thought to be unique to species from this genus, but was classified within this genus based on phylogenetic analysis of both the 16S rRNA and DNA gyrase B (*gyrB*) genes (Goto *et al*., 2003). The presence of the ω-alicyclic fatty acids in the cell membrane was thought to be one of the main reasons these micro-organisms survive the high temperatures of pasteurisation (Kannenberg *et al*., 1984; Moore *et al*., 1997; Chang & Kang, 2004). The fact that this species also survived

pasteurisation despite the absence of these fatty acids in the membrane, indicates that other factors should also be considered as reasons for the survival of TAB at high temperatures. These results confirmed the presence of TAB, in particular species of *Alicyclobacillus*, in South African white grape juice.

Orange, apple and pear juice

Orange, apple and pear juices sampled before and after pasteurisation were analysed for their microbial content and the DGGE fingerprints are presented in Figure 2. No *Alicyclobacillus* spp. were detected in the orange juice samples, pasteurised at 90˚C for 30 s. Band e present in all three fruit juices (Fig. 2A, B and C) showed a 98% sequence similarity to an uncultured soil bacterium from the genus *Bacillus*, related to *B. megaterium* (165 out of 167 bases identical) (GenBank Accession number AF423224) (Valinsky *et al*., 2002). *Bacillus megaterium* is a Gram-positive, endospore-forming bacteria, isolated from soil (Vary, 1994; Scholle *et al*., 2003). Soil-borne organisms enter the fruit juice production line on the surface of contaminated fruit and survive unfavourable conditions, such as high soluble solids content in fruit juice concentrates or low pH conditions, in the form of endospores. The occurrence of this microbe in fruit juice is, therefore, not surprising, as fruit juice is an acidic growth media (Darland & Brock, 1971; Cerny *et al*., 1984; Deinhard *et al*., 1987; Wisotzkey *et al*., 1992). The presence of *B. megaterium* in fruit juice and its survival of pasteurisation is of concern, since this may lead to spoilage and economic losses.

Band f was also present in all three fruit juices analysed (Fig. 2A, B and C). Sequence data of band f in the orange juice showed 94% sequence similarity to that of an uncultured bacterium from an environmental sample (GenBank Accession number AB184980) found to be closely related to *Alcaligenus faecalis* (158 out of 166 bases) (GenBank Accession number AM048879). Band f in the apple juice showed 98% sequence similarity to *Alcaligenus faecalis* (142 out of 145 bases) (GenBank Accession number AJ550279) and band f in the pear juice showed 97% sequence similarity to an uncultured bacterium from an environmental sample (GenBank Accession number DQ06862.1), also closely related to *Alcaligenus faecalis* (121 out of 124 bases) (GenBank Accession number AM048879). Band g just below band f on the orange juice fingerprint was also sequenced and showed similar results to band f (96% homology, 120 out of 124 bases). *Alcaligenus faecalis* is a Gram-negative, aerobic, mesophilic rod, associated with the psychrotropic

spoilage of raw foods (Anon., 2000). Its presence usually indicates post-process contamination. The presence of this microbe throughout the production line in these samples however, can not be explained, as this Gram-negative organism is mesophilic and can not survive the high temperatures of pasteurisation (Jay, 1998). It may be that the fruit juice was contaminated during sampling, leading to its presence in all the samples.

The apple and pear juice samples represented in Figure 2B and 2C were contaminated, as indicated by band i on the gels. This bacteria was present in the unpasteurised samples and survived pasteurisation, indicating that the apples and pears used was contaminated with a thermophilic bacteria. The sequence data of band i in the apple juice (Fig. 2B) showed 99% sequence similarity to *Alicyclobacillus acidoterrestris* (198 out of 200 bases) (GenBank Accession number AY573797), a strain previously isolated from fruit juice (Connor *et al*., 2004). Band i from the pear juice (Fig. 2C) is also present throughout the processing line and the sequences of the re-amplified PCR products was identified as the thermophilic acidophilic endospore-former *A. acidoterrestris* (100% homology, 192 out of 192 bases). Soil-borne organisms enter the fruit processing plant on the surface of fruits, as some fruits may drop to the ground during harvesting. Cleaning of the fruit before processing may not be effective enough to eliminate all the contamination on the surface of the fruits (Pettipher *et al*., 1997; Chang & Kang, 2004; Gouws *et al*., 2005). Spores of *A. acidoterrestris* are produced at a pH as low as 3.2 and have been shown to survive a temperature of 95˚C for 2.5 min in orange juice (Walls & Chuyate, 1998; Eiora *et al*., 1999; Orr & Beuchat, 2000). Should the conditions become favourable again, for example when the fruit juice concentrate is diluted, the spores germinate and grow and can lead to the formation of taint chemicals, spoiling the juice (Borlinghaus & Engel, 1997; Pettipher *et al*., 1997; Jensen & Whitfield, 2003). *Alicyclobacillus acidoterrestris* spores survived pasteurisation in this case, showing that a temperature of 85˚ for 30 s was insufficient to eliminate contamination and the risk of spoilage of the juices by TAB. This data confirms the presence of TAB in pasteurised South African apple and pear juices.

The fingerprints also revealed the presence of a number of unknown micro-organisms, some of which could survive the pasteurisation temperatures. Band h was only present in the apple juice (Fig. 2B) after pasteurisation and the sequence data showed 96% similarity to that of an uncultured *Lactobacillus* sp. (148 out of 154 bases) (GenBank Accession number DQ028930) related to *L. plantarum* (GenBank

Accession number DQ239699). *Lactobacillus* spp. are mesophilic and can not survive pasteurisation temperatures (Jay, 1998). *Lactobacillus plantarum* is a heterofermentative lactic acid bacterium and can grow at a pH as low as 3.3. It is a common food fermenter and has been found in a variety of environmental habitats (Jay, 1998; De las Rivas *et al*., 2005; Li *et al*., 2005). *Lactobacillus plantarum* has caused the spoilage of many food products, including wine (Jay, 1998) and orange juice, where *L. plantarum* together with other lactic acid bacteria, was found to produce a vinegary to buttermilk off-odour (Hays & Riester, 1952). The elimination of *L. plantarum* is an indication of the quality and the shelf life of many high acid foods (Li *et al*., 2005). As it was present in the final product of the analysed samples, post-process contamination may have occurred. The contamination of the fruit juices with this lactic acid bacteria is of concern as it may lead to the formation of an off-flavour in the juice, leading to spoilage.

Sequence data for band j in the apple juice (Fig. 2B) showed 98% similarity to an uncultured *Acetobacteraceae* bacterium (142 out of 145 bases) (GenBank Accession number AY225457), related to *Acetobacter pasteurianus* (GenBank Accession number AB117968). This organism did not survive pasteurisation, as it is not present in the pasteurised samples. Acetic acid bacteria are Gram-negative, aerobic bacteria and acid tolerant bacteria, that can grow well at low pH conditions (Jay, 1998; Anon., 2001). It is usually present were ethanol is derived from yeast fermentations from sugars or plant material and can also be isolated from damaged fruit. This emphasises the importance of implementing HACCP or similar safety regulations during food processing and to prevent the use of contaminated raw materials.

The sequence data for band k from the pear juice (Fig. 2C) showed sequence similarity to the *Enterobacteriaceae* family. The BLAST results showed the organism to be closest to a *Tatumella* sp. (97% homology, 135 out of 139 bases) (GenBank Accession number AJ233437). The presence of *Enterobacteriaceae* in food products are an indication of inadequate heat-treatment or that post-process contamination from the environment has occurred. It also serves as an indication of the overall quality of food and the hygiene conditions that were present during the processing (Crowley *et al*., 2005). In the pear juice it was only present in the first sample taken after maceration of the fruit and it was absent in the samples taken after pasteurisation, indicating that the pasteurisation temperature of 85˚C for 30 s was sufficient to eliminate this bacterium.

Mango juice

Four samples of mango juice taken before and after pasteurisation were analysed using PCR-based DGGE. The fingerprint for the mango juice samples indicated that the fruit was thoroughly washed and that the pasteurisation temperature used was sufficient, as no bands were visible on the fingerprint for the samples either before or after pasteurisation.

Aloe vera juice

The fingerprint of the aloe vera juice showed a band at the same position as the band formed by the pure isolate of *A. acidoterrestris* SA01. The organisms represented by the DGGE band was confirmed as *A. acidoterrestris* with sequencing of the 1500 bp fragment amplified from the contaminated juice (98% homology, 531 out of 539 bases) (GenBank Accession number AY573797).

The maintenance and improvement of human health by the consumption of specific foods is well-known. *Aloe vera* is a member of the Liliaceae family and has increasingly been used in health foods and for medicinal and cosmetic applications (Grindlay & Reynolds, 1986; Yusuf *et al*., 2004). It has been shown to have antitumor and antidiabetic properties, as well as efficacy in healing wounds and burns (Loadman & Christopher, 2001), adding to its popularity amongst health-conscience consumers. These results confirmed that South African aloe vera juice are also susceptible to spoilage by TAB and that the pasteurisation temperature used was not sufficient for the prevention of spoilage. Optimum time/temperature combinations for aloe vera juice should be determined, as other constituents of the juice, such as the polysaccharides and the barbaloin content, are also influenced by the heat treatment applied (Chang *et al*., 2005).

Conclusions

Analysis of the fruit juice samples showed that a number of unidentified micro-organisms are present in the fruit juice and that some bacteria are able to survive the pasteurisation temperatures used or that post-process contamination may occur. Furthermore, contamination might occur due to contaminated raw material used during processing or because of raw material that is not washed properly and may contain soil-borne organisms on its surface. It is, therefore, recommended that different temperature/time combinations should be evaluated for optimum

pasteurisation and the possible effect it may have on the fruit juice quality. Post-process contamination should be eliminated by having sufficient safety and hygiene practices in place.

PCR-based DGGE analysis can provide a fingerprint of the bacterial community in a sample at any time during the manufacturing process and can thus be used to monitor the bacterial community during the process and identify where contamination occurs. Not just food products, but both fermented and non-fermented drinks may also benefit from this molecular analysis (Ercoloni, 2004).

References

Anonymous (2000). *Alcaligenus feacalis*. The Royal Veterinary and Agricultural University of Copenhagen, Denmark, [WWW document]. URL http://www.microbiologyatlas.kvl.dk. 23 November, 2005.

Anonymous (2001). *Acetobacteriaceae*. Wikipedia, Wikipedia Foundation, [WWW document]. URL http://en.wikipedia.org. 25 January, 2006.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, Z., Miller, W. & Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Research*, **25**, 3389-3402.

Ampe, F., Omar, N., Moizan, C., Wacher, C. & Guyot., J.P. (1999). Polyphasic study of the spatial distribution of micro-organisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Applied and Environmental Microbiology*, **65**, 545-547.

Baumgart, J., Husemann, M. & Schmidt, C. (1997). *Alicyclobacillus acidoterrestris*: occurrence, significance and detection in beverages and beverage base. *Flussiges Obst*, **64**, 178.

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). Fruchtsaftverderb durch *Bacillen*: isolierung und charakterisierung des verderbserrengers. *Zeitschrift feur Lebensmittel-Untersuchung und -Forsuchung*, **179**, 224-227.

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

Chang, X.L., Wang, C., Feng, Y. & Liu, Z. (2005). Effects of heat treatments on the stabilities of polysaccharides substances and barbaloin in gel juice from *Aloe vera* Miller. *Journal of Food Engineering*, article in press.

Cocolin, L., Manzano, M., Cantoni, C. & Comi, G. (2000). Development of a rapid method for the identification of *Lactobacillus* spp. isolated from naturally fermented Italian sausages using a polymerase chain reaction-temperature gradient gel electrophoresis. *Letters in Applied Microbiology*, **30**, 126-129.

Cocolin, L., Innocente, N., Biasutti, M. & Comi, G. (2004). The late blowing in cheese: a new molecular approach based on PCR and DGGE to study the microbial ecology of the alteration process. *International Journal of Food Microbiology*, **90**, 83-91.

Connor, C.J., Hongliang, L., McSpadden, B.B. & Wang, H.H. (2004). Development of a real-time PCR-based system targeting the 16S rRNA gene sequence for rapid detection of *Alicyclobacillus* spp. in juice products. *International Journal of Food Microbiology*, **99**, 229-235.

Crowley, H., Cagney, C., Sheridan, J.J., Anderson, W., McDowell, D.A., Blair, I.S., Bishop, R.H. & Duffy, G. (2005). Enterobacteiaceae in beef products from retail outlets in the Republic of Ireland and comparison of the presence and counts of *E. coli* O157:H7 in these products. *Food Microbiology*, **22**, 409-414.

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

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

De las Rivas, B., Marcobal, Á., Gómez, A. & Muñoz, R. (2005). Characterization of IS*Lpl4*, a functional insertion sequence in *Lactobacillus plantarum*. *Gene*, **363**, 202-210.

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

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.

Felske, A., Rheims, H., Wolterink, A., Steckebrandt, E. & Akkermans, A.D.L. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class Acinetobacteria in grassland soils. *Microbiology*, **143**, 2983-2989.

Gonzalez, J.M., Ortiz-Martinez, A., Gonzalez-delValle, M.A., Laiz, L. & Saiz-Jimenez, C. (2003). An efficient strategy for screeing large cloned libraries of amplified 16S rDNA sequences from complex environmental communities. *Journal of Microbiological Methods*, **55**, 459-463.

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

Gouws, P.A., Gie, L., Pretorius, A. & Dhansay, N. (2005). Isolation and identification of *Alicyclobacillus acidocaldarius* by 16S rDNA from mango juice and concentrate. *International Journal of Food Science and Technology*, **40**, 789-792.

Grindlay, D. & Reynolds, T. (1986). The *Aloe vera* phenomenon: a review of the properties and modern uses of the leaf parenchyma gel. *Journal of Ethnopharmacology*, **16**, 117-151.

Hays, G.L. & Riester, D.W. (1952). The control of 'off-odor' spoilage in frozen concentrate orange juice. *Food Technologist*, **6**, 878-883.

Jay, J.M. (1998). Taxonomy, role, and significance of microorganisms in foods. In: *Modern Food Microbiology*, 5th ed. Pp. 20-22. USA: Aspen Publishers, Inc.

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

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.

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

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.

Lee, J.S., Heo, G.Y., Lee, J.W., Oh, Y.J., Park, J.A., Park, Y.H., Pyun, Y.R. & Ahn, J.S. (2005). Analysis of kimchi microflora using denaturing gradient gel electrophoresis. *International Journal of Food Microbiology*, article in press.

Li, S-Q., Zhang, H.Q., Jin, T.Z., Turek, E.J. & Lau, M.H. (2005). Elimination of *Lactobacillus plantarum* and achievement of shelf stable model salad dressing by pilot scale pulsed electric fields combined with mild heat. *Innovative Food Science and Emerging Technologies*, **6**, 125-133.

Loadman, P.M. & Christopher, R.C. (2001). Separation methods for anthraquionone related anti-cancer drugs. *Journal of Chromatography*, **764**, 193-206.

Moore, B.S., Walker, K., Tornus, I., Handa, S., Poralla, K. & Floss, H.G. (1997). Biosynthetic studies of ω-cycloheptyl fatty acids in *Alicyclobacillus cycloheptanicus.* Formation of cycloheptanecarboxylic acid from phenylacetic acid. *Journal of Organic Chemistry*, **62**, 2173-2185.

Muyzer, G., de Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, **59**, 695-700.

Muyzer, G. & Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, **73**, 127-141.

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.

Pettipher, G.L., Osmundsen, M.E. & Murphy, J.M. (1997). Methods for the detection, enumeration and identification of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in 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.

Scholle, M.D., White, C.A., Kunnimalaiyaan. & Vary, P.S. (2003). Sequencing and characterization of pBM400 from *Bacillus megaterium* QM B1551. *Applied Environmental Microbiology*, **69**, 6888-6898.

Silva, F.V.M., 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. (2001). *Alicyclobacillus acidoterrestris* spores in fruit products and design of pasteurization processes. *Trends in Food Science & Technology*, **12**, 68-74.

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

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

Vary, P.S. (1994). Prime time for *Bacillus megaterium*. *Microbiology*, **140**, 1001-1013.

Valinsky, L., Della Vedova, G., Scupham, A.J., Alvey, S., Figueroa, A., Yin, B., Hartin, R.J., Chrobak, M., Crowley, D.E., Jiang, T. & Borneman, J. (2002). Analysis of bacterial community composition by oligonucleotide fingerprinting of rRNA genes. *Applied and Environmental Microbiology*, **68**, 3240-3250.

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.

Wisotzkey, J.D., Jurtshuk, P., Jr, 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.

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

Yusuf, S., Agunu, A. & Diana, M. (2004). The effect of *Aloe vera* A. Berger (Liliaceae) on gastric acid secretion and acute gastric mucosal injury in rats. *Journal of Ethnopharmacology*, **93**, 33-37.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Fruit juices and fruit juice products are at risk of being spoilt by thermophilic acidophilic bacteria (TAB), in particular species of *Alicyclobacillus.* Previously it was believed that *A. acidoterrestris* was the only species implied in the spoilage of fruit juices, but it has been shown that *A. acidocaldarius* and *A. pomorum* also cause spoilage of fruit juices and fruit juice products (Splittstoesser *et al*., 1994; Goto *et al*., 2003; Gouws *et al*., 2005). As more and more spoilage cases have been reported (Cerny *et al*., 1984; Baumgart *et al*., 1997; Yamazaki *et al*., 1996; Pontius *et al*., 1998; Komitopoulou *et al*., 1999) the need has arisen for the development of a sensitive and rapid method for the detection of *Alicyclobacillus* spp. from fruit juices and fruit juice products (Chang & Kang, 2004). Many different media and methods are described in the literature for the culture-dependent isolation and identification of *Alicyclobacillus* spp., which adds to the confusion as to which is the most suitable media (Darland & Brock, 1971; Deinhard *et al*., 1987; Wisotzkey *et al*., 1992; Yamazaki *et al*., 1996; Pettipher *et al*., 1997; Palop *et al*., 2000; Walls & Chuyate, 2000).

The five media most often cited in the literature was evaluated and compared for the growth and isolation of vegetative cells and spores of *Alicyclobacillus* spp. from sterile saline solution (SSS), pasteurised fruit juice and fruit juice concentrate at different incubation temperatures. Potato dextrose agar (PDA), at pH 3.7 and orange serum agar (OSA), at pH 5.5 and incubation temperature of 50˚C for 3 days, repeatedly gave the highest recovery results. A heat shock treatment at 80°C for 10 min was included if endospores were mainly present in the sample to be enumerated. These media are therefore recommended to use for the presence/absence testing of *Alicyclobacillus* spp. from fruit juice and fruit juice concentrate. This culture-dependent detection method for cells or spores of *Alicyclobacillus* spp. from fruit juice was not sensitive as an indication of contamination of fruit juice and fruit juice concentrates. The inclusion of a filtration step has been shown to be more efficient when recovering low numbers of TAB (Walls & Chuyate, 2000; Chang & Kang, 2004).

A PCR-based DGGE method was also developed and used to analyse the microbial content of various South African fruit juices before and after pasteurisation.

DNA was isolated from various fruit juices throughout the production line and apple, pear, white grape and aloe vera juices were found to be contaminated with species of *Alicyclobacillus*, while uncultured bacteria, some of which survived pasteurisation, were also found to be present. Analyses of fruit juice samples from the complete production line over a season might give a better indication of the microbial content of the specific fruit juice. Better detection results may be obtained when PCR-based DGGE is performed, either alone or in conjunction with isolation on selective growth media, as this culture-independent method has been shown to have a lower detection limit than the culture-dependent method.

The results from this study showed that species of *Alicyclobacillus* were present from the first samples taken after maceration of the fruit, indicating that it enters the production line with the fresh fruit. This research also showed that other organisms survive pasteurisation or contaminate fruit juices after production, emphasising the importance of using clean raw material and minimising the risk for post-process contamination. Much of the contamination can be eliminated by following strict hygiene standards. The addition of substances such as nisin (Komitopoulou *et al*., 1999; Yamazaki *et al*., 2000) or chlorine dioxide (Lee *et al*., 2004) to the water used to wash the fruit are possible control measures. Further research should include analysing the effect of different concentrations of these substances on the survival of *Alicyclobacillus* spp. on the surface of fruits.

Current research is focused on *A. acidoterrestris*, but research should include other species of this genus as other species are also being isolated from spoilt juices and are shown to produce flavour compounds such as guaiacol (Matsubara *et al*., 2002; Goto *et al*., 2003). Furthermore, uncertainty still exists about the heat-resistance of these bacteria. It was previously believed that the ω-alicyclic fatty acids played an important role in the heat-resistance of the endospores of *Alicyclobacillus* spp. (Silva & Gibbs, 2001; Goto *et al*., 2002). *Alicyclobacillus pomorum* does not possess these fatty acids in its membrane (Goto *et al*., 2003), but still survived pasteurisation, indicating that other factors also play a role in the heat-resistance of the spores.

Concluding remarks

The presence of *Alicyclobacillus* spp. in pasteurised South African fruit juices and fruit juice concentrates has important implication for fruit juice manufacturers.

Various fruit juices are at risk for spoilage by TAB, and although it has been shown to be non-pathogenic, it may lead to major economic losses. *Alicyclobacillus* spp. are environmental organisms and it is therefore recommended that both the raw material and the final products be evaluated for the presence of these bacteria in order to prevent the distribution of contaminated products. Even low numbers of *Alicyclobacillus* spp. can cause the formation of flavour compounds and lead to spoilage, therefore a sensitive and accurate detection method should be developed and implemented. The PCR-based DGGE method developed in this study proved to be effective for the detection of low numbers of species of *Alicyclobacillus*. This culture-independent method could serve as a rapid and accurate method for the detection of *Alicyclobacillus* spp. in fruit juices, fruit juice concentrates and fruit juice products.

References

Baumgart, J., Husemann, M. & Schmidt, C. (1997). *Alicyclobacillus acidoterrestris*: occurrence, significance and detection in beverages and beverage base. *Flussiges Obst*, **64**, 178.

Cerny, G., Hennlich, W. & Poralla, K. (1984). Fruchtsaftverderb durch *Bacillen*: isolierung und charakterisierung des verderbserrengers. *Zeitschrift feur Lebensmittel-Untersuchung und -Forsuchung*, **179**, 224-227.

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

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

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

Goto, K., Tanimoto, Y., Tamura, T., Mochida, K., Arai, D., Asahara, M., Suzuki, M., Tanaka, H. & Inagaki, K. (2002). Identification of thermo-acidophilic bacteria and a new *Alicyclobacillus* genomic species isolated from *acidic* environments in Japan. *Extremophiles*, **6**, 333-340.

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

Gouws, P.A., Gie, L., Pretorius, A. & Dhansay, N. (2005). Isolation and identification of *Alicyclobacillus acidocaldarius* by 16S rDNA from mango juice and concentrate. *International Journal of Food Science and Technology*, **40**, 789-792.

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.

Lee, S-Y., Gray, P.M., Dougherty, R.H. & Kang, D-H. (2004). The use of chlorine dioxide to control *Alicyclobacillus acidoterrestris* spores in aqueous suspensions and on apples. *International Journal of Food Microbiology*, **92**, 121-127.

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

Palop, A., Alvarez, I., Razo, J. & Condon, S. (2000). Heat resistance of *Alicyclobacillus acidocaldarius* in water, various buffers and orange juice. *Journal of Food Protection*, **61**, 1377-1380.

Pettipher, G.L., Osmundsen, M.E. & Murphy J.M. (1997). Methods for the detection, enumeration and identification of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in 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.V.M. & Gibbs, P. (2001). *Alicyclobacillus acidoterrestris* spores in fruit products and design of pasteurization processes. *Trends in Food Science & Technology*, **12**, 68-74.

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.

Walls, I. & Chuyate, R. (2000). Isolation of *Alicyclobacillus acidoterrestris* from fruit juices. *Journal of AOAC International*, **83**, 1115-1120.

Wisotzkey, J.D., Jurtshuk, P., Jr, Fox, G.E., Deinhart, 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.

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.

ADDENDUM

FIGURES AND TABLES

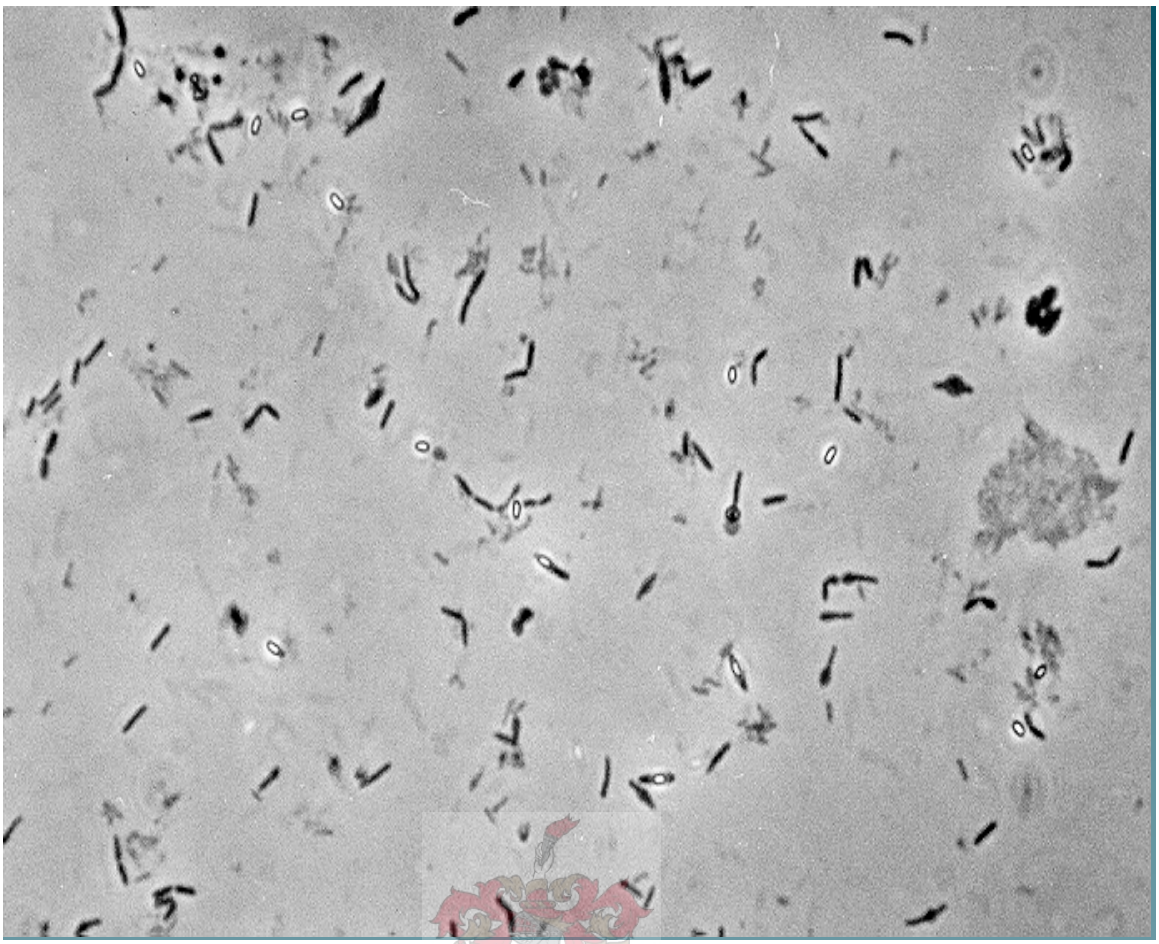


Figure 1 *Alicyclobacillus acidoterrestris* cells as viewed under a light microscope (Jensen, 1999).

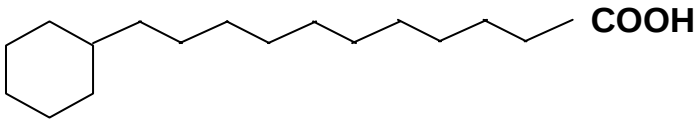
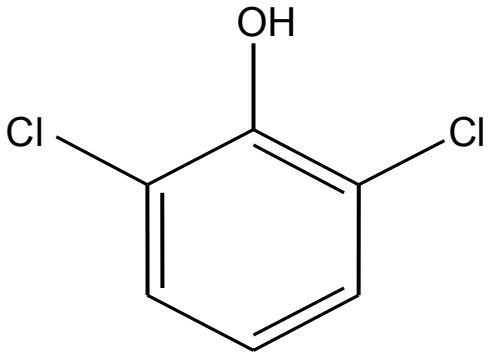


Figure 2 The alicyclic fatty acid, 11-cyclohexylundecanoic acid isolated from the membrane of *A. acidoterrestris* (Jensen, 1999).



B

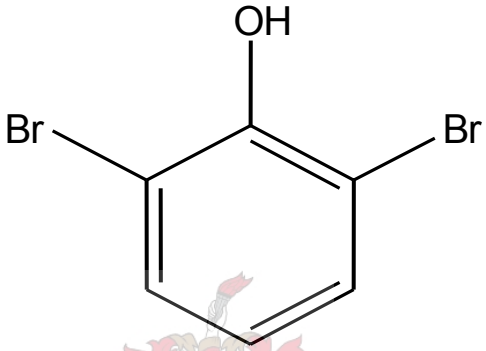


Figure 3 Chemical structures of 2,6-dichlorophenol (2,6-DCP) (A) and 2,6-dibromophenol (2,6-DBP) (B) produced by *Alicyclobacillus acidoterrestris* that contribute to taints in contaminated fruit juices (Jensen, 1999).

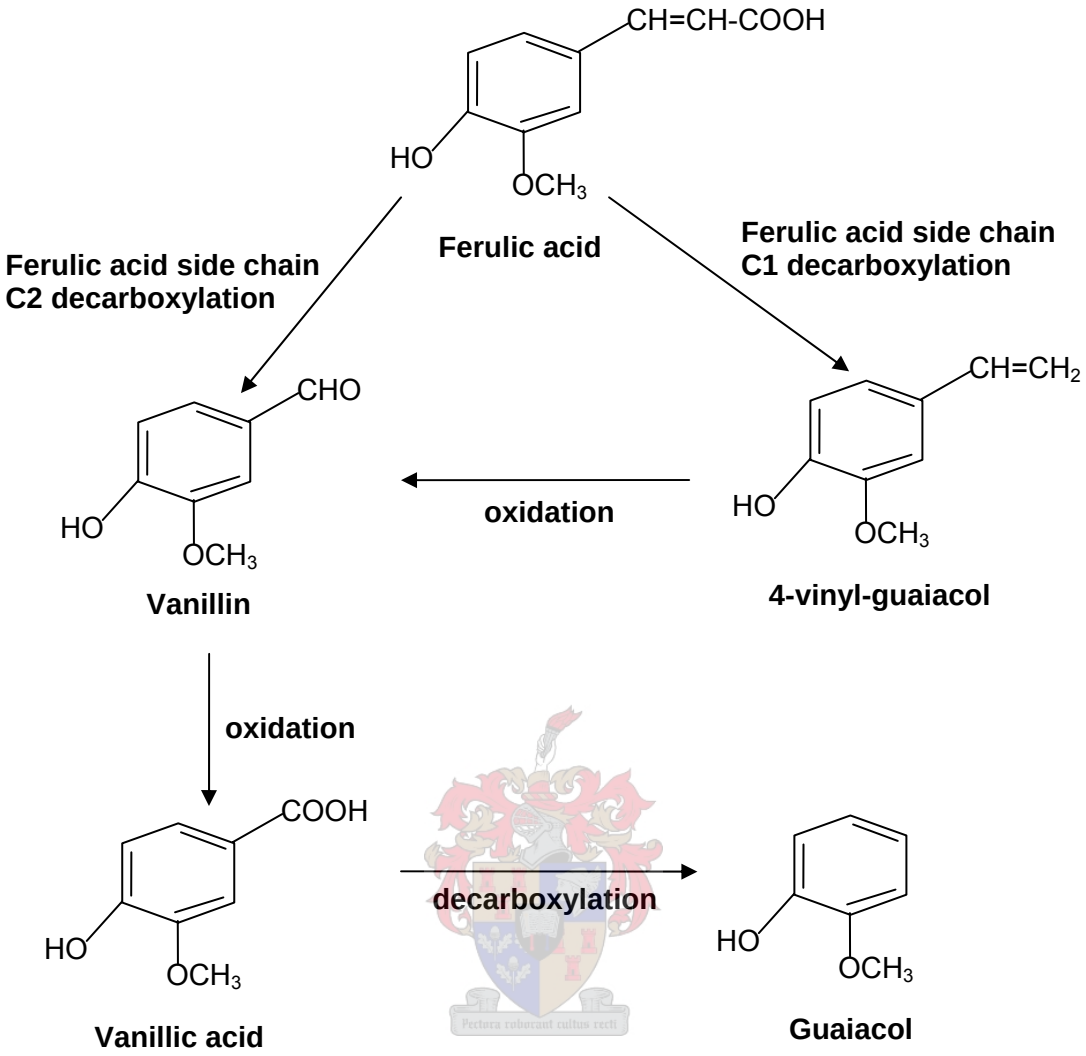


Figure 4 Simplified synthetic pathway for the formation of guaiacol from lignin (Crawford & Olson, 1978; Pometto *et al.*, 1981; Huang *et al.*, 1993).

Table 2 Heat resistance of *Alicyclobacillus* spores shown as *D*- and *z*-values, with varying pH, temperature or soluble solid content in different fruit juices (adapted from Silva *et al*., 1999)

Heating medium	pH	Soluble solids (˚Brix)	Temperature (˚C)	D value ± SD (min)[a, b]	Z (˚C)	Reference
Apple juice	3.5	11.4	85	56 ± 14	7.7	Splittstoesser *et al*., 1998
			90	23 ± 7.5		
			95	2.8 ± 0.7		
Grape juice	3.3	15.8	85	57 ± 13	7.2	Splittstoesser *et al*., 1998
			90	16 ± 4.1		
			95	2.4 ± 0.9		
Orange juice drink	4.1	5.3	95	5.3 ± NR	9.5	Baumgart *et al*., 1997
Fruit drink	3.5	4.8	95	5.2 ± NR	10.8	Baumgart *et al*., 1997
Fruit nectar	3.5	6.1	95	5.1 ± NR	9.6	Baumgart *et al*., 1997
Model fruit juice	3.1	NR	91	31 ± NR	10.0	Pontius *et al*., 1998
			97	7.9		
	3.7	NR	91	54 ± NR	7.7	Pontius *et al*., 1998
			97	85		
Apple juice	3.5	NR	80	41.2 ± 0.2	12.2	Komitopoulou *et al*., 1999
			90	7.3 ± 0.85		
			95	2.3 ± 0.03		
Orange juice	3.9	NR	80	54.3 ± 0.4	12.9	Komitopoulou *et al*., 1999
			90	10.3 ± 0.3		
			95	3.59 ± 0.04		
Orange juice	3.5	11.7	85	65.6 ± 5.5	7.8	Silva *et al*., 1999
			91	11.9 ± 0.6		

[a]SD = standard deviation, [b]NR = not reported

Table 1 The isolated and described species of *Alicyclobacillus*

Species	Source	Unique characteristics	Reference
A. acidocaldarius	Soil	Higher optimum growth temperature	Jensen, 1999 Albuquerque, 2000
A. acidoterrestris	Soil	Known to cause spoilage of fruit juices	Jensen, 1999
A. cycloheptanicus	Soil	Predominant fatty acid in the cell membrane is ω-cycloheptane instead of ω-cyclohexyl or ω-heptyl	Poralla & König, 1983
A. herbarius	Herbal tea	16S rRNA gene sequence	Goto *et al.,* 2002b
A. hesperidum	Solfataric soil	16S rRNA gene sequence	Albuquerque *et al.,* 2000
A. acidiphilus	Acidic beverages	Causes an off-flavour in orange juice	Matsubara *et al.*, 2002
A. pomorum	Mixed fruit juice	Does not possess ω-alicyclic fatty acids	Goto *et al*., 2003
A. sendaiensis	Soil	16S rRNA gene sequence	Tsuruoka *et al.,* 2003
A. vulcanalis	Geothermal pool	Phenotypic characteristics	Simbahan *et al.,* 2004
A. disulfidooxidans	Oxidisable lead-zinc ores	16S rRNA gene sequence	Karavaiko *et al*., 2005
A. tolerans	Waste water sludge	Use elemental sulfur and pyrite as sole energy sources	Karavaiko *et al*., 2005

Table 3 Pre-treatments and enumeration methods used for the isolation and identification of *Alicyclobacillus* spp.

Sample	Pre-treatment			Media[a]	Media pH[b]	Incubation temperature (˚C)	Incubation time (d)	Reference
	Membrane filtration	Heat treatment (˚C, min)	Pre-incubation (˚C, d)					
Soil	Not used	80, 10	Not used	Complex media	3 – 7	50	2	Hippchen *et al*., 1981
Apple and apple-cranberry juices	0.45 μm filters	Not used	Not used	PDA	3.5	30	5 – 7	Splittstoesser *et al*., 1994
Orange and apple juice	0.45 μm filters	Not used	44, 2	OSA	4	44	2	Pettipher *et al*., 1997
Diluted fruit juice concentrate	Not used	80, 10	50, 1-2	BAM	NR	50	1	Pinhatti *et al*., 1997
Fruit juice	Not used	80, 10	37, 7	MEA	4	50	1	Previdi *et al*., 1997
Fruit juice	Not used	60, 60	Not used	PDA	3.5	43	5 – 7	Splittstoesser *et al*., 1998
Orange surface	Not used	90, 20	45, 1-2	ALI-agar	3.5	45	10	Wisse & Parish, 1998
Orange juice	Not used	70, 20	50, 14	BAM OSA	4 4	44 44	2 2	Eiora *et al*., 1999
Apple juice	Not used	80, 10	43, 2	K-agar	3.7	43	5	Walls & Chuyate, 2000
Dried hibiscus flowers	0.45 μm filters	Not used	Not used	YSG	3.7	50	5	Goto *et al*., 2002

[a]PDA – potato dextrose agar, OSA – orange serum agar, BAM – *Bacillus acidocaldarius* medium, MEA – malt extract agar, ALI-agar – modified media from Darland & Brock (1971) and Cerny *et al*. (1984), YSG – yeast extract agar, [b]NR – Not reported

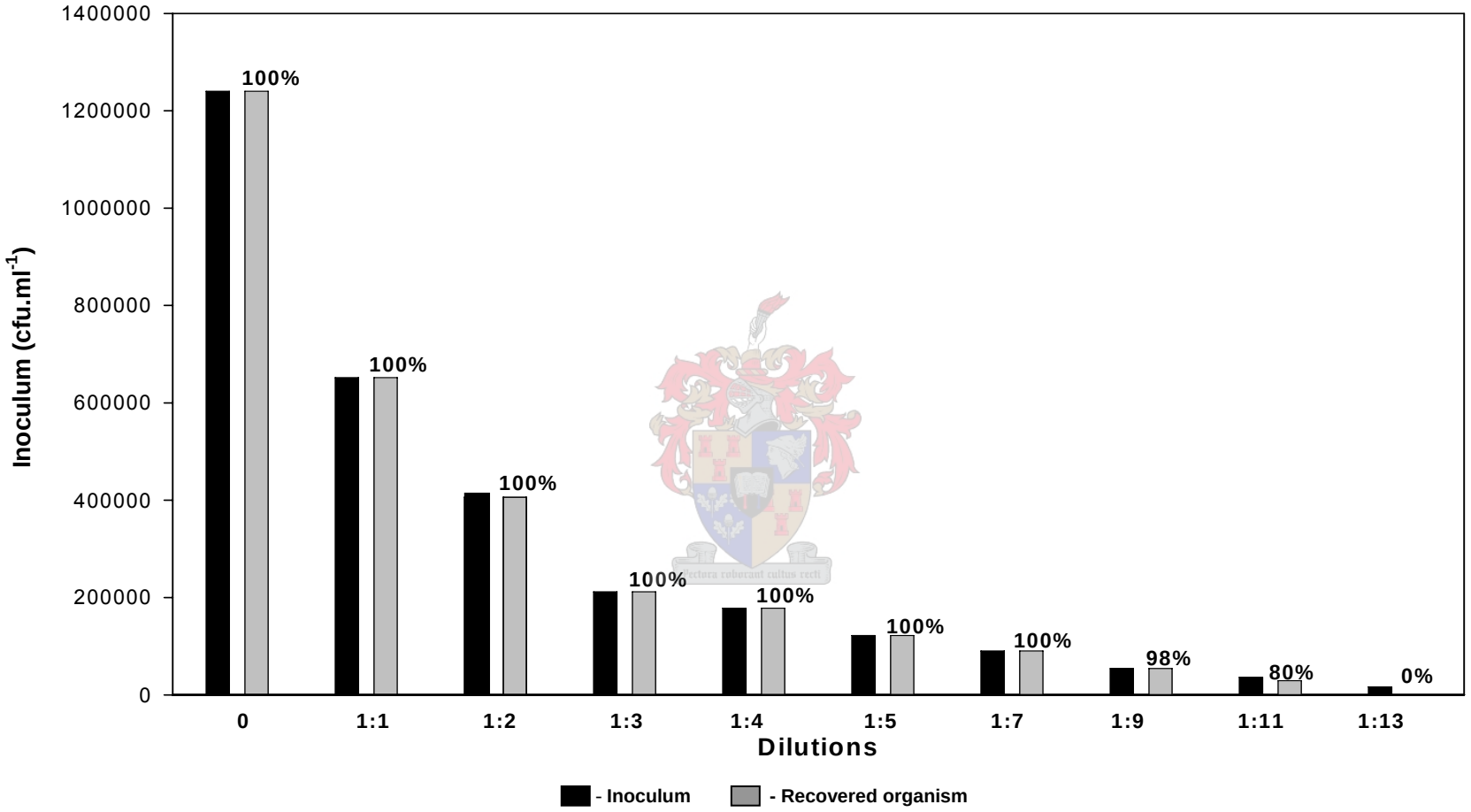


Figure 1 Recovery of vegetative cells of *Alicyclobacillus acidoterrestris* from sterile saline solution (SSS) on PDA, pH 3.7 at an incubation temperature of 50˚C for 3 days.

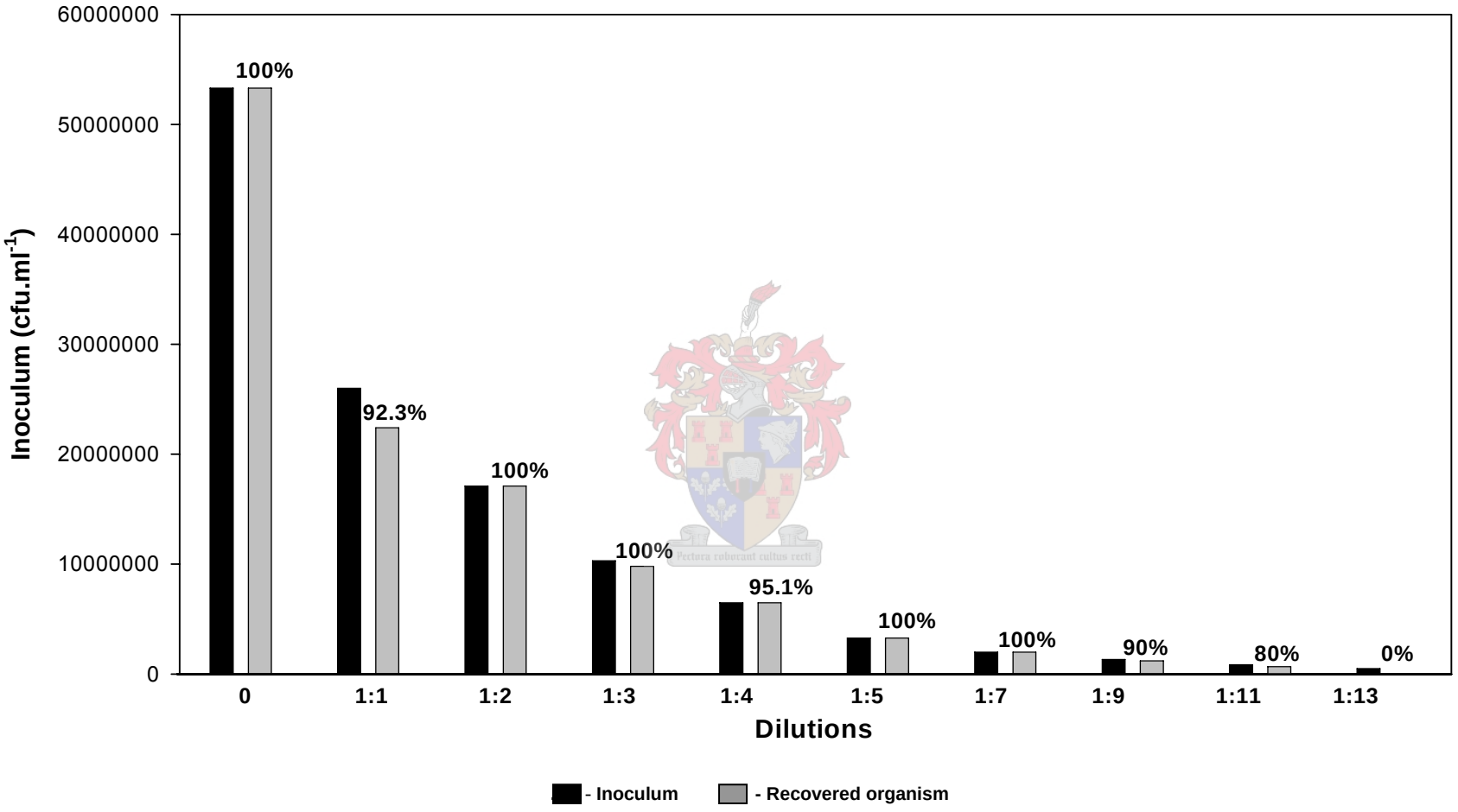


Figure 2 Recovery of vegetative cells of *Alicyclobacillus acidocaldarius* from sterile saline solution (SSS) on PDA, pH 3.7 at an incubation temperature of 50˚C for 3 days.

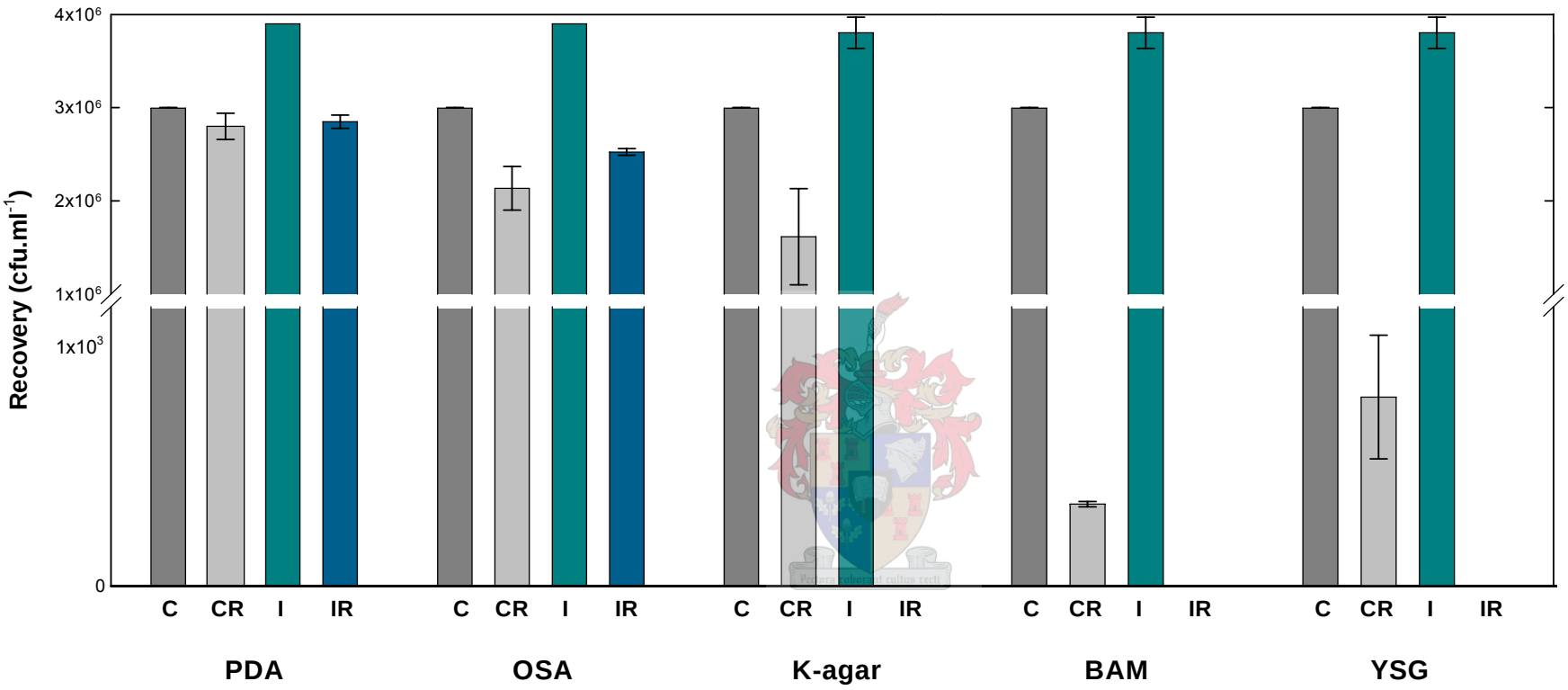


Figure 3 Recovery of *A. acidoterrestris* from diluted pear juice concentrate, on different isolation media at an incubation temperature of 50°C for 5 days, done in triplicate. C – control (in sterile saline solution (SSS)), CR – control recovery, I – inoculum in fruit juice, IR – inoculum recovery, PDA – potato dextrose agar, OSA – orange serum agar, BAM – *Bacillus acidocaldarius* medium, YSG – yeast-starch-glucose agar. The experiment was done in triplicate and the standard deviation (SD) is indicated. Statistical analysis indicated no significant difference between recovery on PDA and OSA ($p = 0.64$).

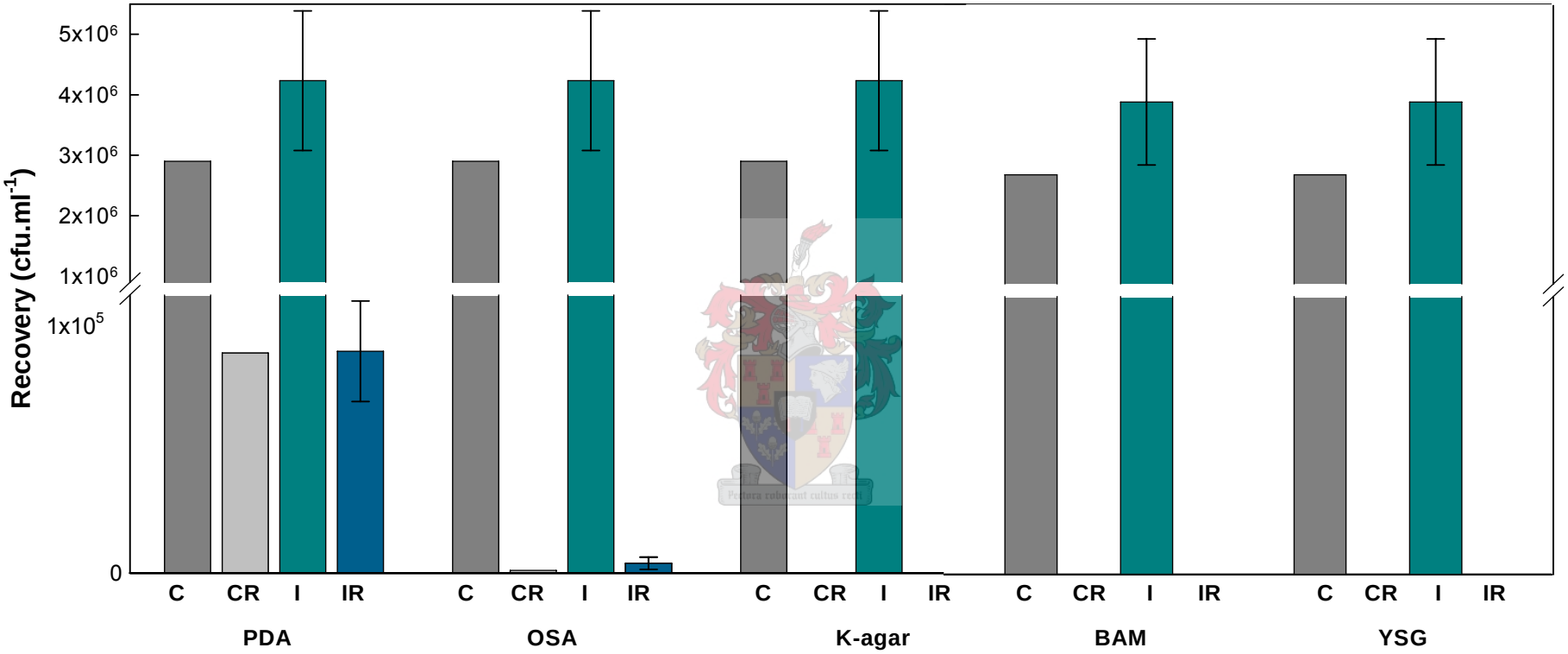


Figure 4 Recovery of vegetative cells of *A. acidoterrestris* from inoculated pear juice concentrate on the different media. C – control (in sterile saline solution (SSS)), CR – control recovery, I – inoculum in fruit juice, IR – inoculum recovery, PDA – potato dextrose agar, OSA – orange serum agar, BAM – *Bacillus acidocaldarius* medium, YSG – yeast-starch-glucose agar. The experiment was done in triplicate and the standard deviation (SD) is indicated. Statistical analysis indicated a significant difference between recovery on PDA and OSA ($p = 0.002$).

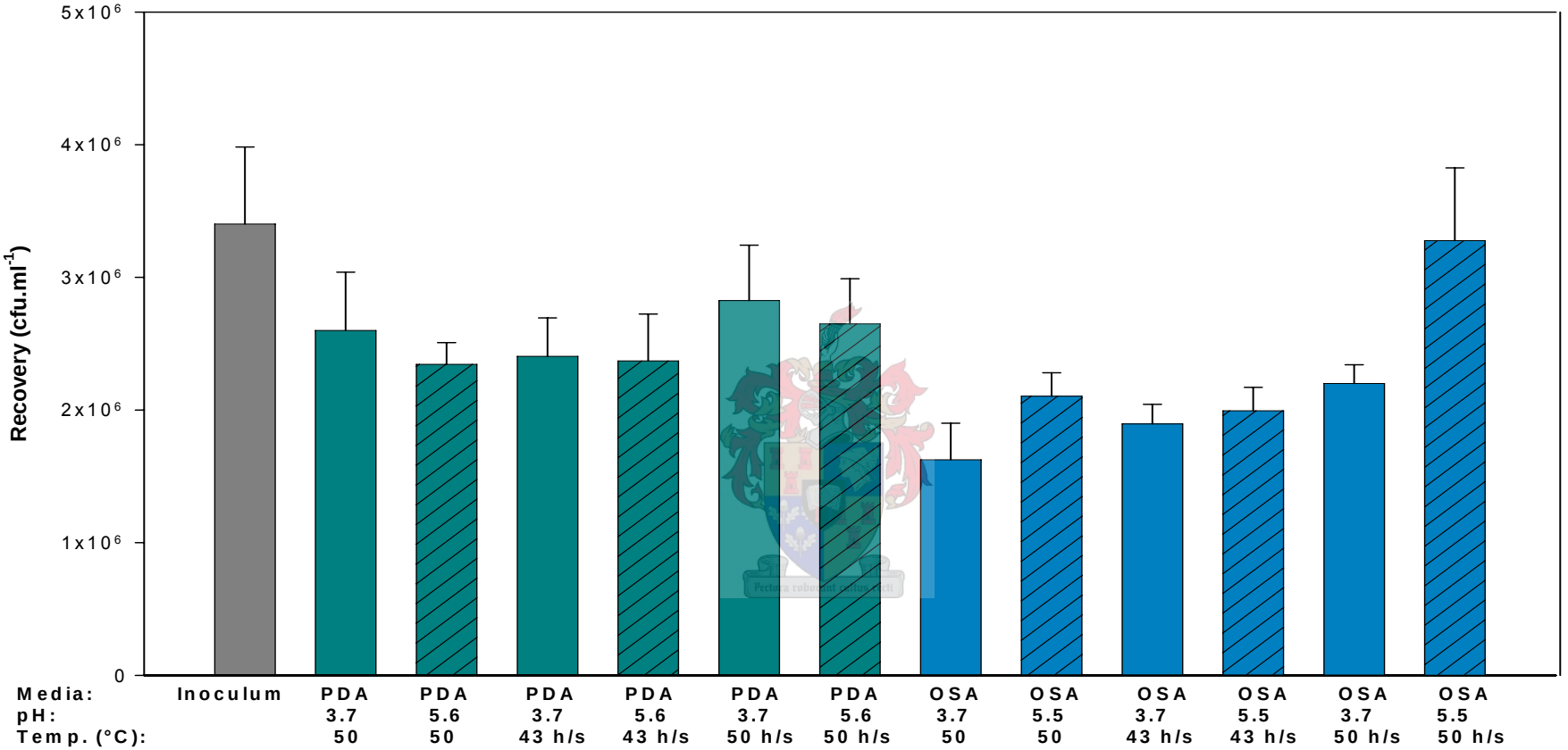


Figure 5 Average recovery of *A. acidoterrestris* on PDA, pH 3.7 and 5.6 and OSA, pH 3.7 and 5.5, at incubation temperatures of 43˚ and 50˚C. PDA – potato dextrose agar, OSA – orange serum agar, h/s – heat shock treatment at 80˚C for 10 min was applied. The experiment was done in triplicate and the SD is indicated as error bars. Statistical analysis indicated a significant difference between the highest recovery after a heat shock treatment on OSA, pH 5.5 and PDA, pH 3.7 at 50˚C ($p = 0.021$).

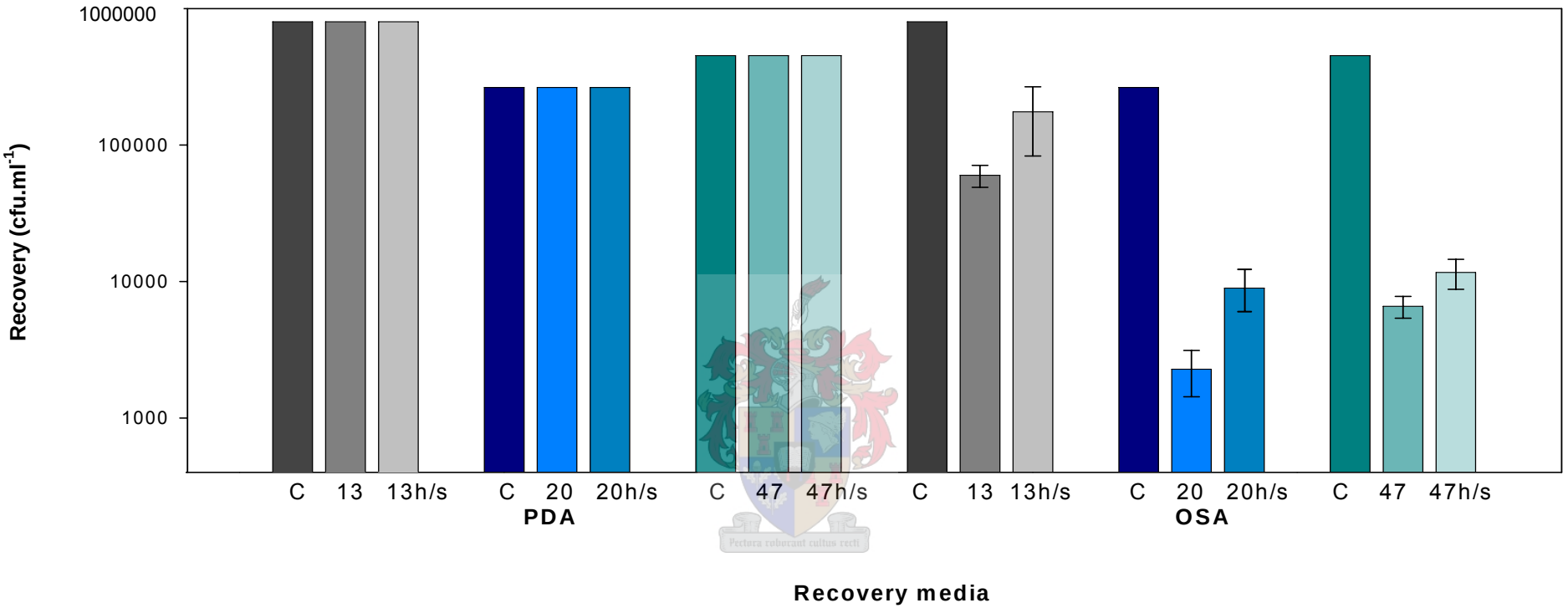


Figure 6 Recovery of *A. acidoterrestris* K13, *A. acidoterrestris* K47 and *A. pomorum* K20 from white grape juice concentrate on PDA, pH 3.7 and OSA, pH 5.5 at an incubation temperature of 50˚C. PDA – potato dextrose agar, OSA – orange serum agar. Column 1 of the three – Amount recovered on PDA included as the control (C), as the original level of contamination was unknown, Column 2 of the three – Recovery before heat shock, Column 3 of the three– Recovery after heat shock at 80˚C for 10 min. The experiment was done in triplicate and the standard deviation (SD) is indicated. Statistical analysis indicated a significant difference for all three samples between recovery on PDA and OSA before a heat shock treatment ($p = 0.000$) and $p = 0.006$ (K20) and $p = 0.008$ (K47) indicated a significant difference between recovery on PDA and OSA after a heat shock treatment for the specific strains.

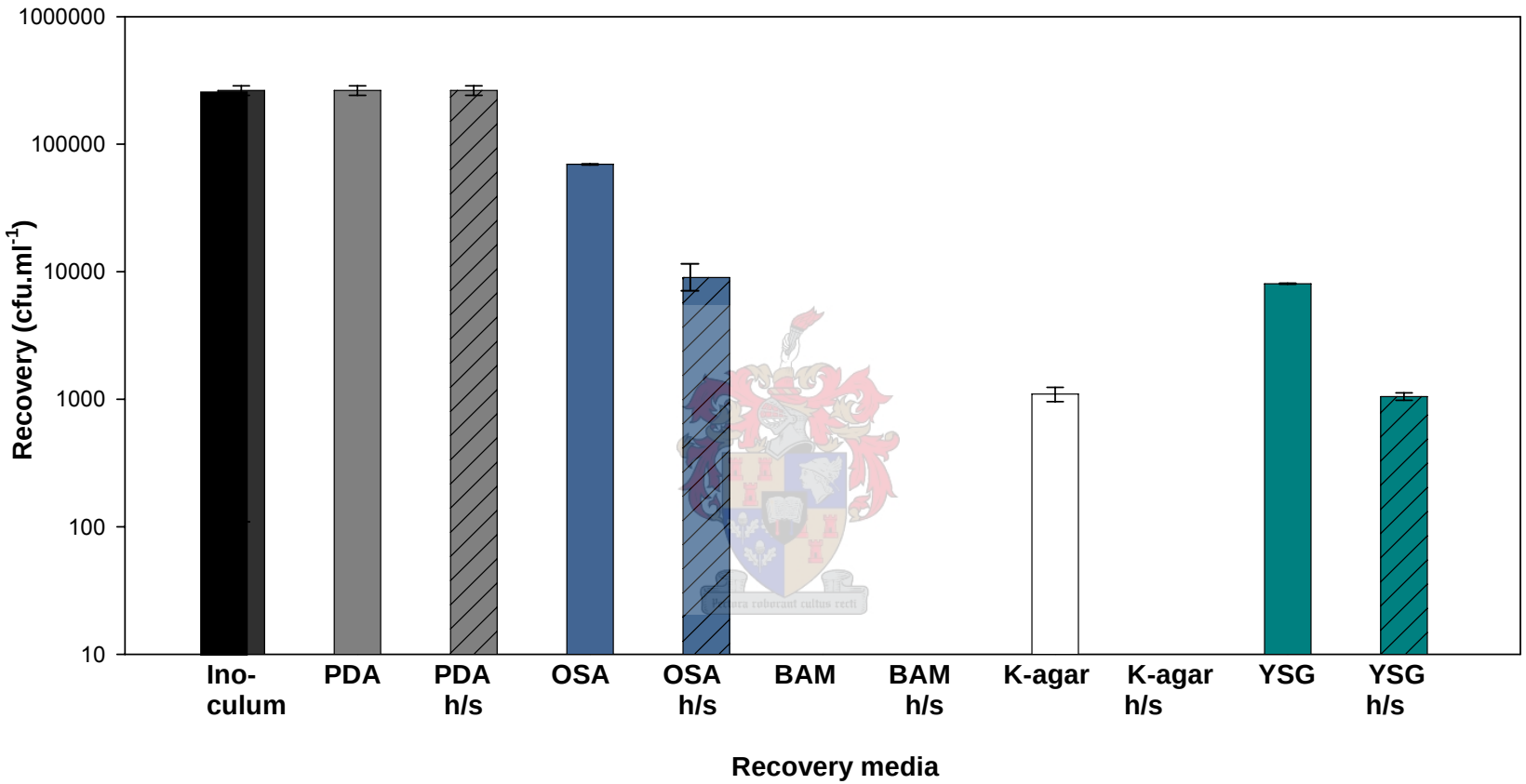
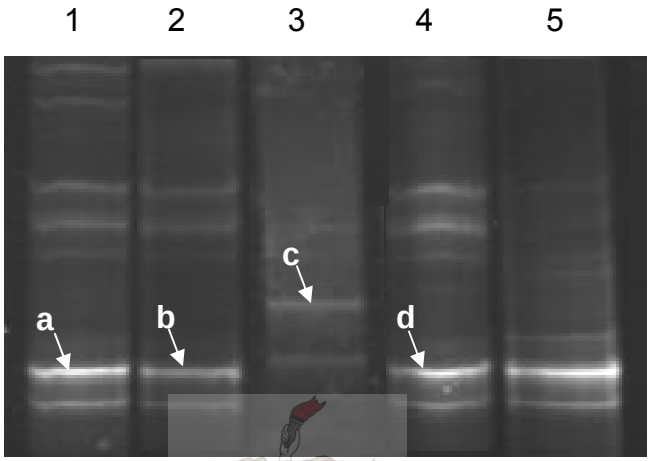
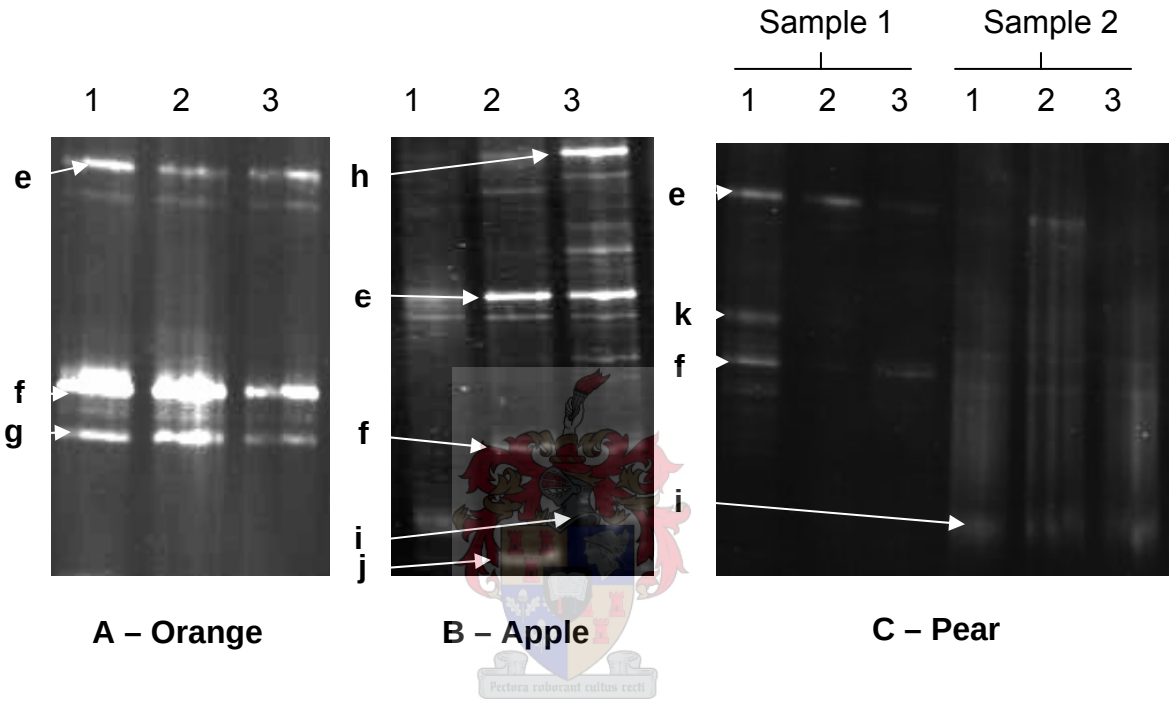


Figure 7 Recovery of *A. pomorum* on different isolation media at an incubation temperature of 50˚C for 5 days. PDA – potato dextrose agar, OSA – orange serum agar, YSG – yeast-starch-glucose agar, BAM – *Bacillus acidocaldarius* medium, h/s – heat shock treatment at 80˚C for 10 min. The experiment was done in triplicate and the standard deviation (SD) is indicated as error bars. Statistical analysis indicated a significant difference between recovery on PDA and the other four isolation media (p = 0.000).



a, b, c, d – DGGE bands identified using DNA sequencing

Figure 1 PCR-based DGGE analysis of pasteurised white grape juice concentrate. Lane 1 – PCR-based DGGE fingerprint of a white grape juice concentrate sample K7, Lane 2 – PCR-based DGGE fingerprint of sample K13, Lane 3 – PCR-based DGGE fingerprint of sample K20, Lane 4 – PCR-based DGGE fingerprint of sample K47, Lane 5 – *A. acidoterrestris* SA01 as a reference strain.



e, f, g, h, i, j, k – DGGE bands identified using DNA sequencing

Figure 2 PCR-based DGGE analysis of South African orange, apple and pear juices. Lanes 1 – Before pasteurisation, Lanes 2 – After evaporation, Lanes 3 – The final product after pasteurisation.