

**The Prevention of Spoilage
in Fruit Juices by
Alicyclobacillus acidoterrestris
and
*Propionibacterium cyclohexanicum***

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Thesis submitted to the University of Northampton in partial fulfilment of the
requirements for the degree of Doctor of Philosophy

The University of Northampton

Northampton, UK, 2006



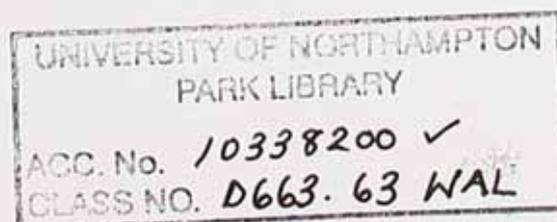
Abstract

During the past two decades several novel spoilage micro-organisms have emerged. Raw materials and products have been contaminated in increasing numbers of spoilage incidents causing widespread problems within the juice and beverage industry. This study investigates two such spoilage micro-organisms, *Alicyclobacillus acidoterrestris* and *Propionibacterium cyclohexanicum*, both isolated from pasteurised contaminated fruit juice.

A variety of media were tested to determine which supported optimal growth of *A. acidoterrestris* with Orange Serum Agar providing consistently high plate counts. The presence of *A. acidoterrestris* in raw materials and shelf stable products was monitored and the effects on its growth and survival of temperature, headspace and movement of containers during storage were investigated. The survival of *P. cyclohexanicum* after pasteurisation was assessed and growth determined at a variety of temperatures. The survival of each bacterium was investigated in different fruit juices, when challenged by the preservatives sodium benzoate and potassium sorbate and the bacteriocin nisin and when grown in the same juice container and co-cultured on the same solid medium.

17% of samples tested were contaminated by *A. acidoterrestris*; however *P. cyclohexanicum* was not isolated from any sample. *P. cyclohexanicum* survived 10 minutes at temperatures of 4°C to 95°C and grew in orange, tomato and pineapple juice while *A. acidoterrestris* grew in all juices tested. *A. acidoterrestris* was inhibited by sodium benzoate (500ppm), potassium sorbate (500ppm) and nisin (5IU/ml). *P. cyclohexanicum*, although not inhibited by nisin (1000IU/ml), was susceptible to sodium benzoate (500ppm) and potassium sorbate (1000ppm). Headspace, movement of containers and storage temperatures affected detection rates of *A. acidoterrestris*. Co-cultures demonstrated that if found within the same environment, both bacilli can survive and cause spoilage.

A. acidoterrestris is a world wide contaminant within the soft drinks industry and, considering the results of these studies *P. cyclohexanicum* with its heat resistance and tolerance to nisin may also emerge as a major spoilage micro-organism.



Acknowledgements

It is with pleasure I thank the many people who made this thesis possible:

Belmay flavours Limited for the sponsorship, time and laboratory use without which this would all have been impossible.

I gratefully acknowledge the inspirational, enthusiastic support of my supervisor Professor Carol Phillips and thank you for keeping everything on track during the difficult times.

All at the Knowledge Exchange for your assistance, patience and friendliness

David Goodman for his support and kindness

Tracey for the cups of tea, Lin for her 'helping hands' in the laboratory and Skeg for the lifts home

Everyone at Belmay Flavours Limited, no-one completes a thesis alone, everyone at Belmay has at sometime had an input into this work and to you all I say thank you.

My family Becki and Phil, Chris and Amelia, and Cookie for their understanding, endless patience and encouragement when it was most required; especially mum 'who understood' and my late father who would be proud.

Margie a wonderful sister and Terry always just a phone call away. My sister Jenny and my brother Steve and their families with love

Lastly and most importantly to my husband Mike – you inspired me, supported me, proof read for me, laughed with me, I love you, to you I dedicate this thesis.

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Chapter 1

Introduction

During the latter part of the last century a rapid increase in fruit juice consumption was recorded in the United Kingdom, with no decline noted in the amount of fresh fruit consumed (Southgate *et al.*, 1990). However consumption of fresh fruit in the USA increased by nineteen per cent between 1980 and 2001 partly due to the advent of new technologies that have enabled the shelf life of produce to be extended (Clemens, 2004). In the USA 6,769 million litres of fruit juice was consumed in 1987 and fruit juice that was traditionally consumed at breakfast and was initially considered a luxury item, is now a drink consumed at any time of the day by young children and adults alike (Jeffries, 1990).

Fruit juice is a sweet tasting, convenient, inexpensive, natural and nutritious part of a healthy diet (Dennison *et al.*, 1997). With nutritional advice to consume more fruit and vegetables daily, and fruit juice counting as part of the required amount, there would seem good reason to believe this trend will continue (Jeffries, 1990).

Formerly surplus, sub-standard or reject fruit would be considered acceptable for processing into fruit juice. Now however, standards of fruit for juicing are expected to mirror the high standards of the fresh fruit market. Due to the demand for good quality, reliable raw material sources a number of fruit growers now grow fruit specifically for the juice industry (Moulton and Whewell, 1990).

Many processes are carried out before fruit becomes a juice or a fruit product, including, storage of raw materials, transportation to and storage at the processors. There may be more than one processor involved depending on the

product and this necessitates packaging and storage at each facility as well as further transportation. Once processing is complete further packaging and storage is involved before delivery to retailers, then shops where it will finally be purchased by the consumer who will store the product until it is to be used (Gould, 1989).

Processors use various methods to preserve the quality of juice products at all times, but any area of production including packaging, storage and transportation within the fruit juice industry can affect the quality of the final product (Brackett, 1992). The objective of preservation is to regulate and control quality loss, including delaying or inhibiting the growth of any micro-organisms that may lead to product spoilage and most importantly those micro-organisms that may cause food poisoning (Russell and Gould, 2003).

In the soft drinks industry there is ongoing product development and many new products are now available. These new soft drinks are not always afforded the same microbiological protection as traditional products, even when two very similar products are involved. Examples of these are red lemonades and orange lemonades, both have similar compositions and contain approximately 6 g/l of CO₂ and have a pH of 3.7. An 'off-flavour' has often been noted in red lemonades made from cherries, red grapes and various other red berries but not in the orange lemonades. The red lemonade is susceptible to contamination with lactic acid bacteria (*Lactobacillus perolens*) leaving the lemonade tasting strongly of diacetyl, this does not however occur in the orange lemonade (Back, 2001).

During the last two decades several novel spoilage micro-organisms have emerged causing concern within the fruit juice industry. *Alicyclobacillus acidoterrestris* has warranted much scientific and industrial attention and a new genus of bacillus, *Alicyclobacillus* has been identified (Wisotzkey *et al.*, 1992). Several other new species; *Alicyclobacillus acidiphilus* (Matsubara *et al.*, 2002); *Alicyclobacillus herbarius* (Goto *et al.*, 2002) and *Alicyclobacillus pomorum* (Simbahan *et al.*, 2004) have been isolated from a variety of beverages.

Other novel species isolated from fruit juices include *L. perolens* sp. nov. (Back *et al.*, 1999) and *Propionibacterium cyclohexanicum* (Kusano *et al.*, 1997) isolated from fruit juices in separate spoilage incidents.

Traditional preservation techniques such as pasteurisation, low acidity, carbonation and refrigeration have been challenged by these novel micro-organisms. Further research and new preservation regimes must be developed for the soft drinks industry to keep up with changes in consumer requirements, new product development and to identify and control novel species of spoilage micro-organisms.

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Chapter 2

Literature Review

2.0. Introduction

The rate at which fruit loses quality after harvesting depends on many factors including chemical, enzymatic and structural changes within the fruit itself, to the growth of spoilage micro-organisms within the fruit and on the fruit surface (Khurdiya, 1995). Most of these micro-organisms are harmless to humans such as lactic acid bacteria or coryneforms (Gras *et al.*, 1994) however, some spoilage organisms may go on to spoil the final product making it unacceptable for market.

2.1. Spoilage in the fruit juice industry

Spoilage of fruit juices and soft drinks by bacterial contamination may result in a variety of undesirable reactions within a product. In the competitive world of soft drinks manufacturing taste, odour, appearance and shelf life are of prime importance to both consumers and producers. Shelf life can be described as the time interval throughout which a product will retain its organoleptic qualities, maintain its physical, chemical and microbial parameters while conforming to any nutritional labelling statements (Özdemir, 1998). Spoilage, which may occur at any point from the orchard soil to consumption of the final product, can alter any one, or all, of the organoleptic characteristics rendering the product unusable. This contamination may prove very costly to the manufacturer (Jensen, 1999), not only in the resulting economic loss (McIntyre *et al.*, 1995) but also in terms of reputation damage in very competitive markets. An example of this was a product

recall in August 2005, which was reported nationwide involving 100,000 cans of a well known soft drink marked with a shelf life of June/July 2006. The products were recalled due to the probability of them containing naturally-occurring yeast which could generate enough carbon dioxide to distort or burst the packaging which probably resulted in a substantial financial loss to the company involved.

The major microbial contaminants of unpasteurised fruit juices have generally been recognised as low-heat-resistant micro-organisms such as yeasts, moulds and lactic acid bacteria as these organisms prefer or tolerate the acidic (pH <4.0) nature of fruit juices (Kusano *et al.*, 1997; Pontius *et al.*, 1998; Eiroa *et al.*, 1999). Most bacterial spores will fail to germinate at pH <4.1 therefore spoilage has been limited to a small number of organisms that can grow at pH 3.8 such as *Bacillus coagulans* and *Clostridium pasteurianum* (Pontius *et al.*, 1998).

One hot-fill and hold pasteurisation process used by the fruit beverage industry involves holding the product at 88°C to 96°C for approximately two minutes and is sufficient to destroy most non-spore forming micro-organisms, the exception being heat resistant moulds. The product is then considered commercially sterile (Pontius *et al.*, 1998). Other processes use temperatures of 90°C to 95°C for between 30 seconds and one minute prior to packaging to achieve a similar effect (Kusano *et al.*, 1997). However, during the past two decades several novel spoilage micro-organisms have emerged. Incidents have occurred involving the contamination of acidified vegetables, fruits and wines by *Alicyclobacillus acidoterrestris* a rare spore forming bacterium that is able to survive at pH3.7 (Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996a; Pettipher *et*

al., 1997; Walls and Chuyate, 1998; Pettipher and Osmundson., 2000) and *Propionibacterium cyclohexanicum* sp. nov., a rare non spore-forming, heat tolerant, acid tolerant bacterium (pH3.2) that was isolated from spoiled pasteurised orange juice (Kusano *et al.*, 1997).

The emergence of these novel micro-organisms, together with increasing consumer demand for the exclusion of many chemically synthesised preservatives, whilst maintaining safety, quality and extended shelf-life, has necessitated further research to ensure product quality and safety (Roller, 1995).

2.2. *Bacillus acidocaldarius*

Although the genus *Alicyclobacillus* was first recognised in 1992 (Wisotzkey *et al.*, 1992), initial reports of acidophilic, thermophilic spore-formers isolated from hot, acid (pH2-3) springs in Tokohu district, Japan, where temperatures can reach 75°C to 80°C, were reported in 1967 by Uchino and Doi. Three strains of acidophilic, thermophiles were isolated from the hot, acid spring waters. All strains were aerobic spore-forming bacilli with terminal to sub-terminal spores. These bacilli were compared to other known acido-thermophiles such as *Bacillus stearothermophilus* and *Bacillus coagulans* and found to be dissimilar to the former, but possibly related to the latter. However, when compared more closely to *B. coagulans* the isolates were more acidophilic and more aerobic and, as they were capable of growth at 55°C and not 37°C, using the classification proposed by Cameron and Esty (1926), were categorised as

'obligate thermophiles' of a strain closely related to *B. coagulans* (Uchino and Doi, 1967).

Brock and Darland (1970) also demonstrated that bacteria could be isolated from acid, hot springs and, in subsequent studies, fourteen cultures of acido-thermophiles were isolated from a selection of thermal, acid environments. Attempts to isolate such acido-thermophilic bacteria from alkaline or neutral (pH5 to pH7) environments were unsuccessful. However, when using media adjusted to neutral pH, *B. stearothermophilus* was successfully isolated from these environments. When comparing the isolates' growth temperatures to that of *B. coagulans*, which is capable of growth at up to 55°C at pH4, none grew at 40°C, all isolates grew at 45°C to 65°C, and six grew at 70°C. Of the six that grew at 70°C none were able to grow at 75°C. Most isolates grew at pH2; all at pH3-5, and eight at pH6, none grew at pH6.4. The eight isolates that grew at pH6 had all lowered the pH to pH5.2 by the time visible growth was observed, in contrast to those that were initially at pH5 or below where no pH change was observed. Many physiological and morphological similarities were revealed when these isolates were compared to those of Uchino and Doi (1967). The isolates proved to be Gram-variable spore-forming rods, a classification that describes organisms of the genus *Bacillus*. However, the DNA composition of the isolates was approximately 62mol% (G+C), higher than that of the genus *Bacillus*, that is between 45 and 50mol%. The difference in DNA base composition to the genus *Bacillus*, plus the observation by Uchino and Doi (1967) that the isolates were more acidophilic and more aerobic than *B. coagulans* and formed spores that were

terminal to sub-terminal, resulted in the proposal of a new species *Bacillus acidocaldarius* (Darland and Brock, 1971).

Cell membrane structures have been considered substantial contributors to the thermo-stability of thermophilic bacteria possibly acting as a barrier in extreme environments (Suzuki *et al.*, 1981). Examination of the membrane structures of *B. acidocaldarius* revealed that the principal components were 11-cyclohexylundecanoic and 13-cyclohexyltridecanoic acids, which can comprise up to 65% of the membrane fatty acids: also found were anteiso- and iso-heptadecanoic fatty acids. No unsaturated fatty acids were discovered (De Rosa *et al.*, 1971). The effects of pH and temperature on these structures seemed to be interdependent, the proportion of iso and anteiso acids increased at low pH (pH2 to pH4) and higher temperatures (50°C to 70°C), and the cyclohexyl acids decreased. At the same temperatures, but at pH5, the reverse occurred in that the iso and anteiso acids declined and the cyclohexyl acids increased, thus proving temperature and pH are interdependent (De Rosa *et al.*, 1974).

Apart from *B. acidocaldarius* the only other source of 11-cyclohexylundecanoic acid has been found as a minor component of rumen bacteria (De Rosa *et al.*, 1974). In 1981 Suzuki *et al.*, demonstrated that *Curtobacterium pusillum*, a mesophilic, Gram positive, catalase negative bacterium isolated from oil brine contained ω -cyclohexyl undecanoic acid as part of its cellular fatty acid composition. As with *B. acidocaldarius* the cyclohexyl acids increased with temperature, but the pH at which this occurred was not recorded, an increase in cyclohexyl acids was also observed with the increase of

glucose in the culture medium. The optimal temperature for growth of *C. pusillum* has been recorded as 27°C and no growth has been observed above 45°C, this is lower than the temperature for growth of *B. acidocaldarius*. The optimal pH for growth of *C. pusillum* which has been recorded as pH7.0 is higher than that of *B. acidocaldarius*. No other *Curtobacteria* spp. exhibit ω -cyclohexyl fatty acids as a feature of the cell membrane (Suzuki *et al.*, 1981).

Hippchen *et al.*, (1981) investigated the possibility that there might be other thermo-acidophilic bacteria possessing ω -cyclohexane fatty acids in their membrane. Two isolation methods were used, the first was a pasteurisation regime (80°C for 10 minutes) for soil samples from neutral environments which were then streaked directly onto a complex agar with differing pH values, followed by incubation at 50°C for 2 days. No colonies grew at pH4 and on examination of the colonies that grew at pHs5, 6 and 7 no ω -cyclohexane fatty acids were detected. An enrichment method was then used where the pasteurised soil samples were incubated for 24 hours at 50°C in a medium adjusted to pH4, followed by further incubation at pH3 to pH3.5. Using agar with the same composition the suspensions were streaked out and 50 thermo-acidophilic spore forming strains were isolated; ω -cyclohexane fatty acids were found in 23 strains and six strains contained hopane lipids, which had previously been found in *B. acidocaldarius* (Langworthy and Mayberry, 1976). As in *B. acidocaldarius* the spores were terminal to sub-terminal and in three strains almost central. These investigations confirmed the existence of thermo-acidophilic bacilli similar to *B. acidocaldarius* in neutral soils. As *B. acidocaldarius* was not detected in every

soil sample the possibility exists therefore of acidic, soil micro-environments that may support growth of thermo-acidophilic bacilli, the detection of which may be dependent on particular minerals present in soil samples. Since *B. coagulans* had not previously been analysed for the presence of ω -cyclohexane fatty acids, seven American Type Culture Collection (ATCC) strains of *B. coagulans* were analysed for the presence of these lipids and all proved negative. Only branched-chain fatty acids, normal for bacilli were present. The strains isolated in these investigations therefore have no relationship to *B. coagulans* (Hippchen *et al.*, 1981).

2.3. *Bacillus acidoterrestris*

During the early 1980s in Germany, an acidophilic '*Bacillus*' strain was isolated from spoiled apple juice. This organism, which had survived pasteurisation, grew between pH 2.5 to 5.5, and analysis of the membrane lipids revealed the presence of ω -cyclohexane fatty acids and hopanoids. A relationship was suggested between this organism and *B. acidocaldarius* and a warning issued that these findings indicated that some fruit juices stored at temperatures of $\geq 26^{\circ}\text{C}$ could be spoiled by this bacilli (Cerny *et al.*, 1984).

Following the research by Hippchen *et al.*, (1981) together with the spoiled apple juice incident in Germany (Cerny *et al.*, 1984), Deinhard *et al.*, (1987) undertook further investigations to identify these bacilli. The G+C composition of the *B. acidocaldarius* strains was found to be similar to that detected in other studies, i.e. 57.3mol% to 61.1mol% (Darland and Brock, 1971)

but the G+C content of the newly discovered bacilli was found to be about 7% lower than *B. acidocaldarius* (51.0mol% to 53.3mol%). Further differences between the two bacteria were revealed in that thirteen of the 'unidentified' bacterial strains formed acid from different carbon sources (Appendix 1, Table a) and the temperature range for growth was 35°C to 53°C with an optimal growth temperature of 42°C to 53°C, in comparison with the temperature range for *B. acidocaldarius* which is approximately 45°C to 70°C. Strains of *B. acidocaldarius* have been isolated from mainly hot, acidic sources world wide (Darland and Brock, 1971) whereas the unidentified species were isolated from garden and forest soils and an apple juice. Following these taxonomic investigations the isolates of Hippchen *et al.*, (1981) and Cerny *et al.*, (1984) were found to differ significantly from *B. acidocaldarius* and a new species was proposed, *Bacillus acidoterrestris* spp. nov. (Deinhard *et al.*, 1987).

Further taxonomic investigations revealed three related species: *B. acidocaldarius*, *B. acidoterrestris* and *B. cycloheptanicus*. Comparative sequence analyses on the 16S rRNA gene of these three species indicated that the sequences of *B. acidocaldarius* and *B. acidoterrestris* were very similar (98.8%) but that of *B. cycloheptanicus* was lower than *B. acidocaldarius* (93.2%) and *B. acidoterrestris* (92.7%). Comparing the levels of similarity between the 16S rRNA of these three species and the three thermoacidophiles *Bacillus subtilis* (84.3% to 85.3%), *Bacillus coagulans* (85.0% to 85.2%) and *Bacillus stearothermophilus* (86.2% to 86.8%) indicated that *B. acidocaldarius*, *B. acidoterrestris* and *B. cycloheptanicus* were specifically related and markedly

different from other *Bacillus* species. On the basis of this and previous evidence, it was proposed that these three bacilli should be reclassified in a new genus, *Alicyclobacillus* gen. nov., in the family *Bacillaceae* (Wisotzkey *et al.*, 1992).

2.4. *Alicyclobacillus* genus

The genus *Alicyclobacillus* therefore comprises thermoacidophilic, heterotrophic, endospore forming bacteria. The original species *A. acidocaldarius*, *A. acidoterrestris* and *A. cycloheptanicus*, have been added to by a number of other species, both newly described and reclassified. Species of the genus *Alicyclobacillus* isolated from soils and geothermal pools include *A. hesperidum* (Albuquerque *et al.*, 2000), *A. sendaiensis* (Tsuruoka *et al.*, 2003) and *A. vulcanalis* (Simbahan *et al.* 2004) and also include a sub-species *Alicyclobacillus acidocaldarius* ssp. *rittmanii*, which exists 2000m above sea level in the crater of Mount Rittmann, Antarctica where the air temperature is -20°C and ground temperature close to active fumarole vents, is 60°C (Nicolaus *et al.*, 1998). Two *Sulfobacillus* spp. (*Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans* and *Sulfobacillus disulfidooxidans*) have recently been re-classified as *Alicyclobacillus tolerans* and *Alicyclobacillus disulfidooxidans* respectively together with an emended description of the genus including a broadening of the temperature growth range to <20°C to 70°C, the optimum to 35°C to 60°C, and the G+C content range to 48.7mol% to 62.7mol% (Karavaiko *et al.*, 2005).

However, what may be of importance to the fruit beverage industry are the species *Alicyclobacillus acidiphilus* isolated from 'off-flavour' orange juice (Matsubara *et al.*, 2002), *Alicyclobacillus herbarius* isolated from spoiled herbal tea made from dried flowers of hibiscus (Goto *et al.*, 2002) and *Alicyclobacillus pomorum* isolated from mixed fruit juice (Goto *et al.*, 2003) together with a putative new species (Chen *et al.*, 2006) isolated from an apple juice processing environment.

2.5. *Alicyclobacillus acidoterrestris*

A. acidoterrestris is a thermo-acidophilic, aerobic, spore forming micro-organism which is catalase positive and Gram positive (with a tendency to Gram variability). One endospore per cell is formed under adverse environmental or nutritional conditions. The spores are located centrally, sub-terminally or terminally and may or may not swell the sporangium (Deinhard *et al.*, 1987; Wisotzkey *et al.*, 1992; Walls and Chuyate, 1998; Jensen, 1999). The rod shaped cells are 2.9 μm to 4.3 μm long, and 0.9 μm to 1.0 μm wide, while the oval shaped spores are 1.5 μm to 1.8 μm long and 0.9 μm to 1.0 μm wide (Deinhard *et al.*, 1987). This acidophilic bacillus does not grow on neutral media; however on Orange Serum Agar (OSA) *A. acidoterrestris* forms distinctive, easily recognisable, translucent, cream coloured colonies, round with flat interiors and raised centres that become darker and more opaque and umbonate with age (Jensen, 1999). On *B. acidocaldarius* medium (BAM) (Deinhard *et al.*, 1987) and

K agar (Walls and Chuyate, 1998) colonies are similarly described with colony size after 5 to 6 days being 3mm to 5mm in diameter.

The optimum temperature for growth of *A. acidoterrestris* has been variously reported as 42°C to 53°C (Pontius *et al.*, 1998; Walls and Chuyate, 1998), 26°C to 50°C (Borlinghaus and Engel, 1997) and 25°C to 60°C (Yamazaki *et al.*, 1997). Spores are able to germinate and grow at pH < 4, and have D values ranging from 16 minutes to 23 minutes at 90°C, greater than the usual pasteurisation treatments used in juice processes (Walls and Chuyate, 1998; Eiroa *et al.*, 1999; Orr *et al.*, 2000). Only small numbers are required to contaminate large volumes of juice as demonstrated by two reported spoilage incidents. In one incident, 14 cfu to 21 cfu in 20ml were recovered from concentrates used to produce a carbonated drink that had an 'off-odour', with the finished product containing 1cfu to 500 cfu/20ml (Pettipher and Osmundson, 2000). In the other, 76 cfu were recovered from 236ml of a pasteurised apple juice and a hot-filled apple-cranberry juice blend (Splittstoesser *et al.*, 1994). *A. acidoterrestris* is difficult to detect in routine laboratory testing of raw materials as there is no substantial change in pH and no extensive gas production in the affected juice. However, odour and flavour taints can develop in shelf-stable products (Splittstoesser *et al.*, 1994; Pettipher *et al.*, 1997; Jensen, 1999).

2.6. Isolation techniques for *Alicyclobacillus acidoterrestris*

A number of methods have been developed for the detection and isolation of *A. acidoterrestris*, using a variety of media, and utilising various incubation times, temperatures and pH. *Bacillus acidocaldarius* sp. nov. was isolated using an enrichment procedure, involving incubating cultures of thermophilic acidophilic bacteria in a basal medium at temperatures of 45°C, 50°C, 55°C and 60°C (Darland and Brock 1971).

A similar medium *Bacillus acidocaldarius* medium (BAM) has been used to isolate *B. acidoterrestris* from soils. The protocol using BAM, which contains low levels of trace elements and is adjusted to pH4, involves incubation for two days or longer at a temperature of 50°C (Deinhard *et al.*, 1987).

Potato Dextrose agar (PDA) and *Alicyclobacillus acidocaldarius* medium (AAM) have been used to isolate and identify *A. acidoterrestris* from acidic beverages. Using a temperature range of 25°C to 60°C, a pH range of pH2.5 to pH6.0, incubation at 40°C for 3 days and AAM with PDA *A. acidoterrestris* was identified for the first time as a causative agent in the spoilage of acidic beverages in Japan (Yamazaki *et al.*, 1996a).

Since spores of *A. acidoterrestris* are present in only low numbers, direct plating does not provide an adequate method for their detection in fruit juices and juice products. However, a pre-enrichment procedure which includes incubation of heat-shocked (80°C for 10 minutes) samples for 48 hours at 40°C to 50°C prior to surface plating onto OSA produces a more realistic spore count. The importance of using heat-shock as part of the protocol for the detection of

Alicyclobacillus in juices was demonstrated by Pinhatti *et al.*, (1997). After diluting industrialised, concentrated juice to approximately single strength juice and heat-shocking at 80°C for 10 minutes, samples were incubated and plated onto BAM medium after 24 and 48 hours. Some samples incubated for 48 hours after heat-shock proved negative for *Alicyclobacilli*, but all samples plated after 24 hours proved positive. When samples from the 48 hour incubation were subsequently heat-shocked all were positive, suggesting that 48 hours after the initial heat-shock many of the vegetative cells had already sporulated. The results indicated that if heat-shock is not used prior to plating out samples the number of *Alicyclobacilli* will be consistently underestimated (Pinhatti *et al.*, 1997).

In order to determine the growth and heat resistance of *Alicyclobacillus* PDA at pH 5.6 has been used. Plates are enumerated after 5 days incubation at 43°C, or 53°C if samples are thought to contain spores of heat resistant mould. Spores are isolated using acidified PDA (pH 3.5) and pre-heating samples at 60°C for 60 minutes, thus eliminating non-heat resistant micro-organisms. Filtration of samples through a 0.45µm pore sized membrane has been used to culture low numbers of spores from large volumes (100ml to 500ml) of juice, if the juice is filterable (Splittstoesser *et al.*, 1994). To determine whether pre-heating samples may improve the recovery of spores, vegetative cells and spores were suspended for 30 minutes in a solution of 70% ethanol in order to eliminate vegetative cells, samples centrifuged and washed in water before heating to 60°C for 30 minutes. Viable counts doubled following this procedure and suggested pre-heating

(Pinhatti *et al.*, 1997) is a requirement to activate dormant spores (Splittstoesser *et al.*, 1998).

A comparison of BAM, PDA, OSA, tryptone soy agar and nutrient agar for the detection and enumeration of *A. acidoterrestris* demonstrated that nutrient and tryptone soy agar were unable to sustain growth. Of the remaining three that did maintain growth OSA was found to be the most effective giving the optimum recovery using a presence/absence method with a 48 hour pre-incubation period at 44°C, followed by streaking onto solid media and incubation at 44°C for a further 48 hours (Pettipher *et al.*, 1997).

An enumeration method has been developed where 0.2ml of product, diluted 1:6.5 (Pettipher *et al.*, 1997) or 1:10 (Pettipher and Osmundson, 2000) with sterile distilled water, is surface plated directly onto OSA and plates incubated at 44°C for 48 hours, any colonies present are considered as presumptive for *A. acidoterrestris*. This method is insufficient however, to detect very low levels of bacteria in juice concentrates. Consequently a more sensitive method has been developed using membrane filtration (Splittstoesser *et al.*, 1994) and heat treatment. Following filtration the membrane is placed on a suitable growth medium when, after incubation at a pre-determined time and temperature, colony counts are carried out. This method allows the detection of low levels of contamination such as that found by *A. acidoterrestris*. Conversely, if colony counts are expected to be high a small amount of product can first be diluted before filtration. The presence/absence and enumeration methods using heat treatment at 80°C for ten minutes encourages germination and growth of spores,

before surface plating and incubation at 44°C for 48 hours (Pettipher *et al.*, 1997) have been successful in isolating *A. acidoterrestris* in fruit juices from the USA, Europe and China (Pettipher and Osmundson, 2000).

Direct methods such as direct epifluorescence microscopy (DEFT) have also been employed to detect *Alicyclobacillus* spp. Fruit juices are filtered through a 0.6µm nucleopore polycarbonate membrane which is subsequently stained with acridine orange and enumerated by epifluorescence microscopy. Very high numbers ($>5 \times 10^5$ cfu/ml) of rod-shaped bacteria have been reported in carbonated fruit juices using this method (Pettipher and Osmundson, 2000). During this study a method was also developed to detect surface contamination by *A. acidoterrestris* which involved swabbing selected surfaces with an appropriate swab, then stomaching the swab in ultra heat treated (UHT) apple juice, followed by incubation of the juice at 44°C for 48 hours. The juice is then surface plated, or if possible membrane filtered onto OSA, and the plates incubated at 44°C for 48 hours. Any growth would indicate a presumptive positive for *A. acidoterrestris*.

A further method for the isolation of *A. acidoterrestris* from frozen, concentrated orange juice has used heat treatment of 70°C for 20 minutes followed by cooling in an ice bath. The samples are inoculated into double concentrated BAM broth, incubated at 44°C for 48 hours, streaked onto the surface of BAM agar and incubated again at 44°C for 48 hours (Eiroa *et al.*, 1999).

An extensive study using isolates obtained from a variety of sources such as spoiled canned diced tomatoes, several spoiled juices, normal juices, juice

blends and pure cultures and a variety of media including OSA, Tomato juice agar (TJA), Special (TJAS), PDA all adjusted to pH 3.5, 4.0, 4.5 and 5.0 as well as Dextrose Tryptone agar (DTA) at pH 7.4 and K medium (pH 3.7). The study investigated the relationship between media, pH and growth temperature. At 20°C and pH 5.0 all isolates grew but at the same temperature and pH 3.5 only two grew. However, the reverse occurred at 55°C with all isolates growing at pH 3.5 and two growing at pH 5.0. DTA at pH 7.4 produced no growth but more rapid growth was described on K medium at an incubation temperature of 43° than at 35°C. K medium and an incubation temperature of 43°C were selected for further studies involving the effects on the growth and survival of *A. acidoterrestris* in the presence of carbonation and preservatives (Walls and Chuyate, 1998).

Bacteria isolated from internal and external areas of a citrus processing plant have been described as spore-forming thermo-acidophilic rod-shaped (STAR) bacteria. These were isolated from the soil from various countries, orange groves and whole oranges, before and after washing, single strength orange juice, concentrated orange juice and juice from bulk storage containers and condensate water. The isolation medium was the modified broth (ALI) of Cerny *et al.*, (1984) and Darland and Brock (1971). Heat activation of spores occurred by placing samples in a water bath for 20 minutes at 90°C followed by rapid cooling in iced water and incubation at 45°C until turbidity or an off-odour was detected. Samples were surface plated onto ALI agar and incubated at 45°C for 24 to 48 hours. The most interesting of the positive samples were those from the surface of washed fruit. During the thermal processing that is used to produce fruit juice

concentrate, water evaporates from the juice which is then condensed and re-used for purposes including washing of fruit. High bacterial spore counts of samples taken from within the condensate water system would indicate that microorganisms could be inadvertently added to juice being processed. This study used samples from various countries and indicates that STAR bacteria are widespread and are not only present in soils and on fruit surfaces but also in processing environments and juice products. A reduction in the incidence of these bacteria is suggested rather than their complete elimination which has been described as difficult and impractical (Wisse and Parish, 1998).

Originally *Alicyclobacilli* were thought to be fastidious organisms failing to grow on most laboratory media used for isolating bacteria. While *A. cycloheptanicus* does require large quantities of yeast extract for growth, *A. acidoterrestris* will grow on most media, including nutrient agar if the pH has been adjusted to <5.8 and under aerobic incubation. *A. acidoterrestris* grows well and forms distinctive colonies on OSA, which is available commercially and requires none of the tedious preparation of some of the agars previously mentioned (Darland and Brock, 1971; Deinhard *et al.*, 1987; Eiroa *et al.*, 1999; Bahçeci *et al.*, 2003).

An acidified diluent containing a protectant to reduce osmotic shock can be used with OSA for the effective recovery of *A. acidoterrestris* containing 0.1% (w/v) peptone, adjusted to pH 4.0 and containing 0.5% (w/v) glucose, glycerol or sucrose (Jensen, 1999). Filtration of products (Splittstoesser, 1994; Pettipher and Osmundson, 2000) has been suggested as larger volumes of juice (100ml –

500ml) can be tested. Most juices however, cannot be filtered so other methods of isolation must be employed. Heat shocking of samples has been recommended at 80°C for 10 minutes (Pinhatti *et al.*, 1997; Splittstoesser *et al.*, 1998; Wisse and Parish, 1998; Eiroa *et al.*, 1999; Pettipher and Osmundson, 2000), once heat shocking has been completed cooling in an iced water bath (Wisse and Parish, 1998; Eiroa *et al.*, 1999), is not recommended; instead samples should be placed in a water bath at 40°C to avoid further shock to the organism and allow more gradual cooling. As spores are present in only low numbers direct plating would not be an efficient isolation method, therefore an enrichment procedure should be used. Incubation of heated samples for 2 days at 40°C to 50°C prior to surface plating or streaking onto OSA, plates should show visible growth after 2 days but may be left for 5 days if growth is not detected. Although *A. acidoterrestris* forms distinctive easily recognisable colonies on OSA, confirmatory tests must still be performed. Inoculation into sterile fruit juice followed by incubation at 40°C to 50°C for several days may produce a detectable taint. As *A. acidoterrestris* will not grow on neutral media, but will grow on more acidic media, testing for the inability to grow on a medium of pH 7.0 or above as well as the ability to grow at pH 4.0 using OSA (adjusted to pH 4.0) are useful confirmatory tests (Jensen, 1999).

Biochemical profiles have been characterised by several authors and a comparison between the isolates of Dienhard *et al.*, (1987) and DSM 2498 and NCIMB 13137 using API 50 CH strips demonstrates the similarity in profiles (Silva and Gibbs, 2001). It is important however, for the fruit juice industry to be

able to detect the presence of *A. acidoterrestris* both quickly and efficiently, as once it has entered processing areas it can prove particularly difficult to eliminate (Jensen 1999).

Developments of new selective media for the isolation of *A. acidoterrestris* continue and Chang and Kang (2005) have tested SK in comparison with OSA, PDA and K agar. Optimum recovery temperature was 43°C and pH was 4.5, but pH4.0 was selected for trials to decrease the possibility of competition from other bacteria that may grow at this pH. In these trials the new SK agar was found to perform better than PDA, OSA or K agar, being able to detect low levels of contamination and isolating significantly higher numbers of *A. acidoterrestris*. An inconsistency in the performance of K agar was noted which may have been due to difficulties experienced dissolving Tween 80 which may be used to increase recovery rates and aid in colony formation. Tween 80 was observed in sterile agar after autoclaving at 121°C for 15 minutes (Chang and Kang 2005). No mention was made of any problems with the Tween 80 used in SK agar although Tween 80 is added as 1.0g in K agar and as 1.0ml in SK agar which may aid solubility. As with other methods (Darland and Brock, 1971; Deinhard *et al.*, 1987; Wisse and Parish, 1998; Eiroa *et al.*, 1999; Jensen, 1999; Pettipher and Osmundson, 2000; Bahçeci *et al.*, 2003) this technique takes a number of days to complete. Researchers are seeking more time efficient methods to identify *Alicyclobacilli* and several rapid methods have been suggested.

2.7. *Rapid detection methods for A. acidoterrestris*

Rapid methods in microbiology involving improved techniques for the isolation, characterisation and importantly the early detection of micro-organisms have advanced considerably during the last three decades (Fung, 2002). Similarly, methods for the detection of *A. acidoterrestris* have improved however, most culture techniques still require at least four days incubation, generally followed by a series of biochemical phenotypic confirmatory tests (Pettipher *et al.*, 1997; Jensen, 1999; Pettipher and Osmundson, 2000; Silva and Gibbs, 2001) with varying efficiencies. Rapid and specific methods of detection for the screening of raw materials quickly before they reach the production line allow time for substitution by uncontaminated materials and the prompt return of contaminated materials to the supplier, thus saving time, warehouse space and money. Final products may also be screened more quickly allowing steps to be taken for the removal of contaminated material from the production line thus minimising waiting times and inconvenience to the customer.

Several molecular methods have been developed for the identification of *Alicyclobacilli*. Randomly amplified polymorphic DNA (RAPD) has been used to discriminate *A. acidoterrestris* from other thermo-acidophilic bacteria. Thermo-acidophilic isolates from food samples and a variety of environments have been identified using standard biochemical and morphological tests (Yamazaki *et al.*, 1996a) and RAPD assay. Although the results from RAPD in the study (Yamazaki *et al.*, 1997) were identical to the standard techniques, the RAPD procedure allowed identification within six hours of the isolation of suspected *A.*

acidoterrestris colonies, considerably reducing the time taken for isolation and identification. The use of reverse transcription-polymerase chain reaction (RT-PCR) as a rapid technique for detecting *Alicyclobacilli* was investigated by Yamazaki *et al.*, (1996b). A detection time of 24 hours may be accomplished with this method and specific primers V2 and V4 for the detection of *Alicyclobacilli* were reported (Yamazaki *et al.*, 1996b). Luo *et al.*, (2004) have developed the first real-time (PCR)-based rapid method for detecting *Alicyclobacillus* spp. using the squalene-hopene cyclase-encoding gene (SHC), a key enzyme in the biosynthesis of hopanoids, important membrane constituents of *Alicyclobacillus* spp. Although this method may be accomplished within three hours to five hours and detection levels have been reported to be less than 10 cells per PCR reaction this assay was only designed for detecting *A. acidocaldarius* and *A. acidoterrestris*. However, more species have now been added to the *Alicyclobacillus* genus (Nicolaus *et al.*, 1998; Albuquerque *et al.*, 2000; Matsubara *et al.*, 2002; Goto *et al.*, 2002; Goto *et al.*, 2003; Tsuruoka *et al.*, 2003; Simbahan *et al.*, 2004; Karavaiko *et al.*, 2005; Chen *et al.*, 2006), therefore the present system requires enlarging to accommodate additional species that may be the cause of spoilage. An expanded assay based on the 16S rRNA gene sequence has been developed and identical primer and probe sequences were identified not only from the species tested, *A. acidocaldarius*, *A. acidoterrestris* and *A. cycloheptanicus*, but also from the newly discovered species *A. hesperidum*, *A. herbarius*, *A. acidiphilus* and *A. sendaiensis* at a detection rate of less than 100 *Alicyclobacilli* cells which can be completed within five hours. This method can

also be used as an indicator of the microbial quality of raw material juices as it is capable of indicating the presence of high numbers of other related bacteria as well as *Alicyclobacilli* spp. and so could be of particular use to the fruit juice/beverage industry (Connor *et al.*, 2005).

Real-time PCR entails the use of specifically designed reagents and instrumentation which although producing precise results involves considerable expense (Li and Drake, 2001). Semiquantitative (RT-PCR) used in conjunction with the Agilent 2100 bioanalyser and the DNA 1000 LabChip kit, is the first commercially available system to utilise chip-based nucleic acid separation technology. This system automatically separates and analyses nucleic acid samples by capillary electrophoresis and detection by laser induced fluorescence. Evaluation of contamination can be achieved within 24 hours, substantially reducing the time for detection by culture techniques with detection limits of about 1cfu/ml (Funes-Huacca *et al.*, 2004).

Fourier Transform Infrared (FT-IR) spectroscopy incorporated with multivariate statistical methods has been used to detect and differentiate *Escherichia coli* O157:H7 cells from *Alicyclobacillus* spp. isolated from apple juice (Al-Qadiri *et al.*, 2006). The system is based on subtle differences in cell wall composition between strains and has been used to differentiate between eight *Alicyclobacillus* strains in apple juice, two of which were closely related. This method can also be used to discriminate between guaiacol and non-guaiacol producing species based on spectral features although it is still in development

phase and requires a comprehensive spectral database to be developed (Lin *et al.*, 2005).

Several 'kits' have been produced that vary in the number of hours (three hours to forty eight hours) for detection time. Although the number of *Alicyclobacilli* cells is often very low in number (Pinhatti *et al.*, 1997; Splittstoesser *et al.*, 1998; Jensen, 1999; Pettipher and Osmundson, 2000; Zierler *et al.*, 2004) one method involves diluting juice concentrates then adding just 1ml of this solution to a specific container followed by incubation for 48 hours, followed by a verification procedure. However due to the small amount of sample tested it is possible to produce false negatives. Another method that takes just three hours to complete only identifies live bacteria although *Alicyclobacilli* are known to survive as spores particularly in juice concentrates that require heat shock to induce growth (Pinhatti *et al.*, 1997; Splittstoesser *et al.*, 1998; Wisse, and Parish, 1998; Eiroa *et al.*, 1999; Pettipher and Osmundson, 2000). Another detection kit uses the presence of guaiacol to detect *Alicyclobacilli* within 2 to 3 days; however, some strains of *Alicyclobacilli* do not form guaiacol (Lin *et al.*, 2005) so this kit may also produce false negatives.

Spoilage off-flavours caused by *Alicyclobacilli* are one of the main problems of contamination by these bacteria, although by the time this off-odour is detected it is too late to prevent the loss of product. Detection of the spoilage compounds, especially at very low levels can provide valuable information to the manufacturer, possibly preventing an expensive product recall. *A. acidoterrestris*

and *Actinomycetes* both produce compounds that may lead to a musty or medicinal off-flavour in apple juice. A method has been validated to detect these 'off-flavours' using gas chromatography-mass spectrometry (GC-MS) following sample preparation/extraction by solid phase micro extraction (SPME). This sensitive method has a limit of detection (LOD) of $0.08\mu\text{g L}^{-1}$ to $7.7\mu\text{g L}^{-1}$ and limits of Quantification (LOQ) of $0.27\mu\text{g L}^{-1}$ to $25\mu\text{g L}^{-1}$, dependent upon compound. The most common spoilage compounds in this study were guaiacol and 2,6-dibromophenol with a LOD of 0.29 and 0.08 respectively, and a LOQ of 1.06 and 0.27 respectively (Zierler *et al.*, 2004).

When *Alicyclobacilli* were first detected there was little choice of isolation method, now more and varied techniques have been developed and each requires careful investigation before a preferred method is selected. Choice will depend on cost-effectiveness and ease of use together with the ability to replicate results with confidence. Sensitive detection methods must be employed for the low number of spores (Jensen, 1999) that may be present to prevent spoilage.

2.8. Spoilage by *Alicyclobacillus acidoterrestris*

The main spoilage characteristic of a 'medicinal' or 'phenolic' offensive smell (Borlinghaus and Engel, 1997; Walls and Chuyate, 1998) was recognised by Yamazaki *et al.*, (1996a) as guaiacol formed from vanillin by this bacterium. Another precursor for the formation of guaiacol is tyrosine of which apple juice contains approximately $4.1\mu\text{g tyrosine/ml}$ of juice. Heat shock, storage

temperature and oxygen concentration are important factors in the formation of guaiacol from tyrosine (Chang and Kang, 2004) and relate to spoilage characteristics. A report by Borlinghaus and Engel (1997) described the flavour taint compound as 2,6-dibromophenol occurring in parts per trillion (ppt) and suggested that spoiled juices may have an increased turbidity and white sediment occurring at the bottom of product.

Bromophenols and chlorophenols are compounds known within the food industry to instil 'disinfectant' like taints in food. *A. acidoterrestris* has been implicated in the production of food taints but trace quantities of these halophenols can easily be formed in the presence of some sanitizers (Saxby, 1996). In one study, in order to identify whether *A. acidoterrestris* was the actual cause of taints, two fruit juice drinks with a medicinal or disinfectant taint were analysed for the presence of micro-organisms and halophenols. Low levels of Gram positive, catalase positive spore-forming bacteria were isolated from both spoiled samples of juice drink, but not from control juices. Cell membrane lipids identified the bacteria as from the genus *Alicyclobacillus*, and following 16S rDNA sequencing of the isolates two strains of *A. acidoterrestris* were identified. Concentrations of 2,6-dibromophenol were within the range of 2 to 4ng l⁻¹, and for 2,6-dichlorophenol was 16 to 20ng l⁻¹. These compounds were not detected in control samples. The taste threshold of 2,6-dibromophenol in water is 0.5ng l⁻¹ (Whitfield *et al.*, 1988) and 2,6-dichlorophenol in water is 6.2ng l⁻¹ (Young *et al.*, 1996), and at these levels would have contributed to the taint in the juice drinks (Jensen and Whitfield, 2003).

2.9. *A. acidoterrestris* a non-pathogen

There is no evidence that *A. acidoterrestris* may pose a human health risk. Neither the organism nor its metabolites have been associated with any form of poisoning. Following discussions with experts, Borlinghaus and Engel (1997) declared *A. acidoterrestris* a non-pathogen. Other investigations have been conducted to provide evidence that this bacterium is not a health risk. A study using mice injected with spores of *A. acidoterrestris* and guinea pigs fed inoculated fruit juices that were cloudy with a pronounced odour and contained 5×10^6 cfu/ml *A. acidoterrestris* demonstrated that none of the animals exhibited any illness symptoms or died at the levels tested (Walls and Chuyate, 2000).

2.10. Spoilage incidents caused by *A. acidoterrestris*

A. acidoterrestris, originally thought to be only of academic interest, was implicated in a large scale contamination of aseptically packed apple juice in Germany (Cerny *et al.*, 1984) followed by several spoilage incidents in Europe, USA and Japan in the 1990s. Spoilage was then thought to happen only occasionally, but spoilage of shelf stable apple juice is now reported more frequently. More diverse spoilage incidents have occurred, for example in shelf stable iced tea containing berry juice and in the ingredients of rose hip and hibiscus teas (Duong and Jensen, 2000), a carbonated fruit drink (Pettipher and Osmundson, 2000) and diced canned tomatoes (Chang and Kang, 2004). Slow cooling of hot-filled products may allow for any spores present to continue to

grow. Spoilage incidents have occurred in Europe when large volumes of hot-filled products were allowed to cool naturally during a hot summer (Pinhatti *et al.*, 1997). Incidents are increasing not only as more varied products are found to be contaminated but also as better isolation techniques are described. The fruit juice industry now acknowledges *Alicyclobacillus* spp. as a major quality control target micro-organism (Chang and Kang, 2004).

2.11. Prevention of spoilage by *A. acidoterrestris*

A. acidoterrestris is already proven to be a significant challenge to the fruit juice industry and may be present in a variety of fruit juices and processed products in extremely low numbers. This micro-organism has been demonstrated to survive the pasteurisation process applied to fruit juices and to have the ability to grow in the 'commercially sterile' environment created and cause spoilage of the product. This is of particular significance to the retail outlet juice industry that uses 100% pasteurised juice and no preservative.

Growth of *A. acidoterrestris* may be affected by components within the juice itself. Apple, tomato and white grape juices are especially liable to contamination whereas red grape juice does not support growth probably due to the presence in the juice of natural phenolic compounds (Splittstoesser, 1998). Resveratrol and ferulic acid at 50µg/ml and 150µg/ml respectively are two grape polyphenols that inhibit the growth of *A. acidoterrestris* and a synergistic effect

between polyphenols such as catechin-gallate and proanthocyanidine has been suggested by Oita and Kohyama (2002).

Alicyclobacilli are known to survive in high acidity so the pH of juices is important (Splittstoesser *et al.*, 1994; Yamazaki *et al.*, 1996b; Pettipher *et al.*, 1997; Walls and Chuyate, 1998; Pettipher and Osmundson., 2000). Inhibition of growth has also been exhibited when concentrations of ethanol exceed 6% or sugar content exceeds 18° Brix, with Brix expressed in terms of percentage sucrose content. However, increasing the Brix of juices also increases the heat resistance of *A. acidoterrestris* suggesting that destroying spores in a juice concentrate would be more difficult than in a single strength juice (Splittstoesser, 1998).

Cerny *et al.*, (2000) observed several known parameters of *Alicyclobacilli*, for example they do not grow well below 25°, but the distribution of refrigerated or chilled juice may cause a notable price increase as refrigerated transport would be required. Although raising the temperature at pasteurisation to sterilisation levels will prevent spoilage this may cause an important loss of juice quality. *A. acidoterrestris* is aerobic so growth may be prevented under anaerobic conditions. Therefore a study was undertaken to investigate reducing the amount of oxygen available in juices and how altering the redox potential may affect the growth of *Alicyclobacilli*. Ascorbic acid is a strong reductant and is able to reduce active and stable oxygen (Niki 1991). 10mg of ascorbic acid added to apple juice was found to stimulate growth of *Alicyclobacilli* but 15mg inhibited growth. The amount of ascorbic acid in apple juice was increased from 1.4 mg/100mL to 31.4

mg/100mL; the original redox-potential of the apple juice was +472mV, after the addition of ascorbic acid the redox-potential measured +374mV. This illustrates the important role played by the redox-potential in juice spoilage concerning *Alicyclobacilli* (Cerny *et al.*, 2000).

Due to the thermal resistance of *A. acidoterrestris* spores it has been suggested that, if this micro-organism becomes a target spoilage organism, processes must be designed to eliminate the spores (Murakami *et al.*, 1998; Silva *et al.*, 1999; Silva and Gibbs, 2001). In order to accomplish adequate processing, D-values must be measured in fruit juices rather than based on broth culture models, since primarily D-values measured in broth are lower than those measured in juices leading to the design of inappropriate pasteurisation processes (Silva *et al.*, 1999). A comparison of the measurement of thermal kinetic parameters of *A. acidoterrestris* by the isothermal method (IM) under batch heating and by the paired equivalent isothermal exposures (PEIE) method under non-isothermal continuous conditions demonstrated that the (PEIE) method provides a more feasible D-value when designing a thermal process for a continuous system and if not used, the risk exists of under-designing the process thus there is a danger that the desired level of spore inactivation will not be achieved and spoilage subsequently occurring (Vieira *et al.*, 2002).

2.12. Pasteurisation in the fruit juice industry

All fruits and vegetables lose quality after harvesting. How much quality is lost depends on several factors such as the initial quality of the fruit, whether it was picked from the plant or if it had previously fallen to the ground or was mechanically harvested. An estimated world-wide loss of approximately 50% of fresh fruits and vegetables has been reported due to postharvest spoilage micro-organisms and fungal pathogens (El Ghaouth, 1997). Stabilising the product by removing micro-organisms that may cause fermentation or spoilage using pasteurisation may help minimise the loss of quality (Downes, 1990).

Comminuted citrus juice (where the whole fruit is used in the product) is unstable under pasteurisation conditions and will in a few days separate into a clear liquid and pulp sediment. Higher pasteurisation temperatures are required to destroy enzymes that are responsible for the loss of cloud in the juice. Cloud is an important attribute of whole juices; comminuted bases are cloudier than whole juices due to the presence of cell fragments of juice sacs, peel oil and needle-like crystals of hesperidin (Houghton, 1984). Cruess (1914) suggested a heat inactivated clearing and clotting enzyme might be present in the juice and in 1933 trials were conducted on cloud retention in citrus juices pasteurised at various temperatures and time periods. These demonstrated that heating citrus juice for eight to ten minutes at 85°C or heating to 90°C for three minutes then cooling was sufficient to ensure pasteurisation and stability of the juice over the maximum storage time of seventeen months. At lower temperatures the same results were observed but only over an eight month period. Navel oranges required around

fifty per cent less heat input to ensure the same results and enzyme inactivity was found to be more efficient at a pH2.5 than at pH4 in all juices tested (Joslyn and Sedky, 1940).

Pasteurised products must be protected against post pasteurisation spoilage. However, at all of the temperatures considered in the Joslyn and Sedky (1940) study neither of the two spoilage micro-organisms under study would have been de-activated. Both *A. acidoterrestris* and *P. cyclohexanicum* have been isolated from spoiled pasteurised juices, suggesting that pasteurisation should be combined with other preservation methods to ensure adequate safety from spoilage by these bacteria.

2.13. Further methods to prevent spoilage by *A. acidoterrestris*

Trials of two preservatives, sorbic acid and benzoic acid, alone and in combination and the use of carbonation in unpreserved soft drinks have prevented spoilage by *A. acidoterrestris* (Pettipher and Osmundson, 2000). Benzoic acid with its broad antibacterial range and non-volatility has been widely used as a fruit beverage preservative. Sodium benzoate is frequently used as it is more water soluble than benzoic acid and its antimicrobial activity develops from the undissociated acid which declines as the pH level increases, (Batchelor, 1984). Although effective against yeasts and bacteria this preservative can be degraded by some bacteria for example *Burkholderia cepacia* (Philippe, *et al.*, 2001) and

some yeasts such as *Zygosaccharomyces baillii* (Leyva and Peinado, 2005) may become resistant to benzoic acid (Batchelor, 1984).

The antimicrobial activity of sorbic acid (frequently used as potassium sorbate) also comes from the undissociated acid but, unlike benzoic acid, this is less affected by pH and consequently can be used at pH levels higher than 3.0. Sorbic acid is also effective against bacteria and yeasts and, like benzoic acid, some yeasts can become resistant to it, for example *Zygosaccharomyces lentus* (Steels *et al.*, 1999). The use of both benzoic and sorbic acids are strictly regulated and levels of no more than 1500 mg/kg singly or in combination are permitted in flavourings (European Parliament, 2004).

The use of sulphur dioxide (Splittstoesser *et al.*, 1998) in non-alcoholic flavoured drinks containing fruit juice is also strictly regulated and only 20mg/kg or 20mg/l is permitted and then only as a carry over from fruit juice concentrates (HMSO, 1996). Therefore, as concentrations of sulphur dioxide and sorbic acid at 100mg/l were unsuccessful in sensitizing *Alicyclobacillus* spores to heat (Splittstoesser *et al.*, 1998) at the present permitted levels, sulphur dioxide would not be effective.

Although carbonation seems to prevent growth of both vegetative cells and spores, a spoilage incident has occurred involving *A. acidoterrestris* and a carbonated fruit drink where low levels of *A. acidoterrestris* were found in the concentrated juices used to make the carbonated drink as well as the soft drink itself. A suggestion is that, following dilution of the juice concentrates, *A. acidoterrestris* increased to levels able to cause taint (43ppb of guaiacol) and

vegetative cells but not spores were killed during the carbonation process (Pettipher and Osmundson, 2000).

Calcium lactate is used in the fruit juice industry to fortify fruit juice and has been shown to effectively inhibit the growth of *A. acidoterrestris*. Calcium lactate has been added to commercial unfortified orange juice, the juice adjusted to pH3.6 or pH4.1 and, following inoculation with *A. acidoterrestris* and other micro-organisms, the juice was incubated at either 4°C or 10°C. The results demonstrated that, in all juice stored at 4°C and in the pH3.6 juice stored at 10°C, growth of *A. acidoterrestris* was inhibited, but at 10°C and pH4.1 juice fortified with calcium lactate at concentrations equivalent to 0 and 5% of the dietary reference intake (DRI), the organism was able to grow. However, although inhibition occurs under these conditions the population remains constant so that if, for example, temperature abuse occurs then the organism might still be able to grow and subsequently produce spoilage (Yeh *et al.*, 2004).

Bacteriocins are produced by many bacteria and generally possess anti-microbial activity against only closely related species. However, the bacteriocin nisin, produced by *Lactococcus lactis* subsp *lactis*, exhibits anti-microbial activity against a broad range of Gram positive bacteria (Delves-Broughton, 1997), including *A. acidoterrestris* (Komitopoulou *et al.*, 1999). It is also active against Gram negative bacteria if the outer membrane is treated with, for example, a chelating agent, such as EDTA (Boziaris and Adams, 1999; Cutter and Siragusa, 1995; Delves-Broughton, 1993).

Yamazaki *et al.*, (2000) demonstrated that the effectiveness of nisin against *A. acidoterrestris* depends on the strain of organism used and the pH and type of juice. The MIC of nisin was higher for vegetative cells (1.5 IU/ml to 50 IU/ml at pH3.4 and 25 IU/ml to 100 IU/ml at pH4.2) than spores (<0.78-12.5 at pH3.4 and 25-100 at pH4.2). Outgrowth of spores was shown to be inhibited by 25 IU/ml to 50 IU/ml in orange and mixed fruit drinks, but not by 600 IU/ml in clear apple juice. The presence of nisin contributes to the reduction of thermal resistance of spores suggesting a 'multiple hurdle' approach is an effective means of control. The presence of nisin during heat treatment can decrease the D value by up to 40% and the MIC for nisin against spores at 25°C is 5IU/ml in apple, orange and grapefruit juices, indicating the usefulness of nisin in controlling this organism in fruit juices and fruit juice products (Komitopoulou *et al.*, 1999).

Enterocin AS-48, produced by *Enterococcus faecalis* A-48-32, is also a broad spectrum cyclic peptide similar in action to nisin and may be considered an alternative to nisin in food biopreservation. At a concentration of 2.5µg/ml enterocin AS-48 has a relatively immediate effect (no viable cells detected at 15 minutes) which is sustained for 60-90 days, dependent on juice, temperature of storage and whether inoculum contained vegetative cells or spores (Grande *et al.*, 2005).

In apple juice a combination of high temperature, time and high pressure eliminates spores so that either 207MPa at 45°C for 10 minutes or at 71°C for 1 minute results in more than a 3.5 log reduction in viable spore numbers; 414MPa or 621MPa at 71°C for 1 minute results in >4 log reduction and 414MPa or

621MPa at 71°C for 10 minutes results in no detectable spore counts, a reduction of >5.5 logs (Lee *et al.*, 2002). The activation of *A. acidoterrestris* by high pressure at different temperatures can be described by a Weibull frequency distribution model and such a pressure-temperature inactivation model, could, it has been suggested, form the engineering basis for the design, optimisation and evaluation of such processes as a preservation technique in the fruit juice industry (Buzrul *et al.*, 2005). High hydrostatic pressure is also effective at inactivating vegetative cells, both in a model broth (BAM) system and in orange, apple and tomato juice. The use of hydrostatic pressure enhances the effect of high temperature alone so that a 4.7 log reduction is seen using 350MPa at 50°C for 20 minutes whereas thermal treatment of 50°C for 20 minutes alone produces a 1.13 log reduction. In juices a similar (<4.0) log reduction was achieved immediately after pressurisation. However on storage at 30°C for three weeks vegetative cells had increased indicating that either a proportion of vegetative cells are able to multiply or that spores formed during the pressurisation procedure may germinate, multiply and subsequently cause spoilage (Alpas *et al.*, 2003).

Another combination, that of temperature and electron-beam and gamma-ray irradiation, has also been shown to be effective in activating spores in dry materials such as dextrin powder used in citrus juice processing. Pre-irradiation reduced the duration of the heat treatment required to produce inactivation. In spore-inoculated juices treated with 1-2 kGy radiation followed by heating at 95°C for 20 minutes no spores could be detected at 7 days at either 25°C or 45°C whereas in samples irradiated with 1-2 kGy but not heat treated between log1.88

and log 4.79 cfu/ml were isolated, suggesting that effect is maintained over at least seven days storage (Nakauma *et al.*, 2004). However consumers, particularly those in Europe, have an antithesis against any food product that has been irradiated which would make it difficult to market in such circumstances.

A further possibility for the inhibition of *A. acidoterrestris* in fruit juices is by developing antimicrobial films for packaging the final product. Such developments are lending themselves to an emerging area of research in the food industry. The active components of such films may be inorganic or organic in origin. The latter are generally based on metal ions such as silver, copper and platinum. Silver ions, in particular, are suited to a wide range of applications including appliances, building products, medical devices, water filtration and food packaging. In the USA the FDA allow the use of a silver ion based food contact substance (AgION®) to be added to food contact polymers. An active film obtained by depositing via plasma a silver-containing polyethyleneoxide-like coating on a polyethylene layer is effective in inhibiting the growth of *A. acidoterrestris* vegetative cells in apple juice with the effect being directly related to the amount of silver released into the juice (Del Nobile *et al.*, 2004).

The ultimate source of *A. acidoterrestris* is soils (Darland and Brock, 1971; Hippchen *et al.*, 1981; Deinhard, 1987 and Norris, 1996) and the organism enters the processing areas most probably on fruit surfaces contaminated from soil during harvesting. Thus, treatment of fruit to reduce contamination before it enters the processing plant is one method of reducing risk of spoilage. Aqueous chlorine dioxide has been used to wash fruit prior to processing. Treatment with

chlorine dioxide for 5 minutes at 40ppm reduced spores by more than 4 log in aqueous solution. On apple surfaces 40ppm reduced spore viability by >4.8 log after five minutes while 120 ppm had a similar effect after just one minute (Lee *et al.*, 2004). Using high, medium and high release chlorine dioxide sachets to measure the effective of the gas on *A. acidoterrestris* spores (Lee *et al.*, 2006) demonstrated that, although high and medium release sachets produced a >5 log reduction in spores, which were therefore undetectable, the visible effects on the fruit were unacceptable. However, low release sachets did produce a measurable effect giving a 2.7log, 3.7log and 4.5 log reductions after 1hour, 2hours and 3 hours exposure respectively, with no visual quality effect on the fruit.

2.14. Conclusion

Although *A. acidoterrestris* and its spores does present a threat to the fruit juice industry in terms of spoilage, contamination may be controlled by monitoring processing regimes and applying HACCP procedures focussing on the control of spores in particular. However, there remain a number of areas that require further research. A robust and well validated isolation protocol needs to be developed in order to assess the extent of contamination within the processing arena by both *A. acidoterrestris*, and other *Alicyclobacillus* spp. that may prove important in terms of spoilage in the future. Rapid methods that reduce time from sampling to identification need to be able to be used in an industrial setting and therefore to be inexpensive, reliable and not time-consuming.

Propionibacterium cyclohexanicum

2.15. Introduction

In 1993 an acid tolerant, non spore-forming coryneform bacterium was isolated from spoiled 'off flavour' orange juice. In common with the *Alicyclobacillus* genus, this strain (TA-12^T) had ω -cyclohexyl undecanoic acid which represented 52.7% of the total cellular fatty acid, but unlike *A. acidoterrestris*, this organism did not form spores. Growth of the isolate was reported to occur at pH 3.2 to pH7.5, with an optimum range of pH 5.5 to pH6.5; the heat resistant cells are reported to be able to withstand a temperature of 90°C for 10 minutes (Kusano, *et al.*, 1997).

2.16. *Propionibacteria* spp

Propionibacteria belong to the class Actinobacteria (high guanine + cytosine content) and the order Actinomycetales defined by Stackebrandt *et al.*, (1997) and comprise two main groups; the cutaneous or acnes group and the classical or dairy propionibacteria (Glatz, 1992). The two groups are found in different habitats; the cutaneous group can be found on the human body and some strains, for example, *Propionibacterium acnes*, and *Propionibacterium granulosum* are involved in certain human diseases, for example, acne vulgaris (Loveckova and Havlikova, 2002). The classical species have been isolated from soil, silage, brines for olive fermentation and rum distilleries and are also found in

milk and dairy products (Cummins and Johnson, 1986). The classical *Propionibacteria* comprise *Propionibacterium freudenreichii*, *Propionibacterium jensenii*, *Propionibacterium theonii* and *Propionibacterium acidipropionici* (Glatz, 1992). *Propionibacteria* ferment sugars and lactic acid to propionic acid, acetic acid and carbon dioxide and are used extensively as propionic acid producers in industry (Ozadali *et al.*, 1996).

2.17. Initial Isolation of *Propionibacterium* genus

The *Propionibacterium* genus was initially isolated from Swiss cheese in 1906 by von Freudenreich and Orla-Jenson, and is important in the dairy industry (Glatz, 1992). Propionic and acetic acid contribute to the flavour of the cheese, producing the characteristic 'nutty' flavour, while the carbon dioxide is responsible for the 'eyes', the distinctive holes in the cheese (Malik *et al.*, 1968; Glatz, 1992). *P. freudenreichii* is used as a starter in Emmental (Swiss) cheese; these bacteria grow in the 'warm' room at 24°C during the ripening process (Meurice *et al.*, 2004). *Propionibacteria* have also been used in industry in the production of vitamin B₁₂, now however faster growing *Pseudomonas* strains are used (Glatz, 1992).

When propionibacteria are grown in a medium such as milk or cheese whey the whole product can be dried, along with any antimicrobial substances and used as a natural preservative. Microgard is a grade A skim milk fermented by *Propionibacterium freudenreichii* subsp. *shermanii* and is used in approximately

30% of cottage cheese produced in the USA. Microgard inhibits the growth of some yeast and moulds, gram-negative species and heterofermentative lactic acid bacteria. This antimicrobial activity can be attributed to propionic, acetic and lactic acids, diacetyl and a heat-stable bacteriocin (Glatz, 1992; Faye *et al.*, 2000). It has not been absolutely established however, that a bacteriocin is the active ingredient in Microgard (Faye *et al.*, 2000).

Bacteria used in food technology such as *P. freudenreichii* are subjected to abiotic stresses surviving by adapting to environmental changes, for example temperature and pH and the presence of toxic compounds. This in turn leads to greater tolerance of these environments. Exposure to sub-lethal acidic conditions can activate an adaptive reaction that can afford protection if a lethal exposure later occurs; this adaptation is known as acid tolerance response (ATR) (Jan *et al.*, 2001). An ATR mechanism has been identified in a number of bacteria such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* spp. and survival studies of *E. coli* and *Salmonella* spp. have shown that they have a range of interlinked genetic regulatory networks that allow adaptation of cells to oxidative, osmotic, acid and thermal stress (Bearson *et al.*, 1998; Brudzinski and Harrison, 1998; Foster, 1995; Garren *et al.*, 1998; Lee *et al.*, 2002; O'Driscoll *et al.*, 1996). An ATR, involving the synthesis of *de novo* polypeptides, has been demonstrated in *P. freudenreichii* in response to acid or bile salts (Jan *et al.*, 2000; Jan *et al.*, 2001; Leverrier *et al.*, 2003).

As well as their use in the dairy industry propionibacteria are considered probiotics because of their capacity to inhibit the growth of undesirable flora

(Lyon *et al.*, 1993) and to modify enzyme activities in the gut and stimulate the growth of *Bifidobacteria* (Bougle *et al.*, 1999). When cultures of *P. freudenreichii* and *Bifidobacterium longum* were simultaneously inoculated into whey and Trypticase Peptone Yeast medium (TPY), higher antimicrobial activity was recorded with the two cultures than with *B. longum* alone (Taniguchi *et al.*, 1998).

2.18. Initial Isolation of *Propionibacterium cyclohexanicum*

Propionibacterium freudenreichii has been considered the most heat resistant of the propionibacteria (Malik *et al.*, 1968), surviving 62.8°C for thirty minutes. In comparison with *P. freudenreichii*, the heat resistance of the newly discovered bacillus (TA-12^T) is very high. Tests on *P. freudenreichii* to further assess its heat resistance revealed its ability to survive 80°C for ten minutes; however survival failed to occur at 90°C for ten minutes. It is possible that TA-12^T could survive the pasteurisation process applied to fruit juices. The fact that it was isolated from pasteurised orange juice, verifies this as a possibility and therefore the heat resistance of the bacterium (Kusano, *et al.*, 1997).

The fermentation products fermented by TA-12^T are lactic, propionic and acetic acids and are produced at a molar ratio of 5:4:2 respectively, in comparison with *P. freudenreichii* that ferments glucose to propionic and acetic acids only. *Propionibacterium propionicus* of the cutaneous group is the only other species of this genus to produce lactic acid. The G + C content of TA-12^T was 66.8mol% and comparative sequence analyses on the 16S rRNA gene values for TA-12^T showed

a similarity level of 97.1% with *P. freudenreichii* and with other propionibacteria similarity levels of 95%. Strain TA-12^T was therefore revealed to be genetically and phenotypically distinct from other propionibacteria and the name *Propionibacterium cyclohexanicum* sp. nov. was proposed (Kusano *et al.*, 1997). This represents along with *Alicyclobacilli* and *Curtobacterium pusillum* (Suzuki *et al.*, 1981) a third group of organisms with cell membranes that contain ω -cyclohexane fatty acids (Kusano *et al.*, 1997).

2.19. *Propionibacterium cyclohexanicum*

P. cyclohexanicum is a Gram positive, acid tolerant, aero-tolerant, heat resistant and non-motile, pleomorphic, rod-shaped bacterium. The cells are catalase and oxidase negative, 1.5 μm to 3.0 μm in length, 1.1 μm to 1.6 μm in width, with some cells club shaped or bent. It is non-spore forming and no specialised structures have been noted during spore staining or by microscopic examination. *P. cyclohexanicum* grows at temperatures of 20°C to 40°C with a reported optimum temperature of 35°C and a pH range of pH3.2 to pH7.5 (optimum pH5.5 to pH6.5). The circular, white to creamy, translucent colonies are 0.2mm to 0.5mm in diameter when grown anaerobically for three days on the surface of peptone-yeast extract-glucose (PYG) medium (Kusano, *et al.*, 1997).

2.20. Spoilage by *Propionibacterium cyclohexanicum*

Only one spoilage incident has so far been documented involving *P. cyclohexanicum* this occurred when pasteurised and packaged orange juice developed an 'off-flavour' (Kusano, *et al.*, 1997). The nature of this incident however, has implications for the fruit juice industry as this micro-organism was isolated from a heat treated product thought to be free of spoilage organisms.

2.21. Conclusion

P. cyclohexanicum has some similar characteristics to *A. acidoterrestris*, for example it has ω -cyclohexyl lipids as a major component of the cell membrane and the ability to tolerate high temperatures and high acidity. However in contrast to *A. acidoterrestris*, *P. cyclohexanicum* does not form spores and is aerotolerant.

A. acidoterrestris is already proving to be a significant challenge to the fruit juice and soft drinks industry and, due to its similarity to *A. acidoterrestris*, *P. cyclohexanicum* may also become a problem.

2.22 Aims

- To investigate suitable isolation techniques for *A. acidoterrestris* and monitor the incidence of *A. acidoterrestris* and *P. cyclohexanicum* in a range of raw materials and shelf stable products

- To assess the growth of *A. acidoterrestris* and *P. cyclohexanicum* in a range of fruit juices and monitor the survival of *P. cyclohexanicum* during pasteurisation and at various temperatures above and below the reported optimum temperature.
- To monitor the effects of preservatives such as sodium benzoate and potassium sorbate and the bacteriocin nisin and their possible use in controlling *A. acidoterrestris* and *P. cyclohexanicum*.
- To investigate the effects on *A. acidoterrestris* at different temperatures during storage in apple juice with varying amounts of container headspace and monitoring the movement or agitation of containers.
- To assess the effects on the growth of *A. acidoterrestris* and *P. cyclohexanicum* when cultured together in fruit juice and to monitor any inhibitory reactions by either or both micro-organisms.

Chapter 3

Methods

3.1. *Materials*

All agar and agar components used were obtained from either Merck KGaA 64271 Darmstadt, Germany or Oxoid Ltd., Basingstoke, Hampshire, UK. Chemicals were obtained from Prolabo Merck Eurolab Z.I. de Vaugereau, 45250 Briare Le Canal.

3.2. *Maintenance of Alicyclobacillus acidoterrestris cultures*

Alicyclobacillus acidoterrestris was donated by Aplin and Barrett, Beaminster, Dorset, UK. Cultures of *A. acidoterrestris* were stored on slopes of Potato Dextrose Agar (PDA). When required, colonies were streaked onto Orange Serum Agar (OSA) enriched with 0.5% (w/v) sucrose and plates incubated at 44°C for five days. After this two colonies of approximately 2mm diameter were aseptically picked from the surface of the plate, suspended in acidified peptone water 0.1% (w/v) bacteriological peptone, 0.5% (w/v) sucrose, (adjusted to pH 4 with concentrated sulphuric acid) and incubated aerobically at 44°C (Jensen, 1999).

3.3. *Maintenance of Propionibacterium cyclohexanicum cultures*

Propionibacterium cyclohexanicum (NCIMB 13575) was obtained from NCIMB Ltd., Aberdeen, UK. The organism was maintained on Peptone Yeast Extract Agar (PYG), containing 0.5% (w/v) bacteriological peptone, 0.25% (w/v)

yeast extract, 1.5% (w/v) agar, (Oxoid, Basingstoke, UK), supplemented with 0.5% (w/v) glucose (VWR International, Poole, UK), anaerobically using Anaerocult[®] A (Merck, Darmstadt, Germany) at 35°C, the optimal temperature suggested by Kusano *et al.*, (1997), sub-culturing at 4 day intervals. Before use cells were grown in peptone yeast extract broth (PYG broth) containing 0.5% (w/v) bacteriological peptone, 0.25% (w/v) yeast extract, 0.5% (w/v) glucose and incubated anaerobically at 35°C (Kusano *et al.*, 1997).

3.4. Determining optimal conditions for growth and isolation of *A.*

acidoterrestris.

3.4.1 Agar Trials

Three commercially available agars were tested for their ability to support growth of *A. acidoterrestris*, all with a pH below 5.8 (Jensen, 1999). The media used were; Potato Dextrose Agar (PDA) pH5.6 ± 0.2, (CM0139), Orange Serum Agar (OSA) pH5.5 ± 0.2, (CM657), and Malt Extract Agar (MEA) pH5.4 ± 0.2, (CM0059).

Each agar was supplemented with 0.5% (w/v) glucose (anhydrous), or 0.5% (w/v) sucrose, or 0.5% (w/v) glycerol or no supplement giving four variations of each agar. The pH of all agars was measured after autoclaving and tempering using an Orion model 410A pH meter. However, no difference in pH from the above values was observed in any agar containing any supplement.

3.4.2 *Broth for dilutions*

An acidified broth was prepared containing 0.1% (w/v) peptone solution adjusted to pH4 using concentrated sulphuric acid and used to perform serial dilutions of cultures containing vegetative cells and spores of *A. acidoterrestris*. The broth was supplemented with 0.5% (w/v) glucose (anhydrous), or 0.5% (w/v) sucrose, or 0.5% (w/v) glycerol or no supplement. 100mls of each broth was decanted into 250ml sterile Duran bottles and each bottle inoculated with either 0.1 ml, 0.2ml, 0.5ml, 1ml or 2ml of a solution containing both cells and spores of *A. acidoterrestris*. Each broth was paired with the corresponding agar (Table 3.1).

Agar	Agar Supplement	Broth Supplement
PDA	0.5% (w/v) glucose	0.5% (w/v) glucose
PDA	0.5% (w/v) sucrose	0.5% (w/v) sucrose
PDA	0.5% (w/v) glycerol	0.5% (w/v) glycerol
PDA	No supplement	No supplement
OSA	0.5% (w/v) glucose	0.5% (w/v) glucose
OSA	0.5% (w/v) sucrose	0.5% (w/v) sucrose
OSA	0.5% (w/v) glycerol	0.5% (w/v) glycerol
OSA	No supplement	No supplement
MEA	0.5% (w/v) glucose	0.5% (w/v) glucose
MEA	0.5% (w/v) sucrose	0.5% (w/v) sucrose
MEA	0.5% (w/v) glycerol	0.5% (w/v) glycerol
MEA	No supplement	No supplement

Table 3.1. Agars and broth combinations used in isolating *A. acidoterrestris* from raw materials and shelf stable products.

3.4.3. Preparation of fruit juices

The agars and broths were also tested to determine their suitability for use in isolating *A. acidoterrestris* from apple and orange juices.

Cartons of ready to drink apple juice and orange juice were each decanted into sterile 1 litre Duran bottles and inoculated with *A. acidoterrestris*. The bottles were shaken and incubated at 44°C for four hours to allow dispersal of the cells within the apple juice.

Samples of un-inoculated juice were tested for the presence of spoilage micro-organisms using Plate Count Agar (PCA) for Total Viable Counts, Oxytetracycline-Glucose-Yeast (Extract) Agar (OGYA) for yeasts and moulds, de Mann, Rogosa, Sharpe agar (MRS) for lactic acid bacteria and OSA supplemented with 0.5% (w/v) sucrose for *A. acidoterrestris*.

3.4.4. Method

Each agar (Table 3.1) was surface plated (0.1ml) in triplicate with each of the inoculated broths or the inoculated apple juice or orange juice in order to determine vegetative cell count. The broths and juices were then heat shocked for 10 minutes as follows: test tubes each containing an aliquot of one of either of the broths or the juices were lowered into a water bath at 80°C. A thermometer was placed into a test tube containing a similar amount of solution and 10 minutes timed from when the temperature reached 80°C in this control tube. The tubes were then removed from the water bath, cooled to 30°C and 0.1ml of the contents

surface plated in triplicate onto each of the agars to determine a spore count. All plates were incubated at 44°C for five days before enumeration.

3.4.5 Results

OSA supplemented with 0.5% (w/v) sucrose recorded significantly higher ($P<0.05$) numbers of *A. acidoterrestris* vegetative cells inoculated in apple and orange juice (Appendix 1, Figure a and b) than all other media combinations. MEA with no added supplement and PDA supplemented with 0.5% (w/v) glucose provided the next highest spore counts (Appendix 1, Figure b) although they were both significantly lower ($P<0.05$) than OSA supplemented with 0.5% (w/v) sucrose. The four OSA combinations all afforded identifiable colonies and, even when the number of colonies were high, they retained their shape and had easily identifiable borders ensuring accurate and reliable counts. Vegetative cell numbers on OSA supplemented with 0.5% (w/v) sucrose were greater than one log higher than those of MEA supplemented with 0.5% (w/v) glucose in the juice trials (Appendix 1, Figure a). However, the differences between the various media in spore numbers were less evident, with all counts within less than one log of each other. Again OSA supplemented with 0.5% (w/v) sucrose provided significantly ($P<0.05$) the highest counts and the most distinctive easily counted colonies (Appendix 1, Figure b). All other media combinations apart from the OSA combinations produced at some time during the trials merged or 'swarming' colonies making counting difficult particularly when counts were high (Appendix 1, Table a and b).

MEA supplemented with 0.5% (w/v) sucrose, OSA supplemented with 0.5% (w/v) sucrose and OSA supplemented with 0.5% (w/v) glycerol and PDA supplemented with 0.5% (w/v) glycerol provided the overall highest plate counts, although none were significantly high, of *A. acidoterrestris* inoculated at low levels (0.1ml, 0.2ml and 0.5ml) in 0.1% (w/v) acidified peptone broth (Appendix 1, Figures c and d).

MEA with no added supplement that provided one of the highest plate counts in the juice trials (Appendix 1, Figures a and b) afforded one of the lower overall counts in the broth trials (Appendix 1, Figures c and d) although not significantly lower than other media combinations. Unlike the juice trials where OSA supplemented with 0.5% (w/v) sucrose demonstrated the highest plate counts each time, the broth trials did not provide a media/broth combination that obviously surpassed any other (Appendix 1, Figures c and d).

None of the media combinations demonstrated vegetative cell counts significantly higher than spore counts (Appendix 1, Figures c and d) which is in contrast to the juices where significantly higher counts ($P < 0.05$) were observed in numbers of spores (Appendix 1, Figures a and b). There was less than one log difference in all three broth trials (Appendix 1, Figures c and d). OSA supplemented with 0.5% (w/v) sucrose displayed consistently high numbers of spores from broths that had been inoculated with higher concentrations of *A. acidoterrestris* suspension (Appendix 1, Tables b and c). Importantly all OSA combinations also provided spore counts whereas other media combinations produced colonies that merged making counting impossible (Appendix 1, Tables

b and c). However, all the media combinations did provide good growth and all are available commercially.

OSA supplemented with 0.5% (w/v) sucrose performed consistently well in both fruit juice and broth giving the highest count of both vegetative cells and spores in the juices (Appendix 1, Figures a and b), high counts in the 0.1ml and 0.2ml broth trials (Appendix 1, Figures c and d) and in the 1ml and 2ml trials (Appendix 1, Tables b and c).

Due to the consistently high counts compared to the other media/broth combinations and the distinctive well-formed colonies OSA supplemented with 0.5% (w/v) sucrose was used in all succeeding experiments.

3.5. Testing for incidence of *A. acidoterrestris* in products and raw materials

Randomly selected shop bought products such as ready to drink juices and raw materials, for example, concentrated fruit juices, fruit esters and single strength juices were tested for the presence of *A. acidoterrestris*.

Fruit concentrates were diluted 1:9 using sterile de-ionised water and tested for the presence of spoilage micro-organisms (Section 3.4.3). After dilution the fruit concentrates and all other un-diluted samples were incubated at 44°C for 48 hours prior to testing.

Also after incubation samples were serially diluted 1:10 using acidified peptone broth before surface plating (0.1ml) onto OSA enriched with 0.5% (w/v) sucrose. 10mls of each sample, was aseptically dispensed into sterile capped test tubes and heat shocked at 80°C (Section 3.4.4). The contents were then surface

plated onto enriched OSA and incubated at 44°C for up to 7 days to allow for any sub lethally injured cells to grow. Plates were examined daily.

Any samples showing evidence of growth required confirmatory tests as follows:

- Gram staining (*A. acidoterrestris* should be Gram variable to Gram positive).
- Catalase test should be positive.
- Failure to grow on neutral agar such as PCA.
- Microscopic examinations for the presence of spores.
- Examination of colony morphology, *A. acidoterrestris* has a distinctive appearance on OSA – a flat interior and raised centre – translucent cream coloured colonies darkening with age.

3.6. Testing for incidence of P. cyclohexanicum in products and raw materials

Randomly selected shop bought products such as ready to drink juices and raw materials, for example, concentrated fruit juices, fruit esters and single strength juices were tested for the presence of *P. cyclohexanicum*.

Samples were serially diluted 1:10 using PYG broth before surface plating (0.1ml) onto PYG followed by anaerobic incubation at 30°C for up to 7 days to allow for any sub lethally injured cells to grow. Plates were examined daily. Any samples showing evidence of growth required confirmatory tests as follows:

- Gram staining (*P. cyclohexanicum* should be Gram variable to Gram positive).
- Catalase test should be negative.
- Growth should be anaerobic.
- Microscopic examinations for the presence of spores using the spore stain method of Schaeffer and Fulton (1990) should show no spores.
- Microscopic examination for motility, cells should be non-motile.
- Examination of colony morphology, *P. cyclohexanicum* cfus are white and dome shaped.
- Inoculation into sterile fruit juice to test for evidence of fermentation.
- HPLC determination to confirm presence of D – turanose and D – fucose.

3.7. Testing for the effect of intermittent shaking, headspace and temperature on the growth of A. acidoterrestris in fruit juice

Colonies of *A. acidoterrestris* at the final concentrations of 3×10^1 vegetative cells per ml and 7.8×10^1 spores per ml (35°C) or approximately 10^2 vegetative cells per ml and 10^3 spores per ml (30°C) were suspended in acidified peptone broth and used to inoculate a ready to drink apple juice. Vegetative cell counts and spore counts were performed before this suspension was used to inoculate the juice samples. Samples of un-inoculated juice were tested for the presence of spoilage micro-organisms (section 3.4.3).

To determine the number of vegetative cells, samples were serially diluted with acidified peptone broth. In order to determine the number of spores present, the culture or sample was heat shocked for 10 minutes (section 3.4.4).

3.7.1 Inoculation of apple juice

Cartons of ready to drink apple juice were decanted into sterile 1 litre Duran bottles and inoculated with *A. acidoterrestris*. The bottles were shaken and incubated at 44°C for four hours to allow dispersal of the inoculum within the apple juice. The inoculated juice was then dispensed into sterile 250ml glass bottles with headspaces of 0%, 25%, 50% and 75% and stored at 35°C or 30°C for the 11-day trials, with sampling occurring on day 4, day 7, day 9 and day 11. Further trials were performed where storage temperatures included 27°C and 44°C and smaller 30ml metal capped glass bottles were used.

On each sampling day, duplicate sets of bottles were removed from the incubator. One set was shaken by upending twenty times and sampled from the middle of the bottle. The other set was handled carefully to minimize mixing of the contents and sampled first from the top and then from the bottom of the juice. Each sample was serially diluted in acidified 0.1% (w/v) peptone broth containing 0.5% (w/v) sucrose and surface plated in duplicate, using at least three separate dilutions, onto enriched OSA. The remainder of each sample was heat shocked as described in Section 3.4.4 and surface plated onto enriched OSA. All plates were incubated at 44°C for 5 days before enumeration.

3.8. The effectiveness of using the preservative sodium benzoate against low levels of A. acidoterrestris

3.8.1 Preparation of sodium benzoate solution

A 10% solution of sodium benzoate was prepared using 10g of benzoic acid and 90g sterile de-ionised water (w/w). The solution was evaluated using Reversed Phase High Performance Liquid Chromatography (HPLC). Mobile Phase (organic) using methanol; C₁₈ or C₈ 250 x 4.6mm 5m at ambient temperature; Detector: UV - vis Spectrophotometer @ 220nm; Perkin Elmer 2001C pump; 235C Diode Array Detector (DE). HPLC reports (Appendix 2).

Cartons of ready to drink apple juice were dispensed into sterile 1 litre Duran bottles and stored for seventeen hours \pm 1 hour at 30°C to represent a hot summer's day in the UK). Before incubation samples of un-inoculated juice were tested for the presence of spoilage micro-organisms (Section 3.4.3).

Colonies of *A. acidoterrestris* suspended in acidified peptone broth were stored in a sterile 250ml Duran bottle also at 30°C prior to inoculating the juice.

3.8.2 Inoculation of apple juice with A. acidoterrestris and sodium benzoate

The bottles of apple juice were inoculated with the acidified peptone broth containing *A. acidoterrestris* at final concentrations of 5.9×10^1 vegetative cells per ml and 1.1×10^2 spores per ml incubated at 30°C for approximately one hour to allow dispersal of solution.

Determination of vegetative cell counts and spore counts in the acidified peptone broth was as described in Section 3.4.4. Samples of un-inoculated juice were tested for the presence of spoilage organisms (Section 3.4.3).

The inoculated bottles of juice were shaken thoroughly and aseptically decanted into sterile 250ml Duran bottles. Sodium benzoate solution was added to each sample bottle at levels of 100ppm, 200ppm, 300ppm, 400ppm and 500ppm and in further trials 500ppm, 1,000ppm or 1,500ppm, in duplicate, with control bottles containing inoculated juice and no preservative. All sample bottles were stored at 30°C for the duration of the study and samples removed from each bottle and tested for survival of *A. acidoterrestris* on days 0, 1, 5, 8, 15, 22 and 29. Preservative levels were analysed on days 0, 15 and 29 using HPLC as described in Section 3.8.1.

On each sampling day, duplicate sets of bottles were removed from the incubator, shaken thoroughly and aliquots aseptically serially diluted using acidified 0.1% (w/v) peptone broth containing 0.5% (w/v) sucrose and surface plated in duplicate, onto enriched OSA. The remainder of each sample was heat shocked as described in Section 3.4.4. and surface plated onto enriched OSA. All plates were incubated at 44°C for 5 days before enumeration.

3.9. The effectiveness of using the preservative potassium sorbate against A. acidoterrestris

3.9.1 Preparation of potassium sorbate solution

A 10% solution of potassium sorbate was prepared using 10g of sorbic acid and 90g sterile de-ionised water (w/w). The solution was evaluated using HPLC (section 3.8.1).

Cartons of ready to drink apple juice were dispensed into sterile 1 litre Duran bottles and stored for seventeen hours \pm 1 hour at 30°. Colonies of *A. acidoterrestris* suspended in acidified peptone broth were stored in a sterile 250ml Duran bottle also at 30°C prior to inoculating the juice.

Before incubation samples of un-inoculated juice were tested for the presence of spoilage micro-organisms (Section 3.4.3).

3.9.2 Inoculation of apple juice with *A. acidoterrestris* and potassium sorbate

The bottles of apple juice were inoculated with the acidified peptone broth containing *A. acidoterrestris* at final concentrations of 6.7×10^1 vegetative cells per ml and 1.1×10^2 spores per ml and returned to 30°C for approximately 1 hour to allow dispersal of solution.

A determination of vegetative cell counts and spore counts in the acidified peptone broth was performed (Section 3.4.4). Samples of un-inoculated juice were tested for the presence of spoilage organisms (section 3.4.3).

The inoculated bottles of juice were removed from the incubator, shaken thoroughly and aseptically decanted into sterile 250ml Duran bottles. The potassium sorbate solution was added to each sample bottle at levels of 100ppm, 200ppm, 300ppm, 400ppm and 500ppm and in further trials 500ppm, 1,000ppm or 1,500ppm in duplicate with control bottles containing inoculated juice and no preservative. All sample bottles were stored at 30°C for the duration of the study and samples removed from each bottle and tested for survival of *A. acidoterrestris* on days 0, 1, 5, 8, 15, 22 and 29. Preservative levels were analysed on days 0, 15 and 29 using HPLC as described earlier.

On each sampling day, duplicate sets of bottles were removed from the incubator, shaken thoroughly and aliquots aseptically serially diluted using acidified 0.1% (w/v) peptone broth containing 0.5% (w/v) sucrose and surface plated in duplicate, onto enriched OSA. The remainder of each sample was heat shocked (Section 3.4.4) and surface plated onto enriched OSA. All plates were incubated at 44°C for 5 days before enumeration.

3.10 The effectiveness of using the preservatives sodium benzoate and potassium sorbate alone and with the bacteriocin nisin against *A. acidoterrestris*.

Cartons of ready to drink apple juice were dispensed into sterile 1 litre Duran bottles and stored for seventeen hours \pm 1 hour at 30°. Colonies of *A. acidoterrestris* suspended in acidified peptone broth were stored in a sterile 250ml Duran bottle also at 30°C prior to inoculating the juice. Before incubation samples of un-inoculated juice were tested for the presence of spoilage micro-organisms (section 3.4.3).

3.10.1 Preparation of nisin solution

Nisaplin (the commercial preparation of nisin) was donated by Danisco, A/S, DK-Grinstad, Denmark.

10mls of a solution of Nisaplin (0.1g) in 0.02N HCl was centrifuged at 2000g for 20 minutes and the supernatant filtered using a Millex Syringe filter

with a 0.22µm pore size to give a stock solution of 10,000 IU/ml. The solution was retained in a sterile container at 4°C until required.

3.10.2 Inoculation of apple juice with sodium benzoate, potassium sorbate and nisin solutions

Bottles of apple juice were inoculated with the acidified peptone broth containing *A. acidoterrestris*, at final concentrations of 1.8×10^3 vegetative cells per ml and 4.2×10^3 spores per ml, shaken thoroughly and returned to the incubator for approximately 1 hour to allow dispersal of solution. A determination was made of vegetative cell counts and spore counts in the acidified peptone broth (Section 3.4.3).

The inoculated bottles of juice were shaken thoroughly and aseptically decanted into sterile 250ml Duran bottles. Sodium benzoate and potassium sorbate solutions were added to selected sample bottles at levels of 500ppm or 1,000ppm and nisin solution at levels of 10IU/ml, 5 IU/ml or 2.5 IU/ml as listed in Table 3.2.

Apple juice	Sodium benzoate	Potassium sorbate	Nisin
200ml	0ppm		
200ml	500ppm		
200ml	1000ppm		
200ml		0ppm	
200ml		500ppm	
200ml		1000ppm	
200ml			10 IU
200ml			5 IU
200ml			2.5 IU
200ml	500ppm		10 IU
200ml	500ppm		5 IU
200ml	500ppm		2.5 IU
200ml	1000ppm		10 IU
200ml	1000ppm		5 IU
200ml	1000ppm		2.5 IU
200ml		500ppm	10 IU
200ml		500ppm	5 IU
200ml		500ppm	2.5 IU
200ml		1000ppm	10 IU
200ml		1000ppm	5 IU
200ml		1000ppm	2.5 IU

Table 3.2. Sample bottles of apple juice pH3.5 (*A. acidoterrestris*) or orange juice pH3.8 (*P. cyclohexanicum*) containing preservatives and bacteriocin combinations and stored at 30°C during preservative challenges to the bacilli.

Sample bottles containing inoculated juice and no preservative served as controls. All sample bottles were stored at 30°C for the duration of the study and samples removed from each bottle and tested for survival or growth of *A. acidoterrestris* on days 0, 1, 5, 8, 15, 22 and 29. Preservative levels were analysed on days 0, 15 and 29 using HPLC as described earlier.

On each sampling day, duplicate sets of bottles were removed from the incubator, shaken thoroughly and 10ml aliquots aseptically serially diluted using acidified 0.1% (w/v) peptone broth containing 0.5% (w/v) sucrose and surface plated in duplicate at each dilution onto enriched OSA. The remainder of each sample was heat shocked (Section 3.4.4) and surface plated onto enriched OSA. All plates were incubated at 44°C for 5 days before enumeration.

3.11 Growth of *A. acidoterrestris* in different fruit juices

Six fruit juices were used to monitor the growth of *A. acidoterrestris* over a period of 29 days at storage temperatures of 30°C and 35°C. The six juices were grapefruit, orange, apple, cranberry, pineapple and tomato juice. All juices were sampled before inoculation and tested for the presence of spoilage microorganisms (section 3.4.3).

Cartons of the ready to drink fruit juices were dispensed into 1 litre sterile Duran bottles. Sample bottles of each juice and *A. acidoterrestris* suspended in acidified peptone broth were stored at 30°C and 35°C overnight to ensure similarity in temperature between the juices and *A. acidoterrestris* cultures.

The bottles of juice were inoculated with the acidified peptone broth containing *A. acidoterrestris* at final concentrations of 1.3×10^4 vegetative cells per ml and 5.5×10^4 spores per ml and incubated at 30°C and 35°C for approximately one hour to allow dispersal of the solution.

Vegetative cell counts and spore counts in the acidified peptone broth were determined (Section 3.4.4) and samples of un-inoculated juice were tested for the presence of spoilage organisms (Section 3.4.3).

The inoculated bottles of juice were shaken thoroughly and aseptically decanted into sterile 250ml Duran bottles. Triplicate sample bottles of each juice were stored at 30°C and 35°C for the duration of the study and samples removed from each bottle and tested for survival or growth of *A. acidoterrestris* on days 0, 1, 5, 8, 15, 22 and 29.

On each sampling day, bottles were removed from the incubator, shaken thoroughly and 10ml removed, 1ml was serially diluted using acidified 0.1% (w/v) peptone broth containing 0.5% (w/v) sucrose and 0.1ml surface plated in duplicate at each dilution, onto enriched OSA. The remainder of each sample was heat shocked (Section 3.4.4) and 0.1ml surface plated onto enriched OSA. All plates were incubated at 44°C for 5 days before enumeration.

3.12. Pasteurisation challenges to *P. cyclohexanicum*

3.12.1 Preparation of fresh orange juice

Whole oranges were 'squeezed' using a domestic juicer allowing juice to be removed leaving skin, pips and pith. The squeezed juice was bottled in sterile 1 litre Duran bottles and kept overnight in a refrigerator to allow for settling of the pulp. The juice was carefully transferred to further sterile 1 litre Duran bottles without shaking leaving the remaining pulp.

Cells of *P. cyclohexanicum* suspended in PYG broth were used to inoculate the orange juice at final concentrations of approximately 10^5 cells per ml. Samples of un-inoculated juice were tested for the presence of spoilage micro-organisms (Section 3.4.3). Samples of inoculated PYG broth were surface plated onto PYG agar (0.1ml) followed by anaerobic incubation for four days at 35°C before enumeration to calculate the number of *P. cyclohexanicum* per ml.

Before use all bottles of juice were removed from the refrigerator and kept at room temperature for approximately 30 minutes before placing at temperatures of 25°C for 1 hour then 35°C for 1 hour prior to pasteurising.

3.12.2 Pasteurisation

An HTST/UHT Heat Exchanger Processing Unit FT74X-A serial number 014226-001 (Armfield Engineering Limited, Hampshire, England) was used with temperatures that can be replicated to $\pm 1^\circ\text{C}$. Samples of inoculated juice were pasteurised for 16 seconds in duplicate at temperatures of 80°C, 85°C, 87°C, 90°C, 93°C, 97°C, 98°C and 102°C. Two samples of unpasteurised inoculated juice served as controls. Immediately after pasteurisation and daily for 6 days 0.1ml of the samples were surface plated onto PYG and plates incubated at 35°C for 5 days. Test samples were stored at 25°C with the exception of the unpasteurised juice that was stored upright in the laboratory at ambient (22°C to 22.5°C) temperature, due to the possibility of yeast growth.

Pasteurisation was also performed after pre-heating juice samples to 60°C in a water bath for 30 minutes; this was to allow for the possibility that the initial

isolation of *P. cyclohexanicum* was post pasteurisation and to give cells an adequate opportunity to grow.

3.13 Determination of temperature growth range of P. cyclohexanicum in orange juice.

Colonies of *P. cyclohexanicum* suspended in PYA broth were used to inoculate a ready to drink orange juice at final concentrations of 10^6 cells per ml. Samples of un-inoculated juice were tested for the presence of spoilage micro-organisms (Section 3.4.3).

Cartons of ready to drink orange juice were dispensed into sterile 1 litre Duran bottles and stored for seventeen hours \pm 1 hour at 35°. Colonies of *P. cyclohexanicum* suspended in PYG broth were stored in a sterile 250ml Duran bottle also at 35°C prior to inoculating the juice.

Aliquots were decanted into 250ml Duran bottles with minimal head space and tightly sealed. Duplicate bottles were incubated at the following temperatures: 4°C, 15°C, 20°C, 30°C, 35°C and 40°C. On days 0, 4, 6, 8, 11, 17 and day 52 triplicate samples were aseptically removed from the bottles and surface plated onto PYG. The plates were incubated anaerobically at 35°C with enumeration carried out after 4 days.

3.14 Investigation into the survival of P. cyclohexanicum after elevated heat treatment in culture broth and orange juice.

Colonies of *P. cyclohexanicum* suspended in PYA broth were used to inoculate 500ml of a ready to drink orange juice or 500ml of PYA broth at final concentrations of 10^8 cfu/ml. Plate counts were performed before this suspension was used to inoculate the 500ml samples (Section 3.12.1) and samples of uninoculated juice were tested for the presence of spoilage micro-organisms (Section 3.4.3).

After anaerobic incubation for 24 hours at 35°C, 10ml aliquots were pipetted into thin-walled test tubes. A tube containing 10ml of PYG broth in which was placed a thermometer acted as a temperature control. Once the temperature in the control tube read the required temperature, timing began.

At 1 minute, 5 minute and 10 minute intervals two tubes were removed and the contents surface plated in triplicate onto PYG and incubated anaerobically at 35°C for 4 days. The procedure was repeated at 60°C, 70°C, 80°C, 85°C, 90°C and 95°C.

In a second set of experiments a different protocol was adopted. All tubes containing *P. cyclohexanicum* were held at 35°C for 2 minutes prior to any heat treatment. For trials at 50°C, 60°C and 70°C, tubes were placed into a water-bath at 45°C until the temperature in the control tube reached this temperature and then for a further 2 minutes before being heat treated. After removal of the tubes from the test temperature they were again placed in the 45°C water-bath for 2 minutes and then held at 35°C as described previously. Samples were then surface plated (0.1ml) onto PYG agar and incubated anaerobically for 4 days.

For trials at 80°C and 85°C or 90°C and 95°C the cultures were treated as described above but with the 'holding temperature' of 45°C replaced by 55°C for 80°C and 85°C and a 60°C holding temperature for 90°C and 95°C samples. Samples were surface plated onto PYG agar and enumerated after anaerobic incubation at 35°C for 4 days.

3.15. Growth of *P. cyclohexanicum* in different fruit juices

Six fruit juices were used to monitor the growth of *P. cyclohexanicum* over a period of 29 days at storage temperatures of 30°C and 35°C. The six juices selected were grapefruit, orange, apple, cranberry, pineapple and tomato juice. All juices were tested for the presence of spoilage micro-organisms (section 3.4.3).

Cartons of the six ready to drink fruit juices were dispensed into 1 litre sterile Duran bottles. Duplicate bottles of each juice were stored for seventeen hours \pm 1 hour at 30° or at 35°C. Colonies of *P. cyclohexanicum* suspended in PYG broth were stored in sterile 250ml Duran bottles also at 30°C and 35°C prior to inoculating the juice.

The bottles of juice were inoculated with *P. cyclohexanicum* at final concentrations of 1×10^7 cells per ml and incubated at 30°C and 35°C for approximately 1 hour to allow dispersal of the solution and a determination was made of cell counts in the PYG broth (section 3.12.1).

The bottles were then shaken thoroughly and aseptically decanted into sterile 250ml Duran bottles. Sample bottles of each juice were stored at 30°C or

at 35°C for the duration of the study and samples removed from each bottle and tested for survival or growth of *P. cyclohexanicum* on days 0, 1, 5, 8, 15, 22 and 29.

On each sampling day, bottles were removed from the incubator; aliquots aseptically serially diluted using PYG broth and 0.1ml surface plated in duplicate on PYG. The 30°C storage samples were incubated anaerobically at 30°C and the 35°C storage samples at 35°C for 4 days before enumeration.

3.16. The effectiveness of using the preservatives sodium benzoate and potassium sorbate alone and with the bacteriocin nisin against P. cyclohexanicum.

Cartons of ready to drink orange juice were dispensed into sterile 1 litre Duran bottles and stored for seventeen hours \pm 1 hour at 30°. Colonies of *P. cyclohexanicum* at final concentrations of 8.4×10^5 cells per ml suspended in PYG broth, were stored in a sterile 250ml Duran bottle also at 30°C prior to inoculating the juice. Before incubation samples of un-inoculated juice were tested for the presence of spoilage micro-organisms (section 3.4.3).

The orange juice was prepared and inoculated with the two preservatives and nisin as described for *A. acidoterrestris* (Section 3.10.2).

Determination of cell counts were performed (section 3.12.1) and samples of un-inoculated juice were tested for the presence of spoilage micro-organisms (Section 3.4.3).

On each sampling day, duplicate sets of bottles were removed from the incubator, shaken thoroughly and aseptically serially diluted using PYA broth and surface plated in duplicate, onto PYA. All plates were anaerobically incubated at 30°C for 4 days before enumeration.

3.17. *Co-culture of A. acidoterrestris and P. cyclohexanicum in orange juice.*

Cartons of ready to drink orange juice were dispensed into sterile 1 litre Duran bottles and stored for seventeen hours \pm 1 hour at 30°. Colonies of *P. cyclohexanicum* suspended in PYA broth and colonies of *A. acidoterrestris* suspended in acidified peptone broth were stored in 250ml Duran bottles at 30°C prior to inoculation. Determination of cell counts was performed (Section 3.12.1) for *P. cyclohexanicum* and vegetative cell counts and spore counts in the acidified peptone broth for *A. acidoterrestris* (section 3.4.4). Samples of un-inoculated juice were tested for the presence of spoilage micro-organisms (section 3.4.3).

The bottles of orange juice were removed from the incubator and 200ml decanted into 250ml sterile Duran bottles which were then inoculated with, for the first study, 5ml *A. acidoterrestris* at final concentrations per ml of (5×10^3 vegetative cells and 3.4×10^4 spores) solution or 5ml *P. cyclohexanicum* at final concentrations per ml of (3×10^7) solution and used as controls. Triplicate samples were then inoculated with:

- 5ml of each solution to give final concentrations per ml in the juice of *A. acidoterrestris* (5×10^3 vegetative cells and 3.4×10^4 spores) and *P. cyclohexanicum* (3×10^7).
- 10ml *A. acidoterrestris* solution and 5ml *P. cyclohexanicum* solution to give final concentrations per ml in the juice of *A. acidoterrestris* (1×10^4 vegetative cells and 6.8×10^4 spores) and *P. cyclohexanicum* (3×10^7).
- 5ml *A. acidoterrestris* solution and 10ml *P. cyclohexanicum* solution to give final concentrations per ml in the juice of *A. acidoterrestris* (5×10^3 vegetative cells and 3.4×10^4 spores) and *P. cyclohexanicum* (6×10^7).

Sample bottles were shaken thoroughly and samples surface plated; *A. acidoterrestris* on OSA enriched with 0.5% (w/v) sucrose, with dilutions performed using acidified peptone broth and heat-shocked (Section 3.4.3); *P. cyclohexanicum* on PYG using PYG broth for dilutions (Section 3.12.1).

Sample bottles were stored in triplicate at 35°C for the duration of the study and plates incubated at 30°C for *P. cyclohexanicum* and 44°C for *A. acidoterrestris*. Samples were removed from each bottle and tested for survival or growth of *A. acidoterrestris* and *P. cyclohexanicum* on days 0, 1, 3, 6, 8 and 10. The pH and Brix of the juices were also measured at each sampling time. The pH was measured using a pH meter (Orion model 410A, Denver, Colorado, USA) and the Brix value (expressed in terms of percentage sucrose content) was obtained using direct readings from a refractometer (Bellingham and Stanley

RFM80), designed specifically for Brix readings at a temperature of 20°C where Brix = w/w% soluble solids as sucrose (Batchelor, 1984).

3.18. Preliminary investigation into the presence of bacteriocins from *P. cyclohexanicum*

Preparation and inoculation of PYG broth with *P. cyclohexanicum* was described in section 3.12.1.

Preparation and inoculation of acidified peptone broth with *A. acidoterrestris* was described in Section 3.4.4.

Inoculated PYG broth was centrifuged at 2000g for 15 minutes in sterile tubes, after which the supernatant was removed and centrifuged a second time. Inoculated acidified peptone broth was also centrifuged in the same manner and the supernatant of each micro-organism was inoculated into the agar wells. *A. acidoterrestris* supernatant was inoculated into wells in PYG and *P. cyclohexanicum* supernatant was inoculated into wells in OSA. *A. acidoterrestris* cultures were also inoculated into wells in PYG and *P. cyclohexanicum* cultures into wells in OSA. Controls consisted of PYG inoculated with *P. cyclohexanicum* and OSA inoculated with *A. acidoterrestris*.

Chapter 4

**Growth of *A. acidoterrestris* and
P. cyclohexanicum in fruit juices
and survival of *P. cyclohexanicum*
following pasteurisation and elevated
temperature treatments.**

4.1. Introduction

Although *A. acidoterrestris* was originally isolated from spoiled apple juice (Cerny *et al.*, 1984), other fruit juices have been investigated for spoilage potential including apple, tomato and white grape (Splittstoesser, 1998), orange juice (Baumgart *et al.*, 1997; Eiroa *et al.*, 1999) and clear apple juice (Previdi *et al.*, 1997).

As *A. acidoterrestris* has now become an industry-wide issue (Duong and Jensen, 2000) its temperature for survival in different media is of concern to the fruit juice industry.

P. cyclohexanicum has a growth temperature range of 20°C to 40°C with a published optimal temperature of 35°C (Kusano *et al.*, 1997). *A. acidoterrestris* has several reported optimal temperature ranges which include 42°C to 53°C (Pontius *et al.*, 1998; Walls and Chuyate, 1998), 26°C to 50°C (Borlinghaus and Engel, 1997) and 25°C to 60°C (Yamazaki *et al.*, 1997). Walls and Chuyate (2000) use a 43°C incubation temperature. This study therefore reflects storage temperatures of 30°C and 35°C that are mid-range for both micro-organisms but also reflect a hot European summer or a normal Australian summer (Jensen and Whitfield, 2003).

P. cyclohexanicum has been recorded as the source of only one spoilage incident to date. However, this incident involved pasteurised orange juice and the potential for the bacterium to survive and grow in different fruit juices has not, so far, been investigated. *P. cyclohexanicum* can survive a temperature of 90°C for ten minutes and investigations at temperatures above and below this may provide

a greater understanding of the ability to survive heat treatment regimes of this micro-organism (Kusano *et al.*, 1997).

4.2. Aims

The studies described in this chapter aimed to investigate:

- The growth of both *A. acidoterrestris* vegetative cells and spores in six popular fruit juices; apple, orange, cranberry, pineapple, tomato and grapefruit stored at temperatures of 30°C and 25°C.
- Investigate the growth of *P. cyclohexanicum* following pasteurisation at temperatures from 80°C to 102°C in fresh orange juice and also in already pasteurised orange juice.
- The ability of the *P. cyclohexanicum* to survive over a temperature range from 4°C to 40°C.
- The ability after 10 minutes exposure to 50°C, 60°C, 70°C, 80°C, 85°C, 90°C and 95°C in culture medium and in orange juice.
- The ability of *P. cyclohexanicum* to grow in other juices stored at temperatures of 30°C and 35°C was also monitored.

4.3. Materials and methods

Methods and materials were as described in Sections 3.11, 3.12 and 3.13.

4.4. Statistical analysis

All statistical analyses were performed using SPSS version 11.5 (SPSS Inc. Chicago, IL, USA) using independent two tailed *t*-test analysis for parametric data, or repeated measures ANOVA for differences between pH and Brix at different temperatures in the various juices tested. Significance was set at $P < 0.05$.

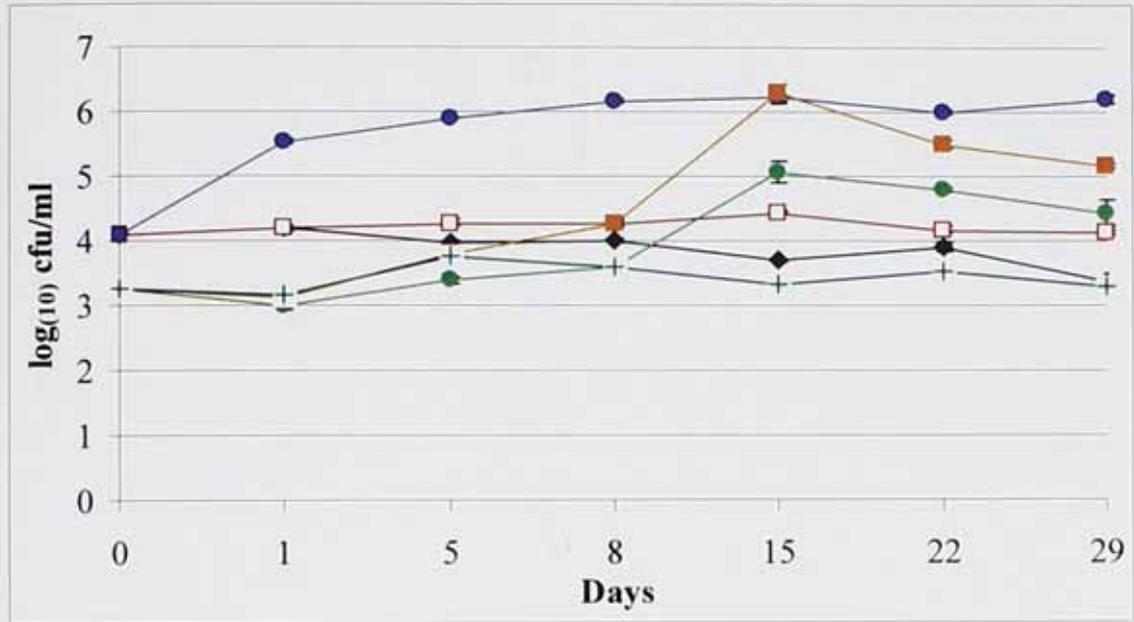
4.5. Results

4.5.1. Growth of *A. acidoterrestris* in fruit juices

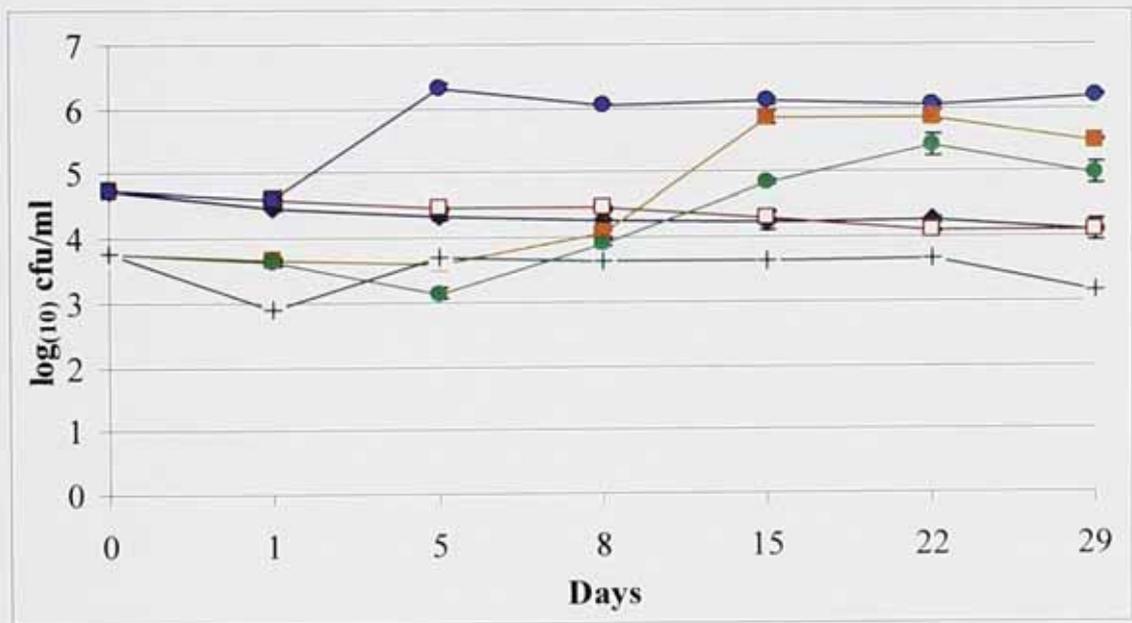
Results are shown as the increase in cfu/ml, Brix and pH (Table 4.1) were recorded on days 1 and 29. No significant difference was noted in either as *A. acidoterrestris* does not cause changes in pH (Brown, 1995; Walls and Chuyate, 1998).

Juice	pH at Day 0	pH at day 29		Brix at Day 0	Brix at day 29	
		30°C	35°C		30°C	35°C
Grapefruit	3.2	3.2	3.2	10.6	10.4	10.5
Cranberry	2.7	2.7	2.7	11.9	11.7	11.8
Tomato	4.0	4.0	4.0	5.7	5.5	5.6
Orange	3.8	3.8	3.9	11.1	10.9	10.8
Pineapple	3.6	3.6	3.6	12.5	12.3	12.2
Apple	3.5	3.5	3.5	11.0	10.9	10.9

Table 4.1. Brix and pH values of fruit juices inoculated with *A. acidoterrestris* at 30°C and 35°C over 29 days. Values are means \pm SEM ($n = 6$).

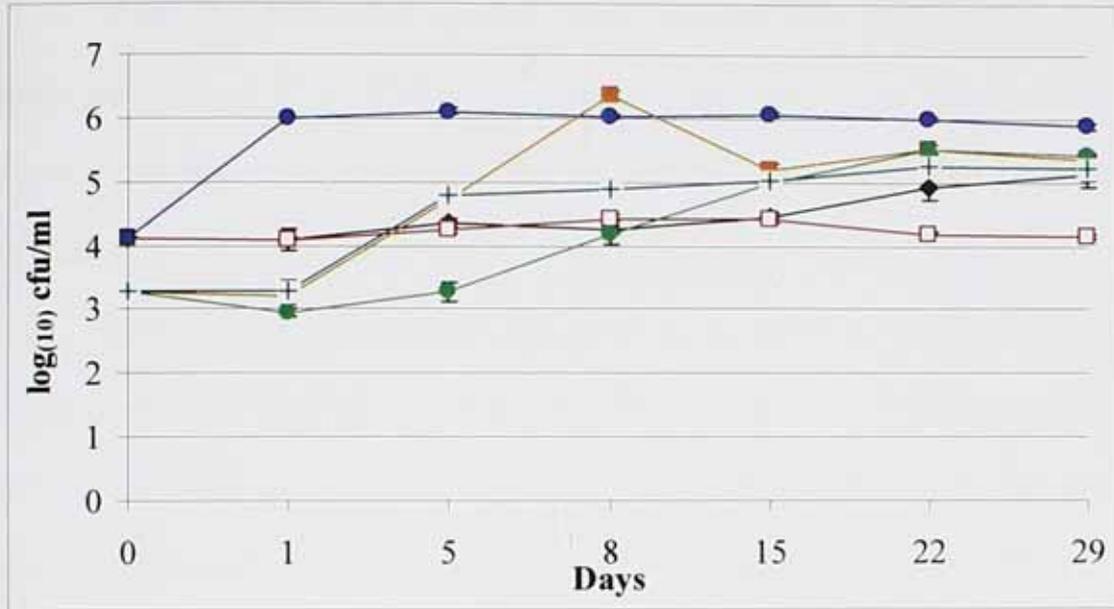


a

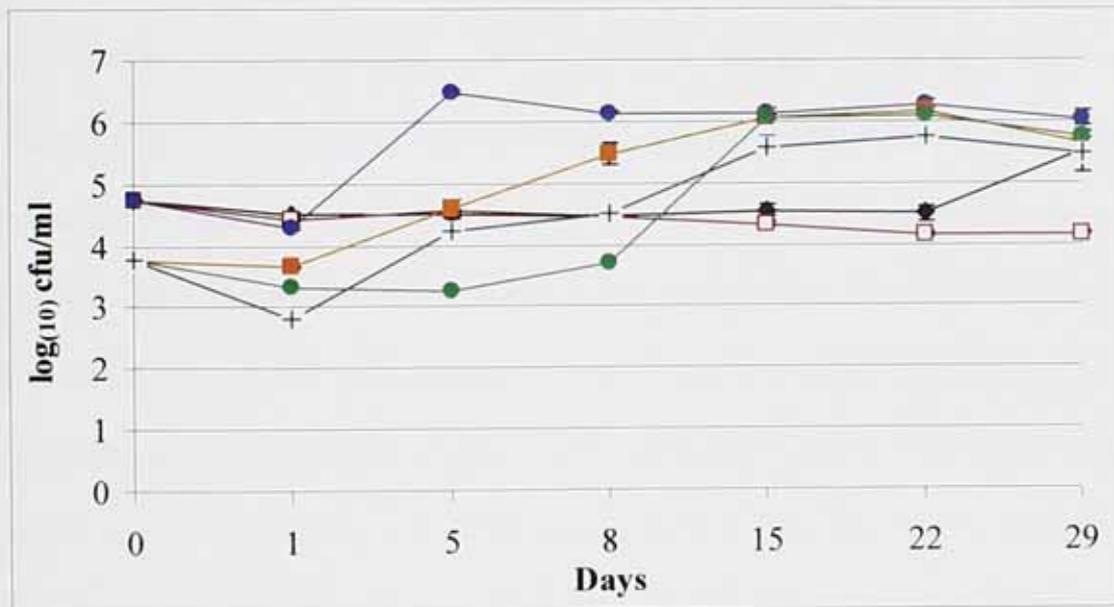


b

Figure 4.1. Survival of *A. acidoterrestris* vegetative cells (a) and spores (b) in various juices at 30°C. Values are means \pm SEM ($n = 6$). (---◆--- grapefruit (pH 3.2); ---□---cranberry (pH 2.7); ---●---tomato (pH 4.0); ---■---orange (pH 3.8); ---●---pineapple (pH 3.6); ---+--- apple (pH 3.5))



a



b

Figure 4.2. Survival of *A. acidoterrestris* vegetative cells (a) and *A. acidoterrestris* spores (b) in various juices at 35°C. Values are means \pm SEM ($n = 6$). (---◆--- grapefruit (pH 3.2); ---□--- cranberry (pH 2.7); ---●--- tomato (pH 4.0); ---■--- orange (pH 3.8); ---●--- pineapple (pH 3.6); ---+--- apple (pH 3.5))

Vegetative cells of *A. acidoterrestris* declined from day 1 to day 29 in grapefruit juice at 30°C (Figure 4.1a) but increased during the same period to significantly higher numbers ($P<0.05$) by day 29 compared to day 1 at 35°C (Figure 4.2a)

Spore counts in grapefruit juice declined from day 1 to day 29 but not significantly at 30°C (Figure 4.1b) and the number of spores did not rise above the level of the initial inoculum until day 29 (Figure 4.2b) and then not in significant numbers. Growth of vegetative cells differed between day 29 at 30°C and day 29 at 35°C with significantly ($P<0.05$) higher counts at 35°C.

In cranberry juice at 30°C and 35°C vegetative cell counts (Figures 4.1a and 4.2a) were not significantly above the level of the initial inoculum and there was no significant difference between days 1 and 29 at either temperature. Spore counts of *A. acidoterrestris* were at the highest on days 1, 5 and 8 but declined between days 15 to 29 at 30°C (Figure 4.1b), however no significant difference was found between the viable counts on any of the days. When spore counts were compared at 30°C and 35°C (Figures 4.1b and 4.2b) numbers were significantly higher on day 1 ($P<0.05$) at 30°C (Figure 4.1b) but then significantly higher on day 5 ($P<0.05$) at 35°C (Figure 4.2b), after this no other significant differences were observed. *A. acidoterrestris* can survive in products at pH2 to pH7 (Borlinghaus and Engel, 1997) and spores can germinate at pH<4. The cranberry juice used in this study was pH2.7 and although vegetative cells grew at both temperatures tested they were not significantly higher than the initial amount of inoculum, spores however, did not germinate on any day at either temperature.

Vegetative cells in tomato juice at 30°C and 35°C (Figures 4.1a and 4.2a) and spores at 35°C (Figure 4.2a) were all significantly higher ($P<0.05$) on days 5 and 29 when compared to day 1. No differences were recorded on any day in vegetative cell viable counts (Figure 4.2a) at 35°C. On day 1 at 30°C and 35°C (Figures 4.1 and 4.2) vegetative cells numbers were significantly higher ($P<0.05$) than spores on the same day, however by day 5 this had been reversed and the spores were significantly higher. Temperature made very little difference to vegetative cell numbers of *A. acidoterrestris* (Figures 4.1a and 4.2a) but spores numbered significantly higher at 35°C (Figures 4.1b and 4.2b) on days 1, 5, 8 and 29 compared to the same days at 30°C. Vegetative cells of *A. acidoterrestris* had multiplied by 2 logs at day 8 at 30°C (Figure 4.1a) and by 2 logs at day 5 at 35°C (Figure 4.2a). Spores (Figures 4.1b and 4.2b) had also increased by day 5 at 30°C and 35°C by 1.5 logs.

Tomato juice supported a greater increase in numbers than apple juice in studies by Splittstoesser *et al.*, (1994). Similarly in the present study at 30°C (Figures 4.1a and b) vegetative cells and spores of *A. acidoterrestris* multiplied in tomato juice but not in apple juice. However, at 35°C both juices supported growth of vegetative cells and spores (Figures 4.2a and b) and, until day 15, the highest counts occurred in tomato juice followed by apple juice. Spore counts also followed this pattern but on days 22 and 29 apple juice had significantly a higher number of spores compared to tomato juice ($P<0.05$).

Numbers of spores in orange juice were significantly higher on days 1, 22 and 29 at 30°C than numbers of vegetative cells (Figures 4.1a and b) and at 35°C

spores were higher in numbers than vegetative cells on days 1, 8, 15 and 22. In both temperatures, on day 29 vegetative cell counts and spore counts (Figures 4.1 and 4.2) were significantly higher ($P<0.05$) than on day 1. Although no differences between *A. acidoterrestris* counts at the two temperatures were observed on day 1, by day 5 spores and vegetative cells (Figures 4.1 and 4.2) were significantly higher ($P<0.05$) at 35°C however, by day 29 there was no significant difference between temperatures. *A. acidoterrestris* grew well in orange juice increasing vegetative cells at 30°C by 3 log at day 15 (Figure 4.1a) and by the same amount at 35°C by day 8 (Figure 4.2a). Spores (Figures 4.1a and b) increased by a 2 log difference at day 15 at each temperature.

In a study by Borlinghaus and Engel (1997) higher viable counts were recorded in apple juice compared with both orange and pineapple juices, but in the studies reported here the four highest counts were in orange, tomato, pineapple and apple at 30°C and pineapple, orange, tomato and apple at 35°C (highest to lowest). However, there was no significant difference between any of the four juices in this study at either temperature (Figures 4.1 and 4.2).

In pineapple juice *A. acidoterrestris* increased in numbers until, by day 29 vegetative cells and spores at 30°C and 35°C (Figures 4.1 and 4.2) were significantly higher ($P<0.05$) than on day 1. There was no significant difference between vegetative cell numbers at 30°C and 35°C (Figures 4.1a and 4.2a) until days 22 and 29 when numbers at 35°C were significantly higher. Spore counts (Figure 4.2a) were significantly higher at 35°C on days 15 and 22 after which no differences in counts due to temperature were observed. At 30°C on days 1 and 8

significantly ($P<0.05$) higher spore counts (Figure 4.1a and b) compared to vegetative counts were recorded after which there was no significant difference. At 35°C significantly higher numbers of spores (Figure 4.2b) compared to vegetative cells (Figure 4.2a) were counted on days 15, 22 and 29. *A. acidoterrestris* vegetative cells and spores increased in numbers by 1 log at days 5 and 8 respectively at 30°C (Figures 4.1a and b) and vegetative cells at 35°C (Figure 4.2a) also increased by 1 log by day 5. However, spore counts (Figure 4.2b), although increasing by almost 1 log at day 5, then increased by over 2 logs by day 15 at 35°C.

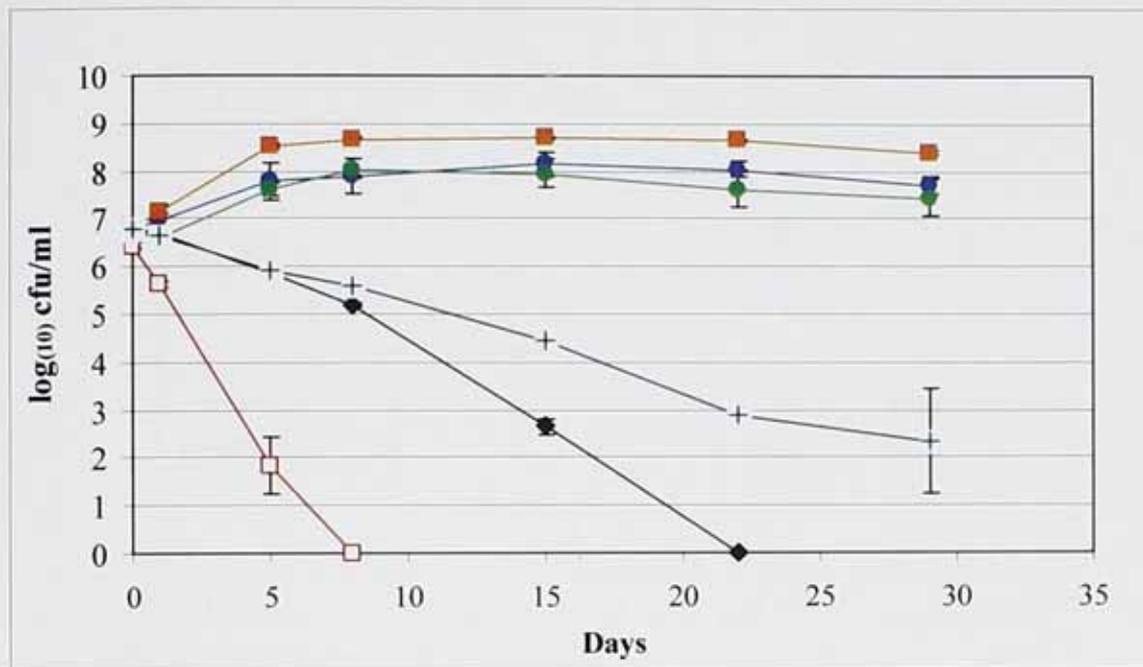
Although numbers of vegetative cells and spores (Figures 4.1a and 4.1b) of *A. acidoterrestris* had declined by day 29 in apple juice there was no significant difference on day 1 from day 29 at 30°C. At 35°C however, counts (Figures 4.2a and 4.2b) on day 29 were significantly higher ($P<0.05$) from day 1 in both vegetative cells and spores. On day 15 at 30°C (Figures 4.1a and 4.1b) spore numbers were significantly higher than vegetative cells no significant difference was noted on any other days. Spore numbers were significantly higher at 35°C than vegetative cells (Figures 4.2a and 4.2b) at day 22 and, although spore counts were higher on days 15, 22 and 29, the numbers were not significant. When numbers of vegetative cells (Figures 4.1a and 4.2a) at 30°C were compared to numbers of cells at 35°C counts were found to be significantly higher at 35°C on all days tested. Spore counts at 35°C (Figures 4.1b and 4.2b) were also found to be significantly higher than at 30°C on all days tested apart from day 1 when spores at 30°C were lower in number but not significantly. *A. acidoterrestris* was

initially isolated from spoiled apple juice (Cerny *et al.*, 1984) and it is in apple juice that spoilage has most often occurred (Chang and Kang, 2004).

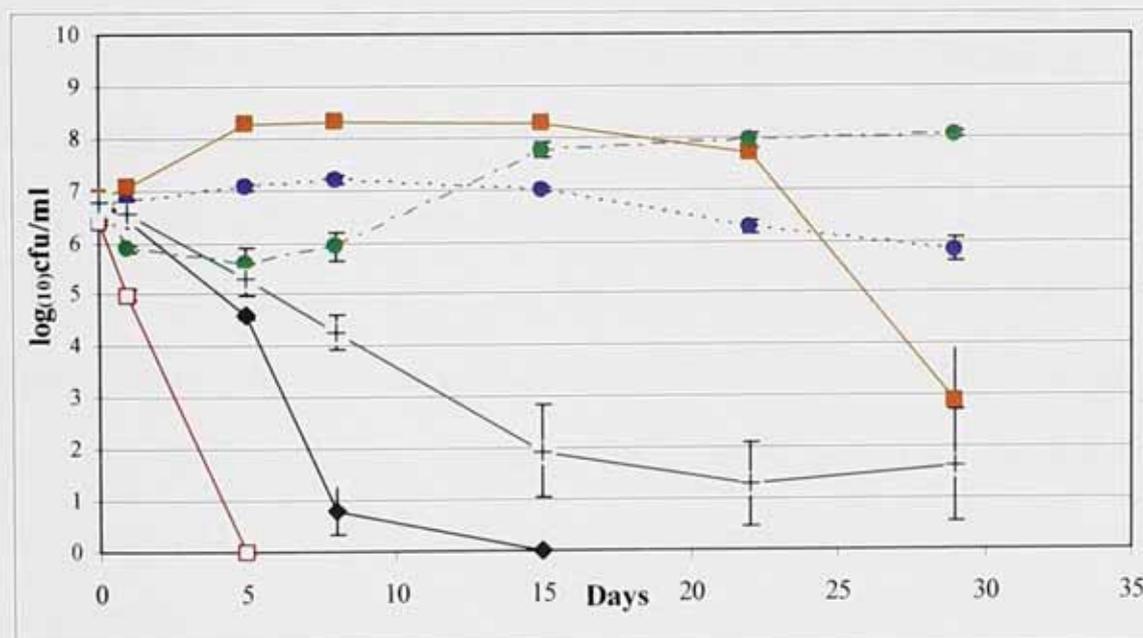
4.5.2. Growth of *P. cyclohexanicum* in fruit juices

Although *P. cyclohexanicum* was first isolated from spoiled orange juice, it is able to survive in other fruit juices (Figure 4.3). At 30°C no viable cells could be detected after 8 days in cranberry juice and 22 days in grapefruit juice (Figure 4.3a) whereas at 35°C none could be detected after 5 and 15 days, respectively (Figure 4.3b). The organism multiplied in tomato juice by approximately 1 log over 8 days at 30°C whereas at 35°C there was no significant increase in numbers. In apple juice viable counts declined over 29 days at 30°C or 35°C with no significant difference between counts.

In pineapple juice there was no significant difference ($P < 0.05$) between numbers of viable cells present at 30°C and 35°C at day 29 and the increase was approximately 1 log overall. Over 15 days in orange juice, the juice from which *P. cyclohexanicum* was first isolated, the bacterium multiplied to significantly ($P < 0.05$) higher numbers at 30°C compared to 35°C increasing by 2 log compared with 1.5 log respectively (Figure 4.3) and this difference continued at 29 days.



a



b

Figure 4.3. Survival of *P. cyclohexanicum* in various juices at 30°C (a) and at 35°C (b) Values are means \pm SEM ($n = 6$) --♦-- grapefruit (pH 3.2); --□-- cranberry (pH 2.7); --●-- tomato (pH 4.0); --■-- orange (pH 3.8); --●-- pineapple (pH 3.6); --+-- apple (pH 3.5)

Juice	pH at Day 0	pH at day 29		Brix at Day 0	Brix at day 29	
		30°C	35°C		30°C	35°C
Grapefruit	3.2	3.2	3.2	10.6	10.4	10.5
Cranberry	2.7	2.7	2.7	11.9	11.7	11.8
Tomato	4.0	4.0	4.0	5.7	5.5	5.6
Orange	3.8	3.8	3.9	11.1	10.9	10.8
Pineapple	3.6	3.6	3.6	12.5	12.3	12.2
Apple	3.5	3.5	3.5	11.0	10.9	10.9

Table 4.2. Brix and pH values of fruit juices inoculated with *P. cyclohexanicum* and incubated at 30°C and 35°C over 29 days. Values are means \pm SEM ($n = 6$).

Changes in pH and Brix values were recorded (Table 4.2) over the incubation period. The Brix values all declined over 29 days, although this was not significant, except in the case of orange juice ($P < 0.05$), whereas pH did not change significantly over the same period. Although there are significant differences between the initial pH and Brix values of the juices tested ($F = 51$, $P = 0.00$), for each juice there are no significant differences between the initial pH or Brix and those at 29 days at either 30°C or 35°C or between the different temperatures ($F = 1$, $P = 0.337$), the only exception being Brix in the case of orange juice.

4.5.3 The effect of pasteurisation procedures on *P. cyclohexanicum*

Overall *P. cyclohexanicum* failed to survive following all pasteurisation treatments attempted. Pasteurising at a lower temperature would be inadequate as *P. cyclohexanicum* has been shown to be able to survive to 90°C. Cleaning regimes were investigated to ensure no cleaning residues were causing cell death

which was not found to be the case. This was performed by running sterile de-ionised water through the pasteuriser at 100°C and testing the pH of the last run-off water to ensure it had returned to neutral pH. Therefore, a series of studies was designed to investigate the survival of *P. cyclohexanicum* at various temperatures.

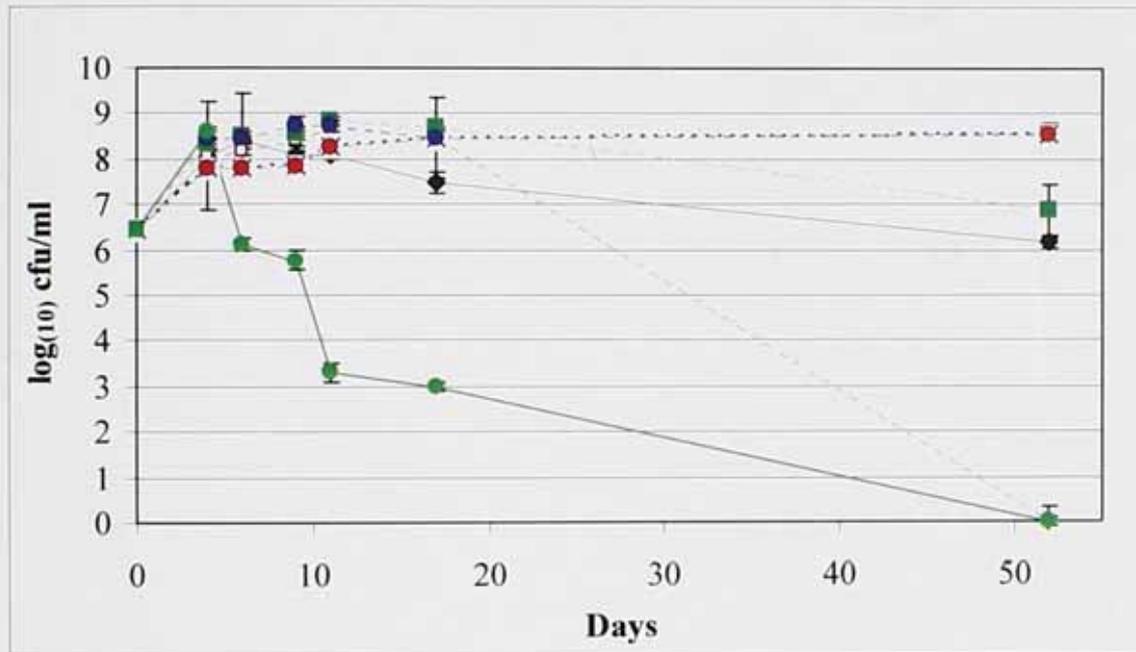
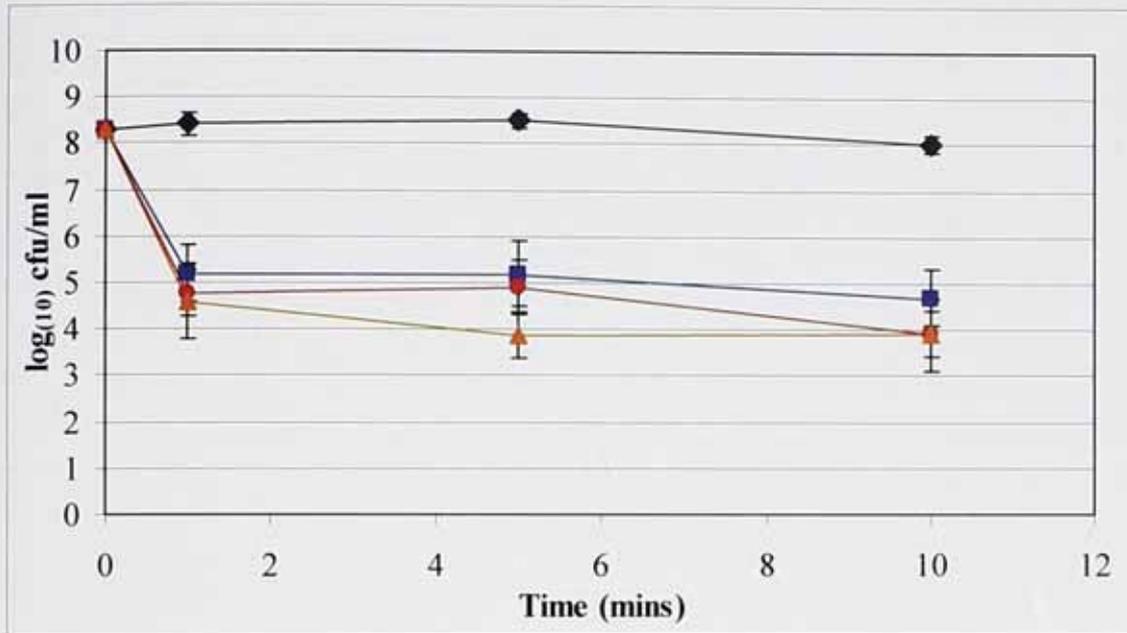
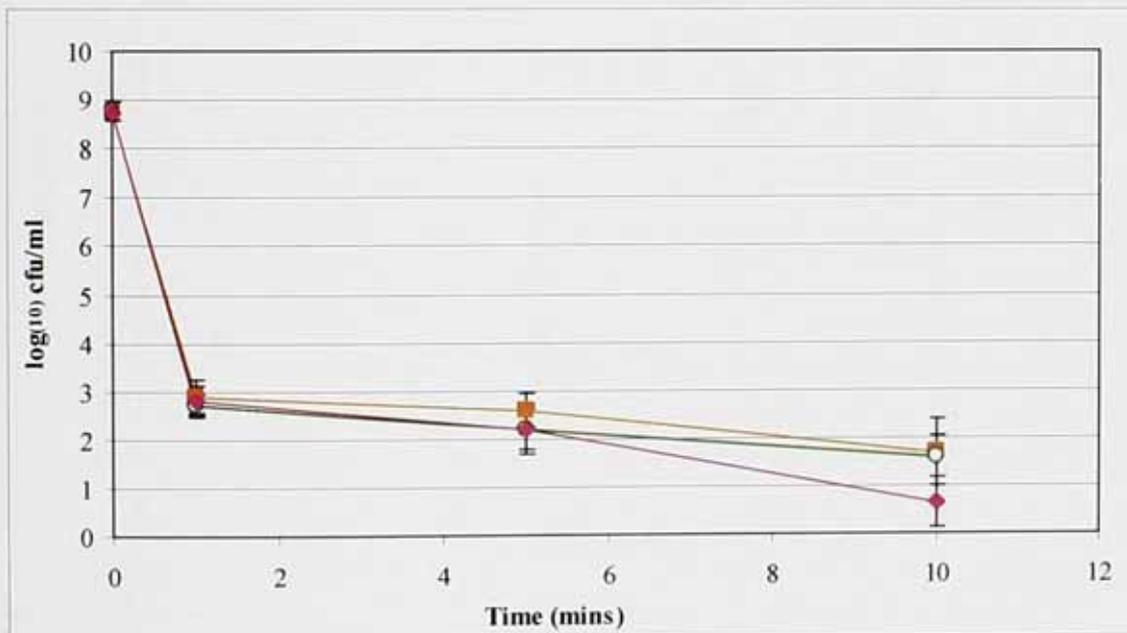


Figure 4.4. Growth of *P. cyclohexanicum* in orange juice (pH 3.8) at different temperatures. Values are means \pm SEM ($n = 6$). --◆-- 4°C; --□-- 15°C; --▲-- 20°C; --●-- 25°C; --■-- 30°C; --●-- 35°C; --●-- 40°C

The growth of *P. cyclohexanicum* was monitored in orange juice at a range of temperatures (Figure 4.4) and was able to survive at all temperatures tested for at least 11 days. By day 6, survival at 40°C was declining but survival at lower temperatures was sustained until day 17. After 52 days no viable cells were detected at 35°C but at all other temperatures at least 6 log cells/ml remained viable.

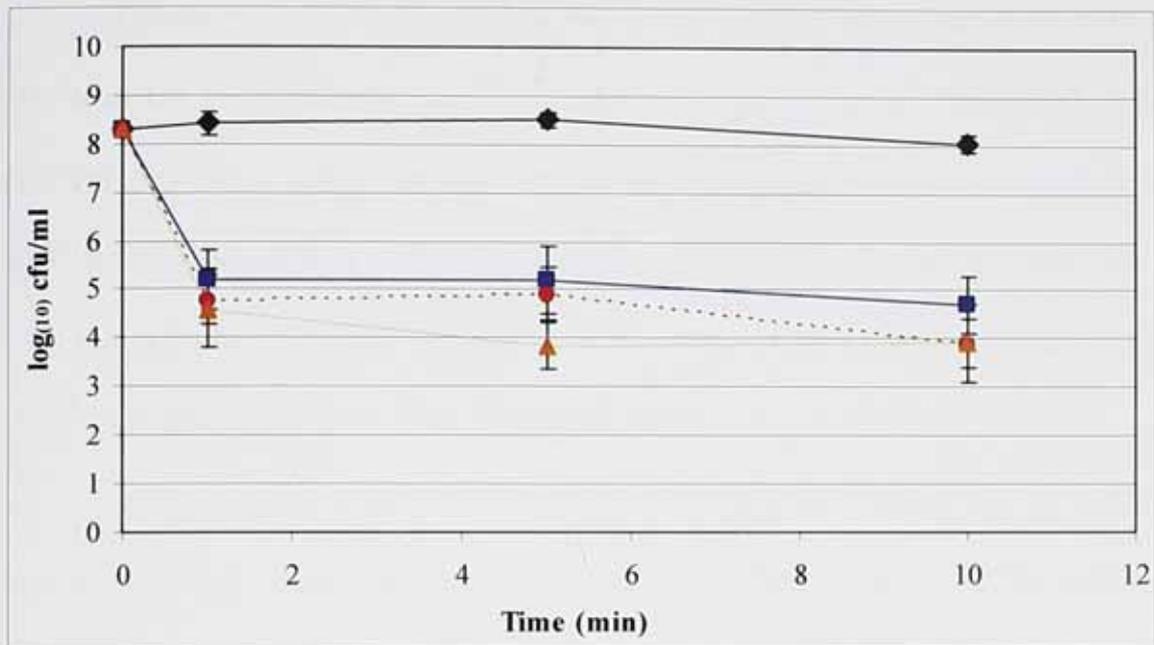


a

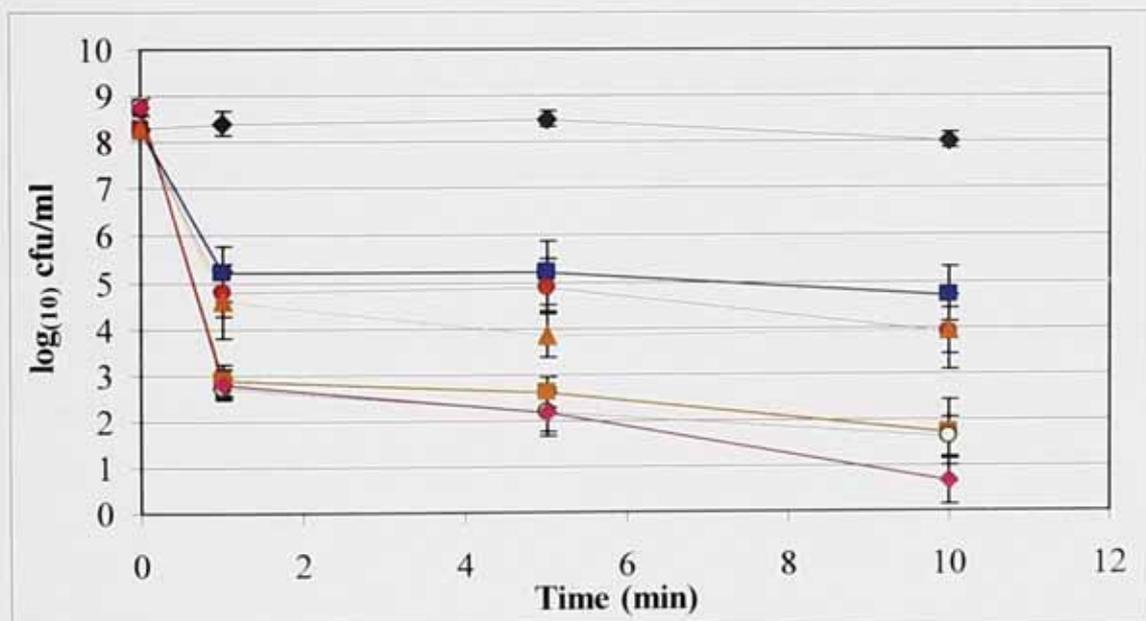


b

Figure 4.5. Survival of *P. cyclohexanicum* exposed to heat treatment for 10 minutes in PYG broth (pH 5.9) (a) non-incremental treatment, (b) incremental treatment. Values are means \pm SEM ($n = 6$). \blacklozenge - 50°C; \blacksquare - 60°C; \bullet -70°C; \blacktriangle - 80°C; \blacksquare - 85°C; \circ -90°C; \blacklozenge -95°C.



a



b

Figure 4.6. Survival of *P. cyclohexanicum* exposed to heat treatment for 10 minutes in orange juice (pH 3.8) (a) non-incremental treatment, (b) incremental treatment. Values are means \pm SEM ($n = 6$). -◆- 50°C; -■- 60°C; -●- 70°C; -▲- 80°C; -■- 85°C; -○- 90°C; -◆- 95°C.

A proportion of the population was able to survive when subjected to heat treatment for 10 minutes at various increasing temperatures in PYG broth, with survival dependant upon the time-temperature combination. Heat treatment at 50°C for 10 minutes had very little effect on viable counts. However, there was a ~3-4 log reduction after 10 minutes at 60°C, 70°C or 80°C, with no statistically significant difference ($P>0.05$) between the responses

Following an initial decline in survival at 1 minute, there was no further statistically significant reduction in survivor numbers after 5 or 10 minutes (Figure 4.5a). No survivors were detected after treatment at 90°C or 95°C, even after 1 minute after non-incremental treatment.

No significant difference was found in the numbers of survivors at 50°C, 60°C, 70°C or 80°C after incremental heat treatment in PYG broth compared to the non-incremental regime. However, after treatment at 85°C, 90°C and 95°C for 10 minutes some cells remained viable (Figure 4.5b) with no significant difference between the effect of these three temperature treatments ($P>0.05$).

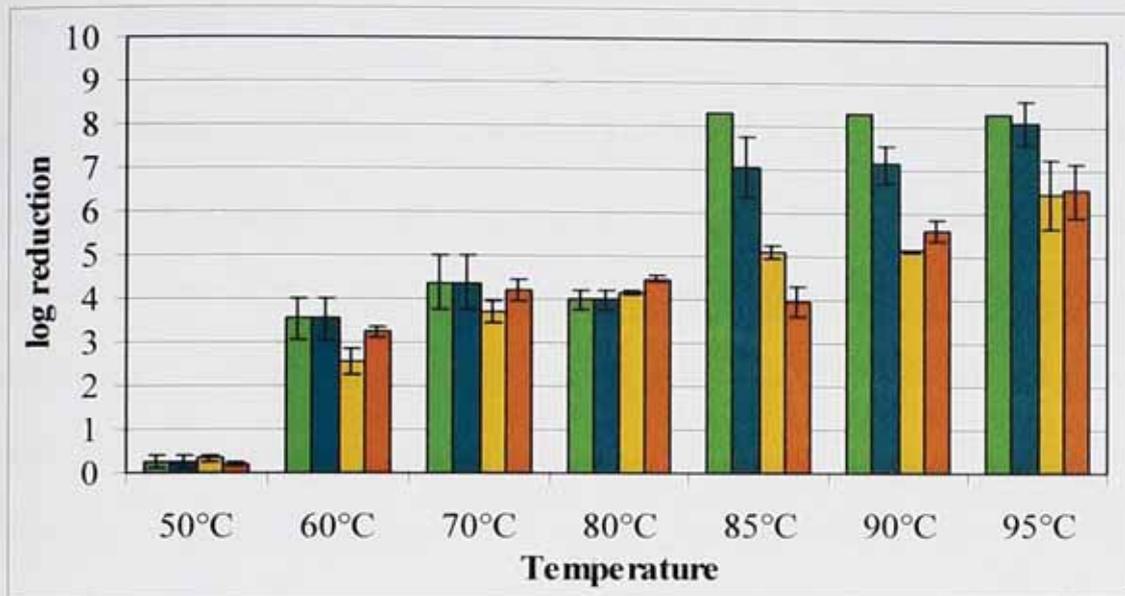


Figure 4.7. Log reductions in viable counts of *P. cyclohexanicum* after 10 minutes exposure to various temperature regimes. Values are means \pm SEM ($n = 6$). ■ non-incremental treatment in PYG broth (pH5.9), ■ incremental treatment in PYG broth (pH5.9), ■ non-incremental treatment in orange juice (pH3.8), ■ incremental treatment in orange juice (pH3.8).

In orange juice (Figures 4.6 and 4.7) some cells survive after both treatment regimes at 95°C for 10 minutes with approximately 1 log viable counts detected. Generally there was no significant difference between the log reductions demonstrated in the non-incremental treatment in orange juice compared with the incremental treatment regime at any of the temperatures tested. There was also no significant difference in the effect of heat treatments in culture medium or orange juice at 50°C to 80°C, although at 85°C, 90°C and 95°C there was a significantly greater ($P > 0.05$) log reduction in culture medium compared with orange juice (Figure 4.7).

4.6. Discussion

The results of this study confirm the ability of *A. acidoterrestris* to survive and grow in a variety of fruit juices at different pH levels. Not all fruit juices support growth of *A. acidoterrestris* and this bacterium failed to grow in prune juice (pH3.7), a cranberry juice with a pH level of 2.4, a Concord grape juice and other red grape juices (Splittstoesser *et al.*, 1998). The red grape juice may have failed to support growth because of natural phenolic compounds which might have been present in the juice. Another reason may have been the higher sugar levels found in grapes with very little growth occurring in Riesling grape juice (Brix 21.6°), but reducing the juice to 16° Brix allowed maximum growth (Splittstoesser *et al.*, 1994). The cranberry juice (pH2.7) in this study did support growth of *A. acidoterrestris* vegetative cells but not spores, (Figures 4.1 and 4.2) suggesting that the higher pH of this cranberry juice compared to the pH2.4 of the other cranberry juice may have aided the growth of *A. acidoterrestris* but outgrowth of spores was prevented.

Tomato juice permitted high levels of growth of *A. acidoterrestris* and this may be explained by its higher pH of 4.0 and the lower amount of soluble solids or possibly the use of a different strain of *A. acidoterrestris* (Splittstoesser *et al.*, 1994).

A. acidoterrestris produced different growth characteristics at the two temperatures tested with significantly higher counts of vegetative cells (Figure 4.1 and 4.2) but not spores ($P < 0.05$) in grapefruit juice at 35°C. Tomato juice

recorded significantly higher numbers of spores at 35°C but not vegetative cells, as did orange juice. Spore and vegetative cell counts of *A. acidoterrestris* were higher in apple juice at 35°C, but temperature seemed to affect the growth of this bacterium in pineapple juice to a lesser extent with counts higher at 30°C on some days then higher at 35°C on other days. *A. acidoterrestris* was affected least by temperature in cranberry juice with very low levels of vegetative cell growth and no outgrowth of spores could be detected.

In contrast to *A. acidoterrestris* that grew in every juice, *P. cyclohexanicum* did not survive at 30°C to day 15 in cranberry juice or day 29 in grapefruit juice and had declined by ~ 4 log by day 29 in apple juice. However, similarly to *A. acidoterrestris* the highest numbers of *P. cyclohexanicum* occurred in orange, pineapple and tomato juice at 30°C. However, at 35°C growth in orange juice had declined by 5 log and in tomato juice by 2 log compared to the levels at 30°C, suggesting that pineapple juice supported the highest growth of *P. cyclohexanicum* at 35°C after 29 days. This may have been partly because during the higher growth levels throughout the study. *P. cyclohexanicum* may have exhausted the nutrients in the sample containers at the higher temperature.

This study therefore demonstrates that *P. cyclohexanicum* is able to survive and in some cases multiply in other juices with pHs varying from 2.7 (cranberry) to pH4.0 (tomato) and Brix values from 5.7° (tomato) to 12.5° (pineapple), with the length of time of survival varying. In cranberry (pH 2.7; Brix 11.9°), grapefruit (pH 3.3; Brix 10.6°) and apple (pH 3.5; Brix 11.0°) juices,

although the organism survives, viable counts decrease, while in tomato juice (pH 4.1; Brix 5.2°), orange (pH 3.9; Brix 9.9°) and pineapple (pH 3.6; Brix 12.0°) juices, the organism multiplies. The results suggest therefore that Brix values at these levels do not have as much influence as pH on the growth of *P. cyclohexanicum* since it is able to grow in juices with Brix values of 5.2° (tomato), 9.9° (orange) and 12.0° (pineapple) while it does not multiply in apple juice (pH 3.5) but does in pineapple juice (pH 3.6) with similar initial Brix values, suggesting the lowest pH sustaining growth is pH 3.6.

The results of this study confirm the fact that *P. cyclohexanicum* is a very heat resistant bacterium, surviving 10 minutes exposure at 95°C in orange juice which corresponds to the original description of the species in which it was reported as surviving 10 minutes at 90°C (Kusano *et al.*, 1997). This makes it the most heat resistant of the propionibacteria, surviving higher temperatures than *P. freudenreichii*, previously considered the most heat resistant of this group of bacteria. The cell membrane of *P. cyclohexanicum* has ω -cyclohexyl undecanoic acid as its major fatty acid and, as in the case of *A. acidoterrestris*; this probably protects the cell from high temperatures and high acidity (Pontius *et al.*, 1997), although unlike *A. acidoterrestris* *P. cyclohexanicum* produces no spores.

Changes in environmental conditions including those of temperature or pH are potential stressors to organisms. An adaptive tolerance response (ATR) involving the synthesis of *de novo* polypeptides such as the universal chaperonins, GroEL and GroES, has been demonstrated in *P. freudenreichii* in response to acid

or bile salts (Jan *et al.*, 2000, 2001; Leverrier *et al.*, 2003) and also in response to heat (Anastasiou *et al.*, 2006). In the latter case both constitutive thermotolerance and adaptive thermal thermotolerance were evidenced in natural isolates from cheese and two separate, but overlapping mechanisms, have been suggested. Thermotolerance involving molecular chaperones, antioxidant thiol compounds and molecular actors of the stringent response and thermal adaptation involving signal transduction, biosynthetic pathways and cell wall maintenance in addition to protein turn-over.

P. cyclohexanicum survived for 10 minutes with non-incremental heat treatment at 95°C in orange juice which suggests that the induction of an ATR is not the mechanism. If this were the case then cells would be more likely to survive after an intermediate heat treatment although the intermediate treatment used in this study (approximately 5 minutes) is relatively short for an adaptive response to take place (Anastasiou *et al.*, 2006). The cell membrane of *P. cyclohexanicum* has ω -cyclohexyl undecanoic acid as its major fatty acid and in the case of *A. acidoterrestris*; this probably protects the cell from high temperatures and high acidity (Pontius *et al.*, 1998) although *A. acidoterrestris* is also a spore-former. An explanation for the heat resistance of *P. cyclohexanicum* may therefore be a mechanism of thermotolerance and further investigations are required in order to elucidate the molecular actors involved as in the case of *P. freudenreichii* (Anastasiou *et al.*, 2006).

P. cyclohexanicum was able to survive and multiply in orange juice at low temperatures such as 4°C and 15°C, commonly considered to reduce growth of food spoilage bacteria such as *A. acidoterrestris* (Walker and Phillips, 2005), suggesting that if the organism is present after pasteurisation, either because it survived the pasteurisation process or due to post-pasteurisation contamination, it will be able to survive for up to 52 days. Industrial heat treatment of fruit juices is for example 90°C to 95°C for 30 seconds to 1 minute. These results demonstrate the ability of *P. cyclohexanicum* to survive 10 minutes at 95°C suggest the original isolation was as likely to be due to pre-heat-treatment contamination as post-processing contamination. Although there is no significant difference at 50°C, 60°C, 70°C and 80°C between the log reductions in PYA broth and orange juice the log reductions are less in orange juice at the highest temperature tested. This could be due to a number of factors intrinsic to the juice, including pH, and reflects the fact that orange juice, the juice from which *P. cyclohexanicum* was first isolated, provides a more suitable growth environment for recovery after heat treatment at these higher temperatures.

Kusano *et al.*, (1997) reported the optimum temperature in culture medium as 35°C. In orange juice (Figure 1) over four days, growth rate is faster at 35°C but over longer time periods, the optimum temperature appears to be 25°C. In the same study the pH growth range was suggested as 3.2 to 7.5. However, the results of this present study demonstrate that in cranberry juice (pH 2.7) a proportion of the *P. cyclohexanicum* population is able to survive for five days at 30°C and at least one day at 35°C.

A. acidoterrestris has already become a significant challenge to the fruit juice industry and if *P. cyclohexanicum*, as suggested by this study, has the ability to survive the acidic environment of fruit juices and the capacity to tolerate temperatures such as those used in the pasteurisation process applied to fruit juices this organism could prove to be another significant challenge to the fruit juice industry. However, after pasteurisation many fruit beverages are subjected to further methods of preservation and studies involving these techniques are the subject of much investigation particularly concerning novel micro-organisms.



Chapter 5

The effect of sodium benzoate

potassium sorbate

and nisin on

***A. acidoterrestris* in apple juice and**

***P. cyclohexanicum* in orange juice**

5.1. Introduction

Pasteurisation does not destroy heat resistant spores such as those of *A. acidoterrestris* and, although *P. cyclohexanicum* does not produce spores, it has been isolated from pasteurised orange juice (Kusano *et al.*, 1997). Therefore, other means of preservation are required to prevent spoilage occurring within the shelf life of a product. In the soft drinks industry preservatives can be used as an aid in preventing or delaying the onset of spoilage due to the growth of micro-organisms (Russell and Gould, 2003).

Types of preservatives and the amounts allowed differ between countries, with each country producing detailed legislation for each permitted preservative. In the UK legislation permits more preservatives in, for example, beverages containing higher levels of glucose syrup (>23.5° Brix) and mixed preservatives may be added to one product as long as the overall permitted level is not exceeded (Hicks, 1990).

The broad range bacteriocin nisin has been demonstrated to have inhibitory effects against Gram positive spore forming bacteria including *A. acidoterrestris* (Yamazaki, *et al.*, 2000). The use of nisin as an inhibitor of *P. cyclohexanicum*, a Gram positive, heat resistant, non spore forming bacterium has not been investigated to date.

5.2. *Aims*

This study aims to investigate:

- The effects of two preservatives, sodium benzoate and potassium sorbate, widely used in the food and soft drinks industry and the bacteriocin nisin, on the growth of *A. acidoterrestris* and *P. cyclohexanicum* in apple juice and orange juice respectively.

5.3. *Materials and methods*

Methods and materials were as described in sections 3.8, 3.9 and 3.10.

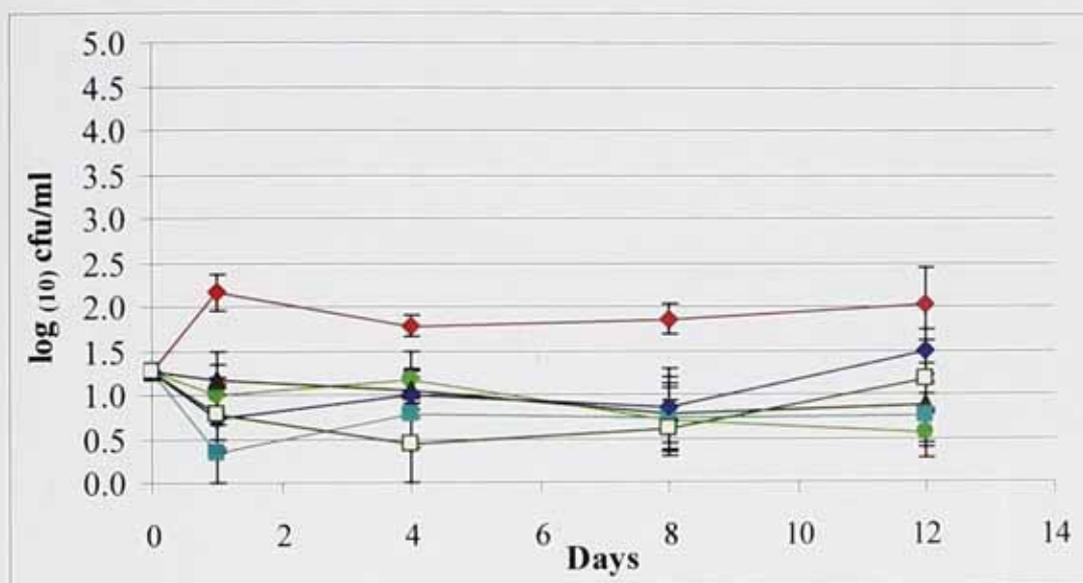
5.4. *Statistical analysis*

Statistical tests were performed by using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) using independent *t*-test analysis. The results are shown as mean \pm SEM. Significance was set at $P < 0.05$.

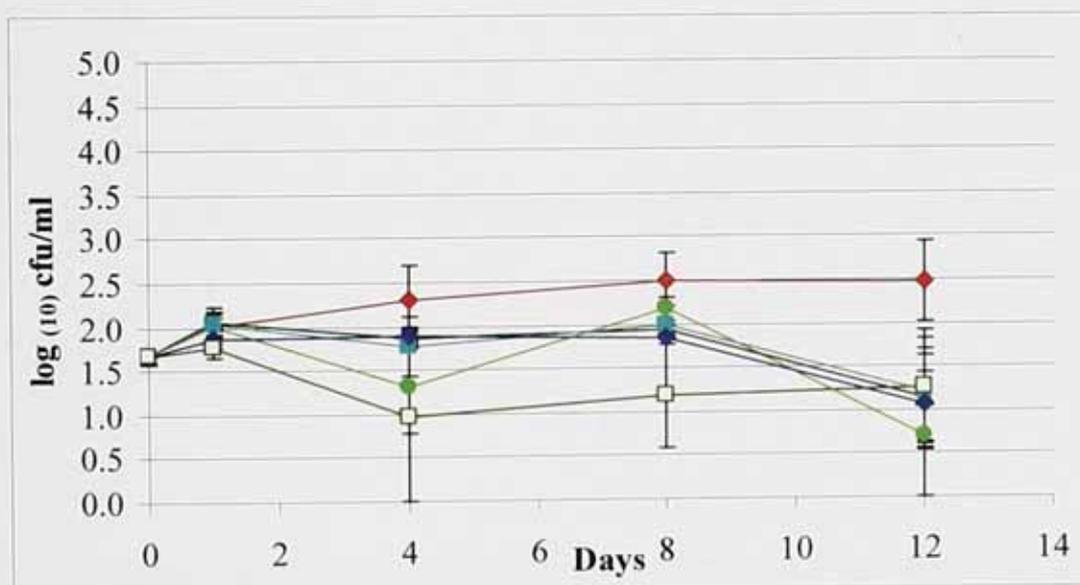
5.5. *Results*

At initial low numbers (10^{-1}) of *A. acidoterrestris* spore counts were lower by day 12 in all concentrations of sodium benzoate although not significantly (Figure 5.1b). However, vegetative cells counts were, slightly higher by day 12 at a concentration of 400ppm of sodium benzoate but not significantly (Figure 5.1a).

There was no significant difference between numbers of vegetative cells or spores (Figures 5.1a and b) at day 12 at any concentration of sodium benzoate.

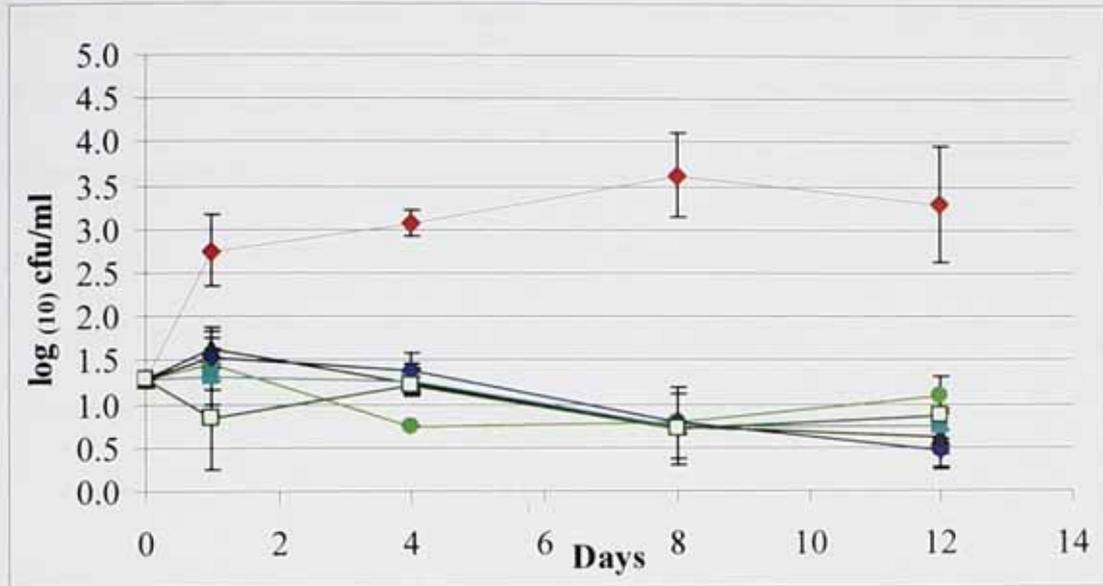


a

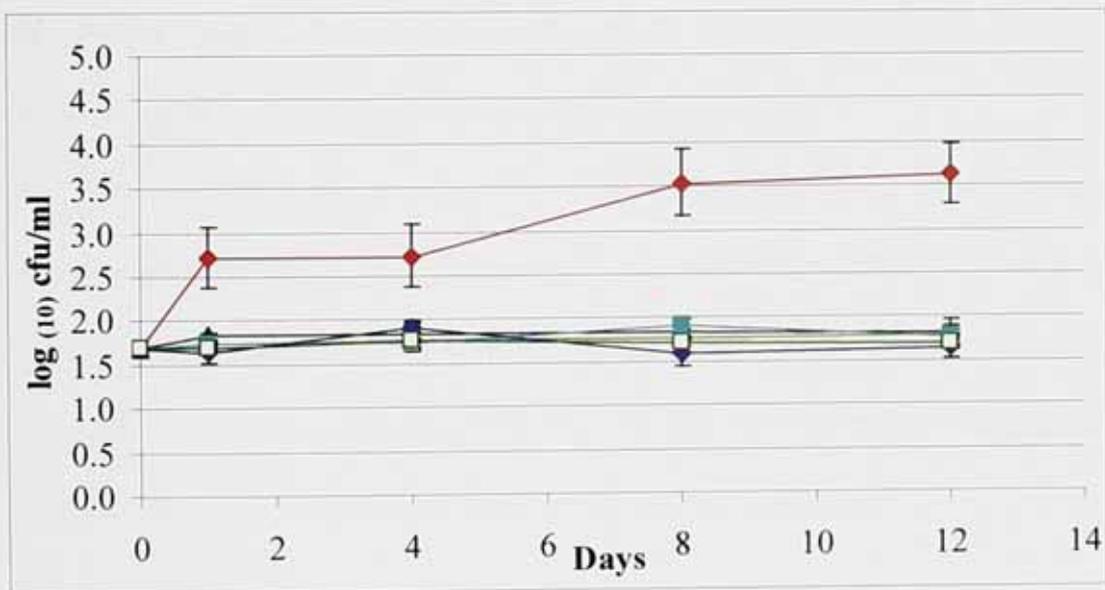


b

Figure 5.1. Survival at 30°C of *A. acidoterrestris*, vegetative cells (a) and spores (b) exposed to sodium benzoate in apple juice (pH 3.5) at 100 – 500ppm. Values are means SEM \pm , ($n = 6$) -♦- 0ppm; -●- 100ppm; -▲- 200ppm; -■- 300ppm; -◆- 400ppm and -□- 500ppm of sodium benzoate.



a



b

Figure 5.2. Survival at 30°C of *A. acidoterrestris*, vegetative cells (a) and spores (b) exposed to potassium sorbate in apple juice (pH3.5) at 100 – 500ppm. Values are means SEM \pm . ($n = 6$) -♦- 0ppm; -●- 100ppm; -▲- 200ppm; -■- 300ppm; -◆- 400ppm and -□- 500ppm of potassium sorbate.

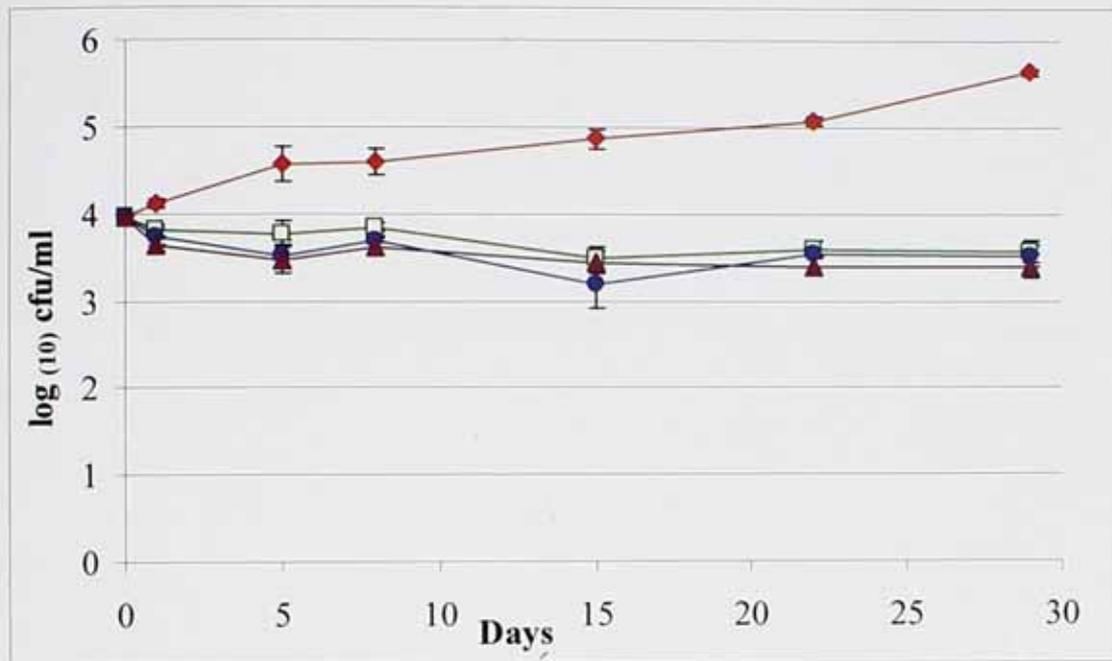
No growth of vegetative cells occurred in any concentrations of potassium sorbate on any days with all counts lower by day 12 although not significantly

(Figure 5.2a). Spore counts (Figure 5.2b) were higher by day 12 but not significantly at all concentrations of potassium sorbate except 400ppm and 500ppm where numbers were very similar to the initial inoculum.

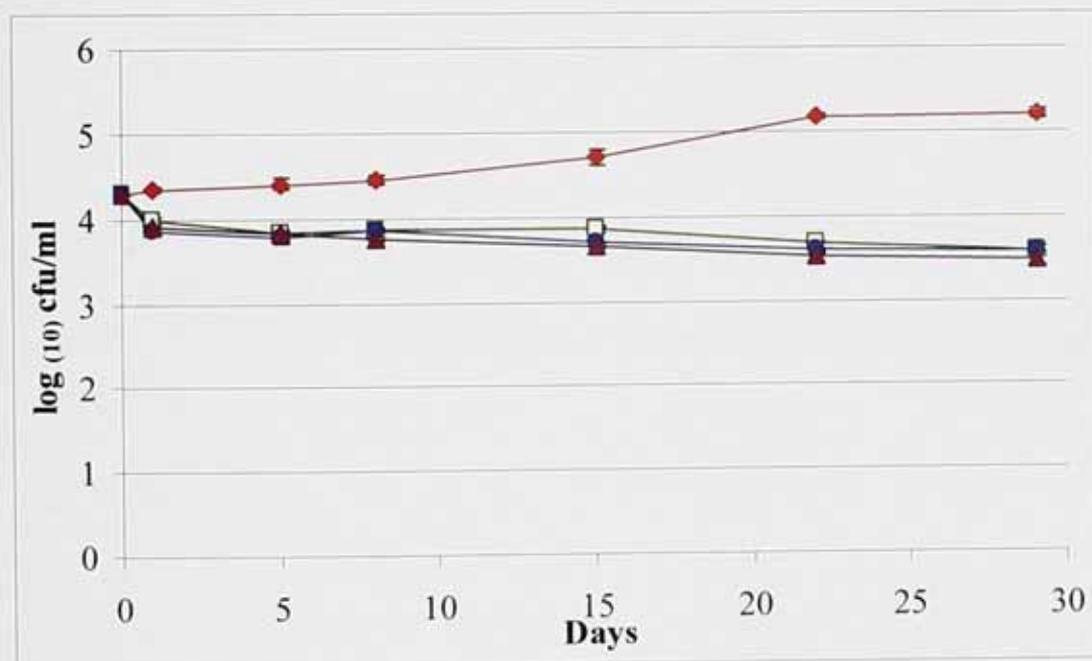
There was no significant difference between the numbers of survivors of *A. acidoterrestris* between the two preservatives (Figures 5.1 and 5.2) although sodium benzoate seemed to be more effective against spores.

The results of this study suggest that low numbers of *A. acidoterrestris* can survive or though not multiply at these levels of preservative and higher levels may be more successful in killing cells or spores. Further studies were performed using higher initial inoculum levels of *A. acidoterrestris* and higher concentrations of the two preservatives (Figures 5.3 and 5.4).

No growth of *A. acidoterrestris* occurred on any day (Figure 5.3) in any of the tested concentrations of sodium benzoate. No significant differences occurred between any days apart from day 8 when vegetative cell counts (Figure 5.3a) were significantly higher than day 15 ($P < 0.05$). By day 29 vegetative cell numbers were lower in all concentrations, although this was not significant.

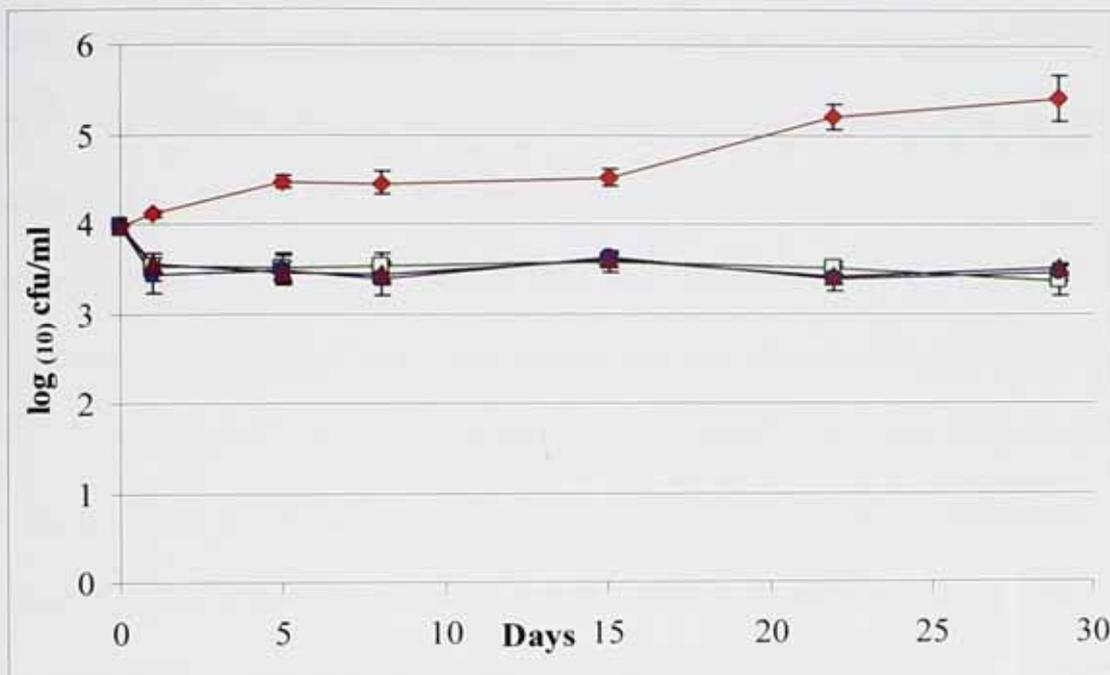


a

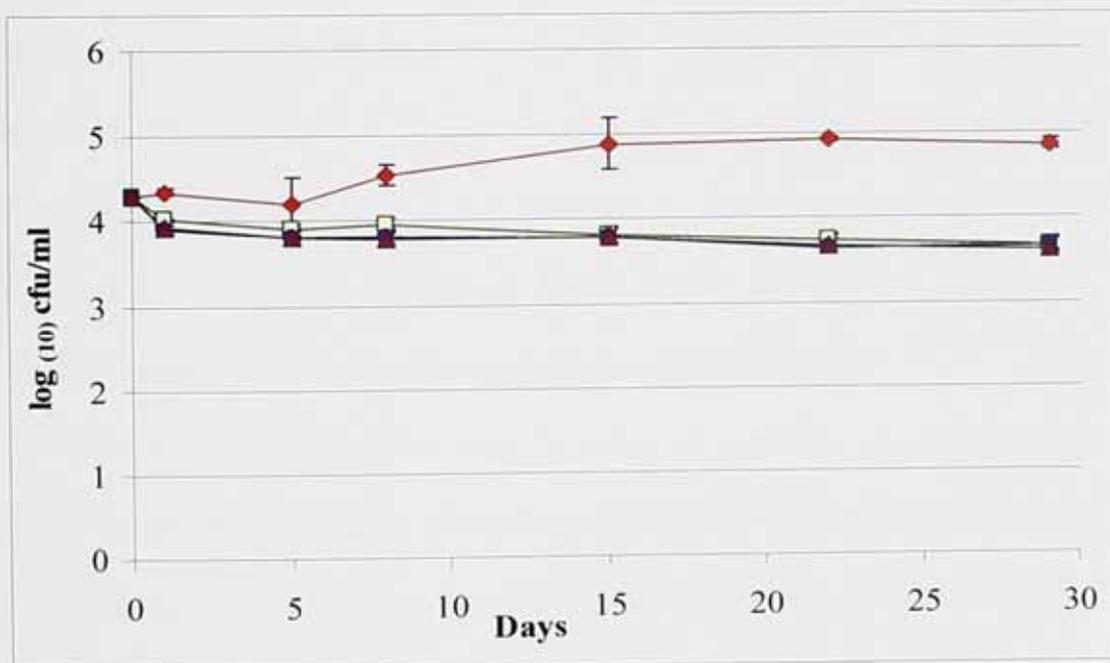


b

Figure 5.3. Survival at 30°C of *A. acidoterrestris*, vegetative cells (a) and spores (b) exposed to sodium benzoate in apple juice (pH3.5) at 500 – 1500ppm. Values are means SEM \pm . ($n = 6$) -◆- 0ppm; -□- 500ppm; -●- 1000ppm; -▲- 1500ppm sodium benzoate.



a



b

Figure 5.4 Survival at 30°C of *A. acidoterrestris* vegetative cells (a) and spores (b) exposed to potassium sorbate in apple juice (pH3.5) at 500 – 1500ppm. Values are means SEM \pm , ($n = 6$) -♦- 0ppm; -□- 500ppm; -●- 1000ppm; -▲- 1500ppm of potassium sorbate.

Spore counts (Figure 5.3b) were significantly higher on day 1 ($P<0.05$) compared to day 29 in all concentrations of sodium benzoate. A general decrease in numbers occurred over 29 days with significantly lower spore counts on day 22 compared to day 15 ($P<0.05$), suggesting that levels of sodium benzoate may prevent both growth of vegetative cells and sporulation of *A. acidoterrestris*, although viable cells and spores still remain and may cause spoilage.

No growth of *A. acidoterrestris* occurred on any days in any concentrations in potassium sorbate (Figure 5.4). No significant differences in numbers of *A. acidoterrestris* were noted between any days at concentrations of 500ppm. However, on day 15 at concentrations of 1000ppm and 1500ppm vegetative cell counts (Figure 5.4a) were significantly higher ($P<0.05$) than on days 8 and 22 which compares to a significant rise ($P<0.05$) in spore counts on day 15 at a concentration of 500ppm sodium benzoate. This increase in numbers, although significant, did not cause numbers to rise above those of the initial inoculum. By day 29 vegetative cells (Figure 5.4a) were lower in all concentrations of potassium sorbate, although not significantly.

No growth of spores occurred in any concentrations of potassium sorbate and no significant differences were demonstrated between any days, however by day 29 all spore counts in all concentrations were significantly lower than on day 1.

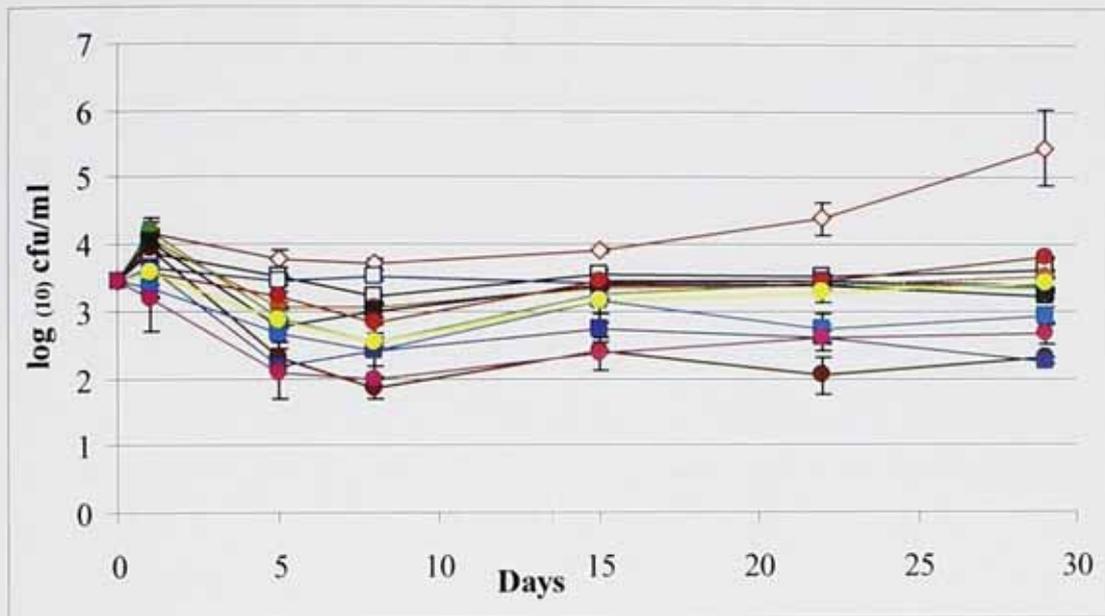
This suggests that potassium sorbate may prevent the growth of *A. acidoterrestris* but that, as with sodium benzoate, remaining cells or spores may still survive and cause spoilage. The use of sodium benzoate or potassium sorbate

may be enhanced by the addition of the bacteriocin nisin possibly allowing lower levels of these two preservatives to be used if nisin provides a synergistic effect.

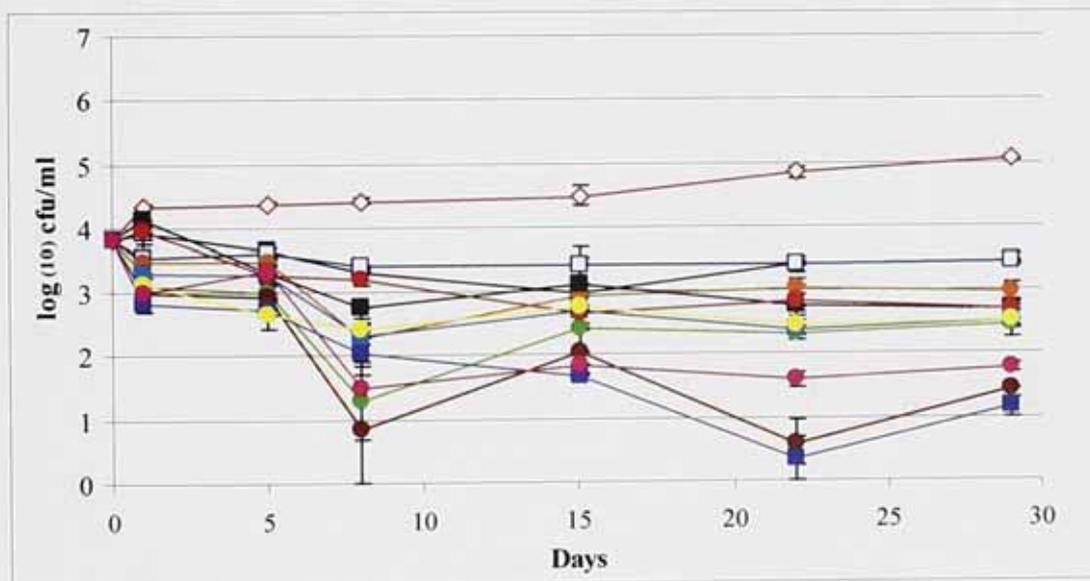
5.5.1 The effect of nisin on *A. acidoterrestris*

In sodium benzoate concentrations of 500ppm no significant differences were observed in numbers of *A. acidoterrestris* (Figure 5.5) apart from day 5 when spores and vegetative cells were significantly higher ($P<0.05$) and day 29 when vegetative cells were significantly higher at benzoate concentrations of 500ppm. In potassium sorbate concentrations of 1000ppm vegetative cell counts were significantly higher ($P<0.05$) on day 1 compared to day 5, however by day 29 numbers of *A. acidoterrestris* (Figures 5.5a and b) had decreased and were lower than day 1 in all preservative samples with no nisin addition.

A comparison of *A. acidoterrestris* counts in sodium benzoate and potassium sorbate demonstrated that sorbate at concentrations of 500ppm recorded higher viable counts of vegetative cells (Figure 5.6a) on day 1 and again on day 29 although these were not significant. However, at concentrations of 1000ppm although sorbate showed the highest counts on day 1, by day 29 benzoate (Figure 5.5a) provided the highest numbers of vegetative cells and again these were not significant. Very little difference was exhibited between numbers of spores (5.5b) in the 1000ppm samples of sorbate and benzoate, however in the 500ppm samples of spore counts in sodium benzoate were significantly higher ($P<0.05$) than in the potassium sorbate.

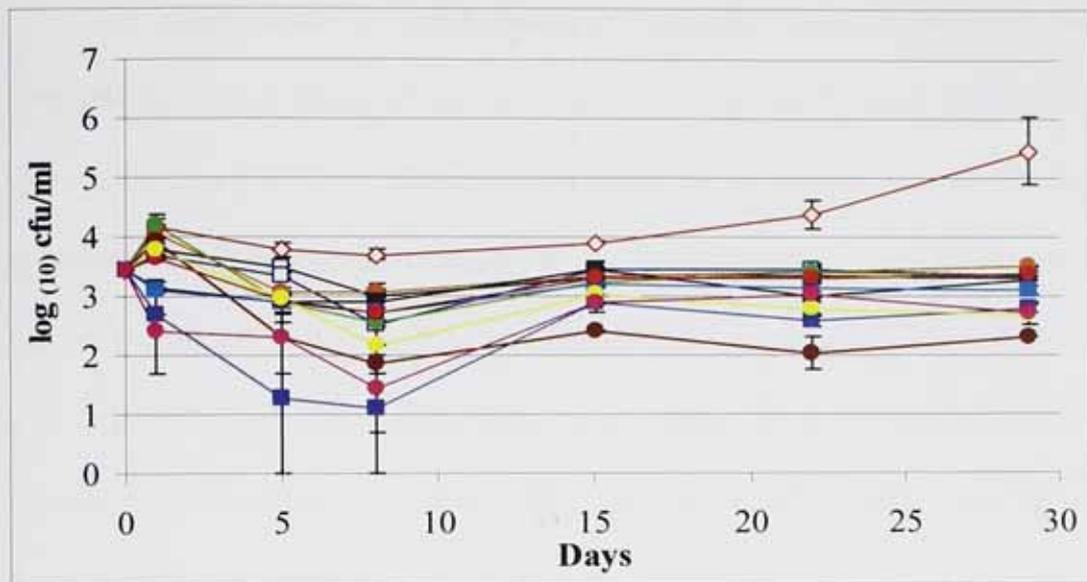


a

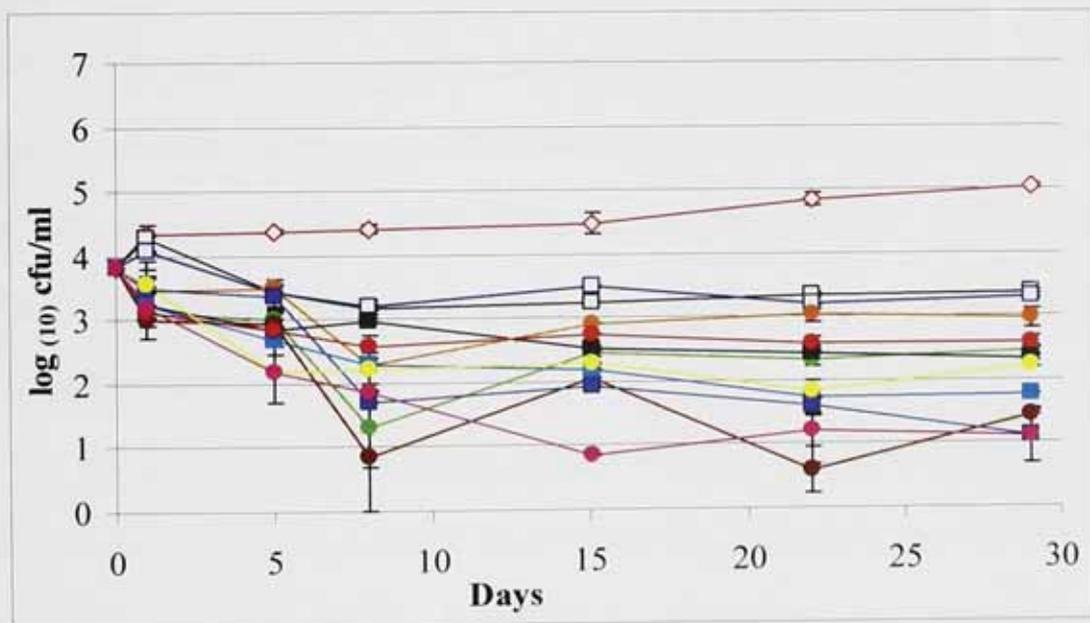


b

Figure 5.5 Survival at 30°C of *A. acidoterrestris* vegetative cells (a) and spores (b) exposed to sodium benzoate and nisin in apple juice (pH3.5). Values are means SEM \pm . ($n = 6$) - \diamond - No preservative - \square - 500ppm; - \square - 1000ppm; sodium benzoate. - \bullet - 2.5 IU/ml; - \bullet - 5 IU/ml; - \bullet - 10 IU/ml nisin; - \blacksquare -500ppm benzoate and 2.5 IU/ml nisin; - \blacksquare -500ppm benzoate and 5 IU/ml nisin; - \blacksquare -500ppm benzoate and 10 IU/ml nisin; 1000ppm benzoate and 10 IU/ml nisin. - \bullet -1000ppm benzoate and 2.5 IU/ml nisin; - \bullet -1000ppm benzoate and 5 IU/ml nisin; - \bullet -1000ppm benzoate and 10 IU/ml nisin.



a



b

Figure 5.6. Survival at 30°C of *A. acidoterrestris* vegetative cells (a) and spores (b) exposed to potassium sorbate and nisin in apple juice (pH3.5). Values are means SEM \pm ($n = 6$) - \diamond - No preservative; - \square - 500ppm; - \square - 1000ppm; - \bullet - 2.5 IU/ml; - \bullet - 5 IU/ml; - \bullet - 10 IU/ml nisin; - \blacksquare -500ppm sorbate and 2.5 IU/ml nisin; - \blacksquare -500ppm sorbate and 5 IU/ml nisin; - \blacksquare -500ppm sorbate and 10 IU nisin; - \bullet - 1000ppm sorbate and 2.5 IU nisin; - \bullet -1000ppm sorbate and 5 IU/ml nisin; - \bullet - 1000ppm sorbate and 10 IU/ml nisin.

In nisin alone *A. acidoterrestris* numbers were significantly higher ($P < 0.05$) on day 1 when compared to day 29 (Figures 5.5 and 5.6) apart from spore counts in 2.5IU/ml nisin concentrations when counts were higher but not significantly. The largest decrease in *A. acidoterrestris* was shown at nisin concentrations of 10 IU/ml where numbers were 1 log lower by day 29 compared with day 1. The only other differences demonstrated were on days 1 and 15 when vegetative cell numbers (5.5a and 5.6a) were significantly higher than days 5 and 8 respectively in nisin concentrations of 2.5IU/ml and 5IU/ml. In all nisin samples numbers of *A. acidoterrestris* show an initial increase on day 1 (Figures 5.5 and 5.6) followed by a decrease in counts by day 5 after which counts increase though not significantly to day 29 by which time at 2.5IU/ml nisin, numbers of *A. acidoterrestris* are higher than the initial inoculum although not significantly. This suggests that nisin levels of 5IU/ml and 10IU/ml may prevent the growth of *A. acidoterrestris*.

A combination of 500ppm sodium benzoate and nisin demonstrated a significant decrease of *A. acidoterrestris* (Figures 5.5 and 5.6) by day 29 at all benzoate/nisin concentrations. Increasing the sodium benzoate concentration to 1000ppm reduced the numbers of vegetative cells by day 29 (Figure 5.5a) in all concentrations of nisin and numbers of cells were significantly ($P > 0.05$) reduced with the addition of 10 IU/ml of nisin. Significant reductions in numbers of spores (Figure 5.5b) occurred at all nisin and benzoate concentrations. This suggests that the addition of nisin to sodium benzoate may provide a synergistic effect and lower levels of the preservative may be used.

When counts of vegetative cells and spores were compared at the benzoate/nisin combinations vegetative cell counts (Figure 5.5a) were all significantly higher ($P<0.05$) than numbers of spores at 1000ppm sodium benzoate at all concentrations of nisin. At 500ppm of sodium benzoate, vegetative cell counts were significantly higher ($P<0.05$) at nisin concentrations 2.5/mlIU and 10IU/ml and although at 5IU/ml nisin cell counts were higher, this was not significant, suggesting that at these concentrations of sodium benzoate and nisin the decrease in spore counts is significantly higher than the reduction of vegetative cells.

Vegetative cell counts were all significantly higher on day 1 in 500ppm of potassium sorbate (Figure 5.6a) compared to the sorbate/nisin combinations. By day 29 this was maintained and, although numbers were still higher in sorbate (500ppm), these were only significant ($P<0.05$) at 10IU/ml nisin. Numbers of spores (Figure 5.6b) were significantly higher ($P<0.05$) in potassium sorbate alone at 500ppm and 1000ppm compared to the sorbate/nisin combinations suggesting that sorbate/nisin combinations were more effective at reducing the numbers of *A. acidoterrestris* than potassium sorbate used alone.

When the survival of *A. acidoterrestris* vegetative cells and spores (Figure 5.6) were compared in the sorbate/nisin combinations vegetative cells numbers were all significantly higher on day 29 in potassium sorbate. This suggests that potassium sorbate and nisin are more effective against spores than vegetative cells of *A. acidoterrestris*.

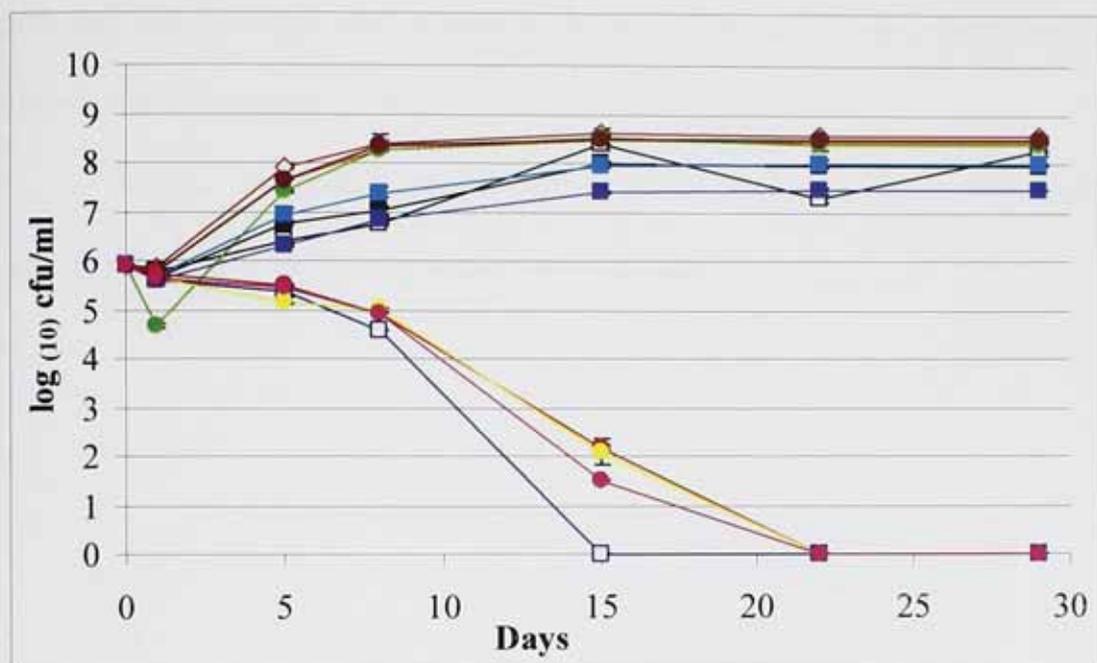


Figure 5.8. Survival at 30°C of *P. cyclohexanicum* exposed to potassium sorbate and nisin in orange juice (pH3.8). Values are means \pm SEM ($n = 3$) - \diamond - no preservative; - \square - 500ppm; - \square - 1000ppm; - \circ - 2.5 IU/ml; - \bullet - 5 IU; - \bullet - 10 IU/ml nisin; - \blacksquare -500ppm sorbate and 2.5 IU/ml nisin; - \blacksquare -500ppm sorbate and 5 IU/ml nisin; - \blacksquare -500ppm sorbate and 10 IU/ml nisin; - \bullet -1000ppm sorbate and 2.5 IU/ml nisin; - \bullet -1000ppm sorbate and 5 IU/ml nisin; - \bullet -1000ppm sorbate and 10 IU/ml nisin/ml.

P. cyclohexanicum failed to grow in any preservative or nisin on day 1 and also failed to survive in sodium benzoate concentrations of 500ppm by day 8, 1000ppm by day 15, all benzoate/nisin concentrations and sorbate at 1000ppm by day 15 (Figure 5.7). *P. cyclohexanicum* did however grow in nisin alone at all concentrations, potassium sorbate (Figure 5.8) at 500ppm and combinations of sorbate and nisin at all concentrations tested. By day 29 numbers had increased by more than 3 log and exhibited significantly higher counts throughout the study (*P*

<0.05) compared to day 1. This suggests that sodium benzoate (Figure 5.7) at 500ppm and 1000ppm and potassium sorbate (Figure 5.8) at 500ppm are very effective in controlling growth of *P. cyclohexanicum*.

Because *P. cyclohexanicum* grew at all the nisin levels tested further studies were performed using higher levels of nisin.

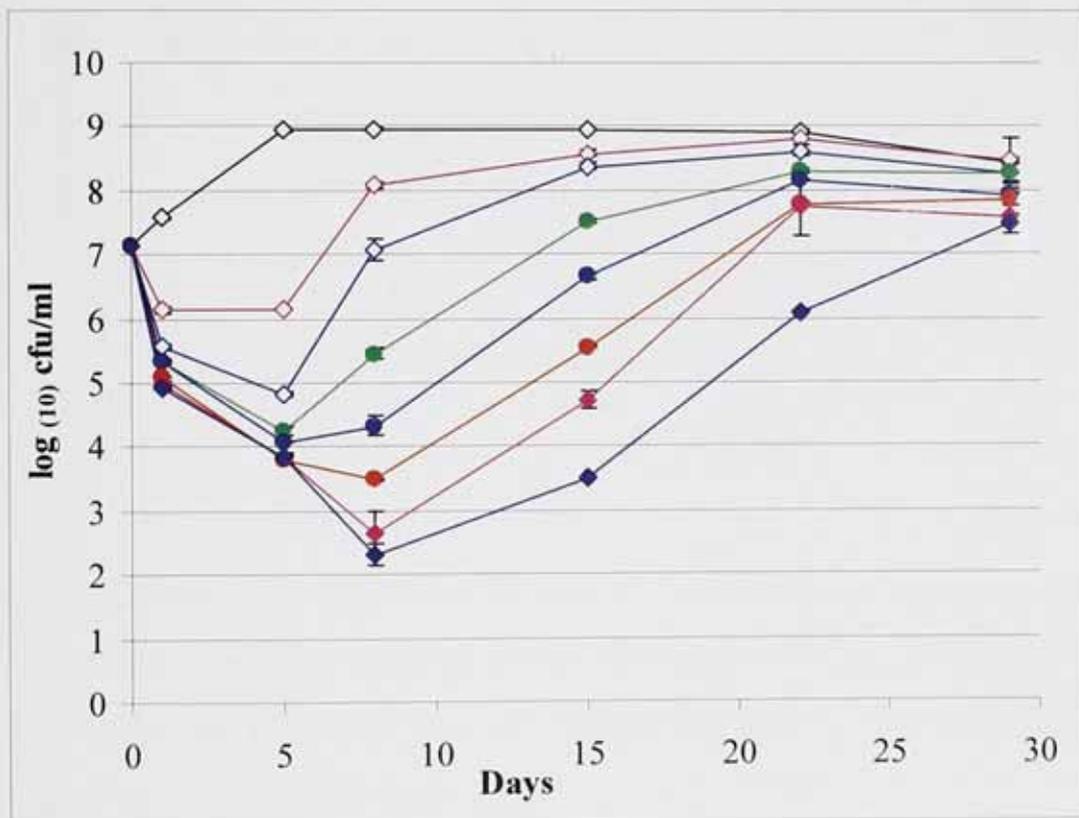


Figure 5.9. Survival at 30°C of *P. cyclohexanicum* exposed to nisin in orange juice (pH3.8). Values are means SEM ±. (n = 3) -◇- No nisin; -◇- 10 IU/ml nisin; -○- 25 IU/ml nisin; -●- 50 IU/ml nisin; -●- 75 IU/ml nisin; -●- 100 IU/ml nisin; -●- 125 IU/ml nisin; -●- 150 IU/ml nisin.

P. cyclohexanicum did not grow in the presence of nisin at concentrations of 10-150IU/ml (Figures 5.9) until day 8 when growth occurred at concentrations of 10IU/ml and 25IU/ml. By day 29 numbers had increased significantly ($P<0.05$)

in all concentrations of nisin up to 125IU/ml and although at 150IU/ml numbers were also higher than the initial inoculum, these were not significant.

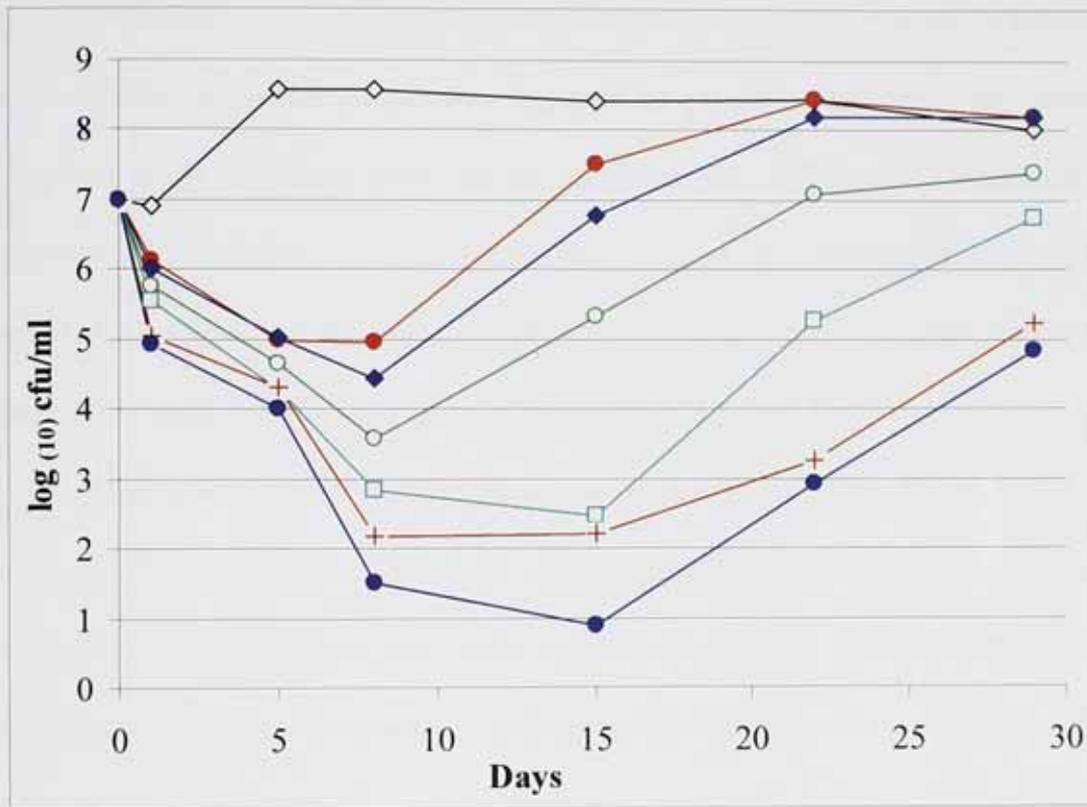


Figure 5.10. Survival at 30°C of *P. cyclohexanicum* exposed to nisin in orange juice (pH3.8). Values are means SEM \pm . ($n = 3$) -◇- No nisin; -●- 100 IU/ml nisin; -◆- 150 IU/ml nisin; -○- 250 IU/ml nisin; -□- 500 IU/ml nisin; -+- 750 IU/ml nisin; -●- 1000 IU/ml nisin.

5.6. Discussion

A. acidoterrestris growth occurred in sodium benzoate at concentrations of 400ppm and in potassium sorbate at concentrations of 300ppm (Figure 5.1) but at higher levels of preservative up to 1000ppm no increase in numbers was observed. However, viable vegetative cells and spores remain so that if, for

example, temperature abuse occurs these remain viable, grow and cause spoilage (Yeh *et al.*, 2004).

Nisin alone has an inhibitory effect against *A. acidoterrestris* (Yamazaki, *et al.*, 2000) but higher levels are required to prevent vegetative cell growth compared to the inhibitory effect on spores. At levels of 5IU/ml the outgrowth of spores was prevented at 25°C (Komitopoulou, *et al.*, 1999). In the studies recorded here a storage temperature of 30°C was used and the same effect was observed at nisin levels of 2.5 to 10IU/ml (Figures 5.5 and 5.6).

The growth of *P. cyclohexanicum* was prevented by 500ppm sodium benzoate (Figures 5.5 and 5.6) and by a combination of sodium benzoate at concentrations of 500ppm with nisin at concentrations of 2.5IU/ml. Therefore, sodium benzoate alone is effective against *P. cyclohexanicum* at levels at or below 500ppm with or without the addition of nisin.

Potassium sorbate however, used at 500ppm (Figure 5.7) was ineffective and *P. cyclohexanicum* had increased by more than 3 logs by day 29. When nisin was combined with potassium sorbate concentrations of 500ppm, growth occurred to almost the same levels at all concentrations of nisin. Higher concentrations of up to 150IU/ml of nisin (Figure 5.9) were used and these proved as ineffective so levels of up to 1000IU/ml of nisin were tested (Figure 5.10). By day 29 increased numbers (compared to the initial inoculum) of *P. cyclohexanicum* were recorded at nisin levels of 1000IU/ml. Therefore the level at which nisin is effective against *P. cyclohexanicum* is undetermined by this study and further research is required to ascertain if *P. cyclohexanicum* is nisin resistant.

Although *P. cyclohexanicum* is able to grow at pH3.2 its optimum pH is higher at pH5.5 to pH6.5 (Kusano *et al.*, 1997). Nisin is most stable at pH3 with activity decreasing above and below this value, and at a lower pH inhibition of the outgrowth of *A. acidoterrestris* spores increased (Yamazaki *et al.*, 2000). The higher optimum pH of *P. cyclohexanicum* growth may have contributed to nisin ineffectiveness although throughout these studies the pH of the orange juice used remained between pH3.6 and pH3.7. *Streptococcus bovis* is a Gram positive, nisin-resistant bacterium and although initially inhibited by nisin the cells that survive grow at the same rate as the untreated cells (Mantovani *et al.*, 2001), as in this study with *P. cyclohexanicum*. There are also nisin resistant strains of *Clostridium botulinum* and stable resistant isolates have been developed by exposing cells to incremental exposure to increasing levels of nisin. Nisin resistance was maintained by spores from these cells (Mazzotta *et al.*, 1997). Before these present studies the effect of nisin on *P. cyclohexanicum* has not been reported but, if as in *S. bovis*, *P. cyclohexanicum* was initially inhibited by nisin, and as nisin levels were increased (Figures 5.8 and Figure 5.9) the time for 'adaptation' increased so that at 2.5IU/ml *P. cyclohexanicum* grew on day 5 but at 1000 IU/ml of nisin cells grew on day 29.

This study suggests that the preservatives sodium benzoate and potassium sorbate are effective against *A. acidoterrestris* but the levels for inhibition of each preservative are higher for vegetative cells than spores. Vegetative cells are less sensitive to nisin than spores and 100IU ml⁻¹ was required to prevent growth of spores and vegetative cells of *A. acidoterrestris* in a mixed inoculum in juices

incubated at 44°C (Komitopoulou *et al.*, 1999). There appears to be an antagonistic effect between sodium benzoate and nisin (Figures 5.5 and 5.6) with higher levels required of each to inhibit vegetative cells compared to spores.

Sodium benzoate and potassium sorbate are also effective against *P. cyclohexanicum*, but the level at which potassium sorbate is inhibitory is between 500ppm and 1000ppm. The use of potassium sorbate would therefore require careful calculation as growth occurred at 500ppm.

This study also suggests that *P. cyclohexanicum* may have an adaptive mechanism towards nisin as initially no growth occurs and, depending on the level of nisin concentration, growth does occur at higher levels. Further investigations are required to establish a preservative regime for *P. cyclohexanicum*.

Chapter 6

**The effect of intermittent shaking,
headspace and temperature on the
growth of *A. acidoterrestris* in stored
apple juice.**

6.1. Introduction

During previous trials assessing the effectiveness of the two preservatives sodium benzoate and potassium sorbate against *A. acidoterrestris*, (Chapter 5) growth of high numbers of vegetative cells were observed in control samples i.e. those without preservatives. Although *A. acidoterrestris* forms spores under adverse environmental or nutritional conditions (Deinhard *et al.*, 1987; Walls and Chuyate, 1998), the amount of headspace available, agitation of containers and whether sampling occurred from nearer the top or bottom of the juice were all considered as possible contributing factors.

6.2. Aims

This study aims to investigate the effects of headspace and the growth and hence detection of *A. acidoterrestris* in apple juice.

6.3. Materials and methods

Methods and materials are described in Section 3.7.

6.4. Preparation of *A. acidoterrestris* cultures

Preparation of *A. Acidoterrestris* is described in Section 3.7.

6.5. Determination of vegetative cell and spore counts

Determination of vegetative cell and spore counts is described in Section 4.4.4.

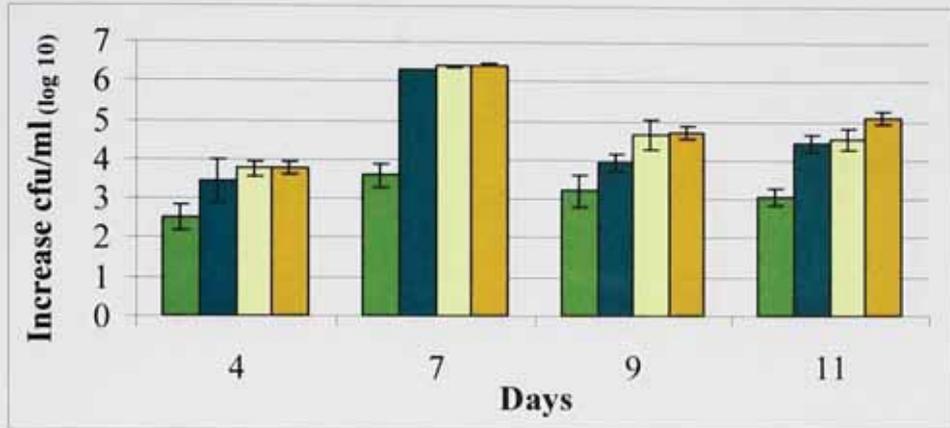
6.6. Statistical tests

Statistical was performed by using SPSS version 10 (SPSS Inc., Chicago, IL, USA) using an independent *t*-test analysis. The results are shown as mean \pm SEM. Significance was set at $P < 0.05$.

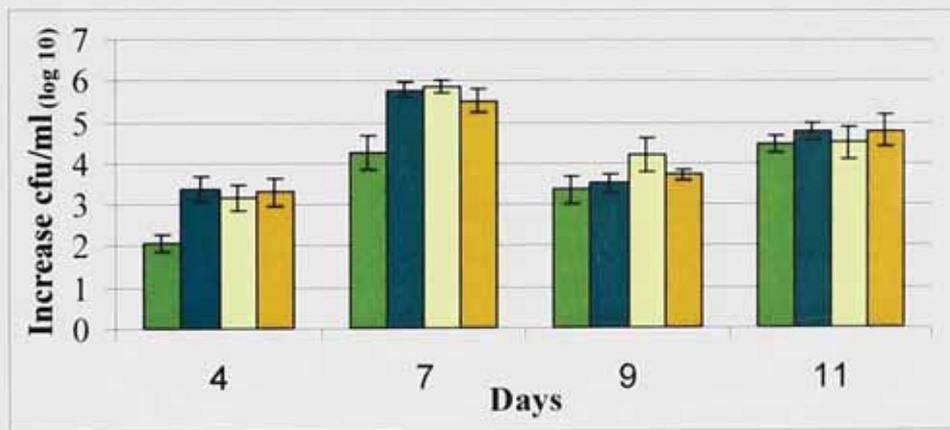
6.7. Results

All results are shown as increase in numbers compared with initial inoculum. At 35°C, vegetative cell growth (Figure 6.1) generally showed a significantly lower level of increase in cell numbers in 0% headspace samples compared with 25%, 50% and 75% headspace ($P > 0.05$). This was particularly the case in the shaken samples (Figure 6.1a) compared with the unshaken samples when sampling occurred from the top (Figures 6.1b and c). The rate of isolation and the numbers of *A. acidoterrestris* isolated from the bottles with 25%, 50% and 75% headspace were not significantly different from each other on day 7 (Figure 6.1).

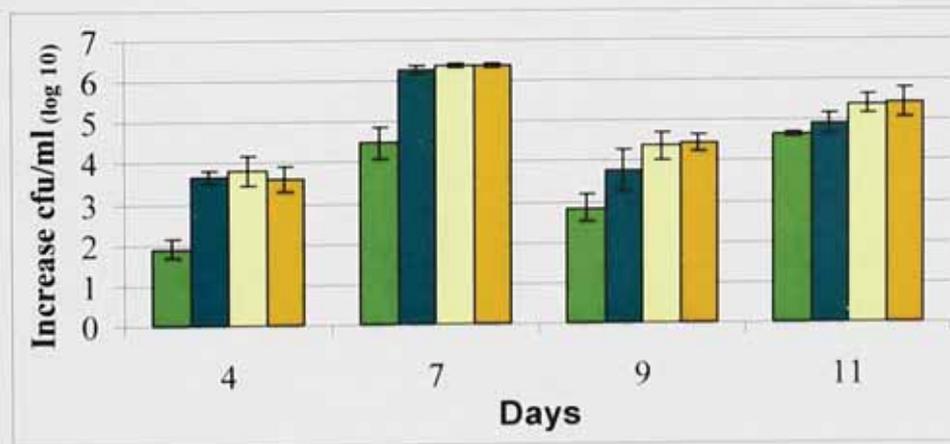
At day 11 there was no difference in vegetative cell count in any of the headspace bottles in the unshaken top sample or the unshaken bottom samples. Therefore, by the day 11, headspace seems to have little effect on growth in the unshaken bottles (Figures 6.1b and c) but continues to have an effect on the shaken bottles (figure 6.1a) where 0% headspace produces numbers of cells and hence growth and hence lower potential detection rate.



a

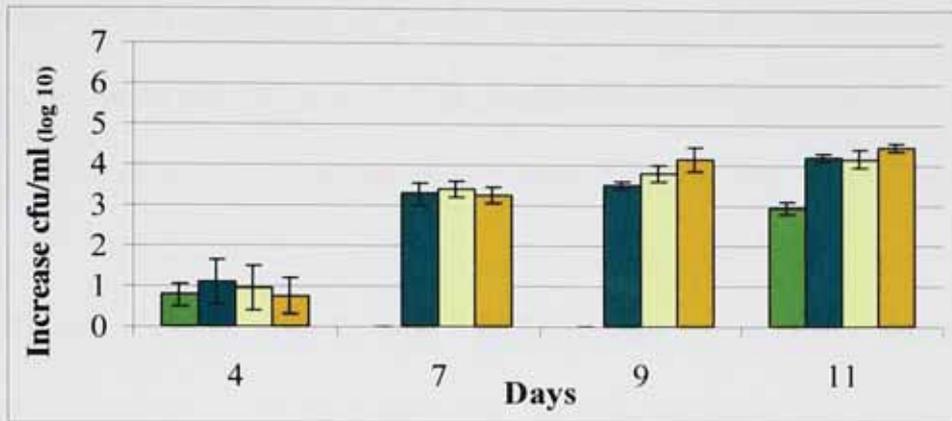


b

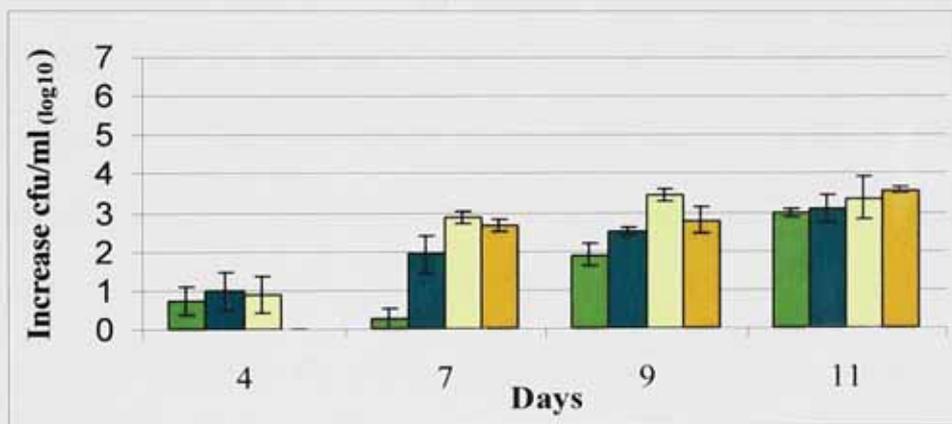


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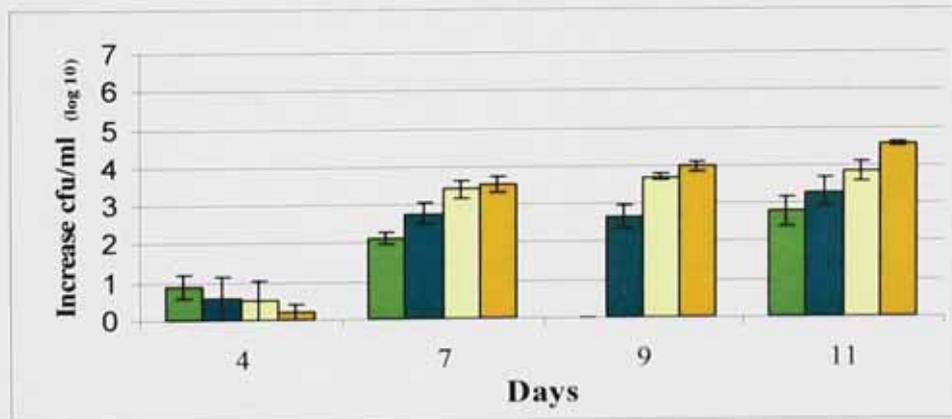
Figure 6.1 Growth of *A. acidoterrestris* vegetative cells in apple juice (pH3.5) at 35°C mean \pm SEM ($n = 6$) (a) shaken sample; (b) unshaken top sample; (c) unshaken bottom sample. -■- 0% headspace; -■- 25% headspace; -■- 50% headspace; -■- 75% headspace.



a



b

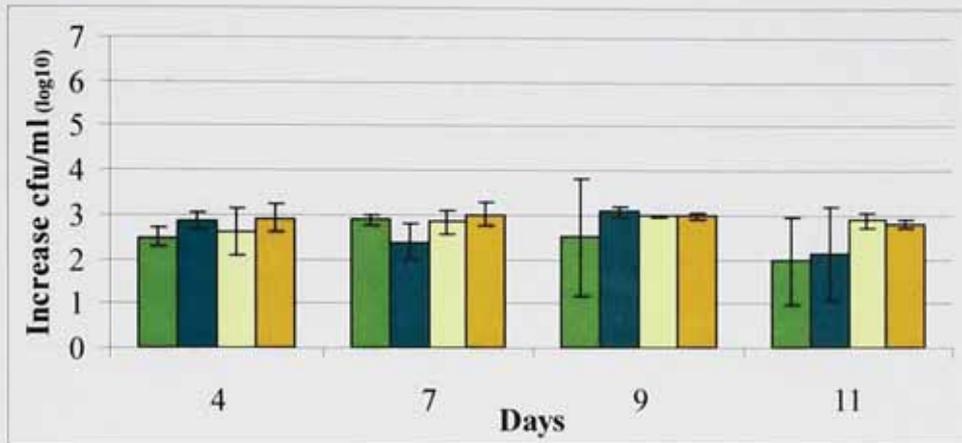


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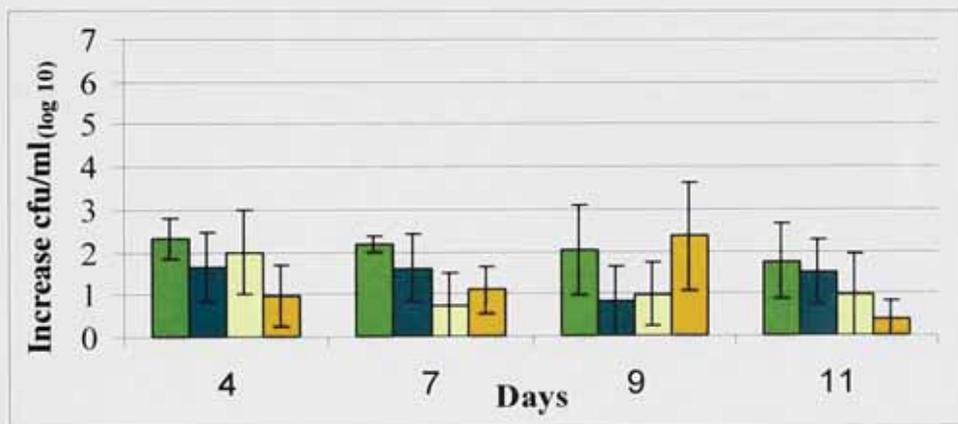
Figure 6.2 Growth of *A. acidoterrestris* spores in apple juice (pH3.5) at 35°C mean \pm SEM ($n = 6$) (a) shaken sample; (b) unshaken top sample; (c) unshaken bottom sample -■- 0% headspace; -■- 25% headspace; -■- 50% headspace; -■- 75% headspace.

There are no significant differences between the isolation rate from the top or bottom unshaken samples at any of the headspace conditions (Figure 6.1) and it seems immaterial whether the sample is taken from the top of the unshaken bottles.

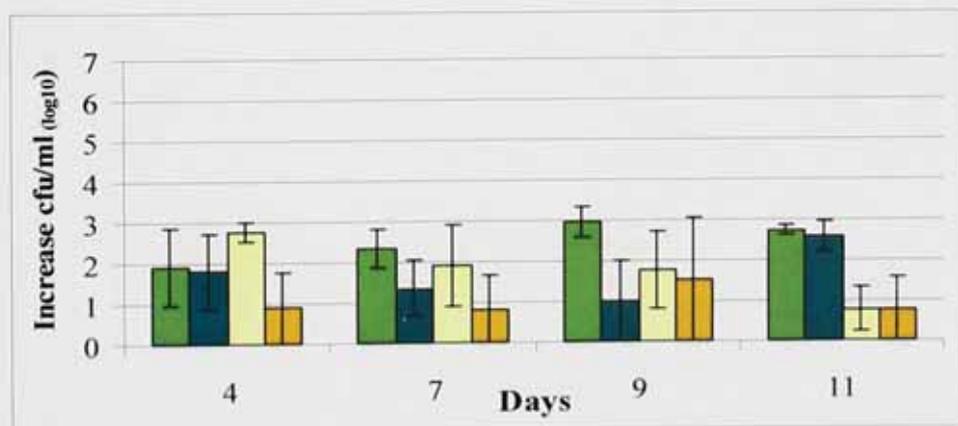
Spore counts (Figure 6.2) were significantly lower than the vegetative cell counts at all times and with all headspaces. As with vegetative cell growth there was no significant difference between numbers of spores and hence isolation rates from 25%, 50% and 75% headspace bottles. 0% headspace bottles again showed the lowest numbers of spores, with no spores being detected on days 7 and 9 in the shaken samples (Figure 6.2a). On day 4 there was no significant difference in spore count between any headspace under any of the three conditions. However, on day 11 spore counts in the shaken samples (Figure 6.2a) were significantly lower than in either the unshaken top or bottom samples (Figures 6.2b and c), with the unshaken bottom samples giving the highest spore counts, suggesting that *A. acidoterrestris* spores tend to settle at the bottom of containers during storage.



a

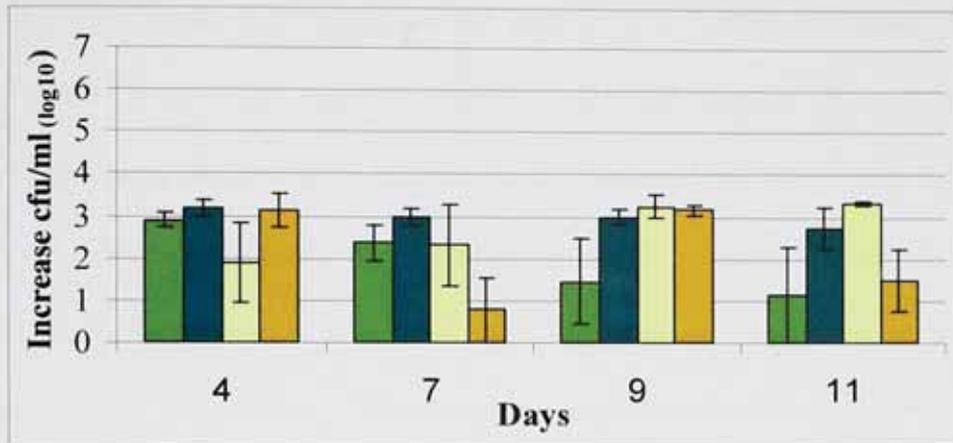


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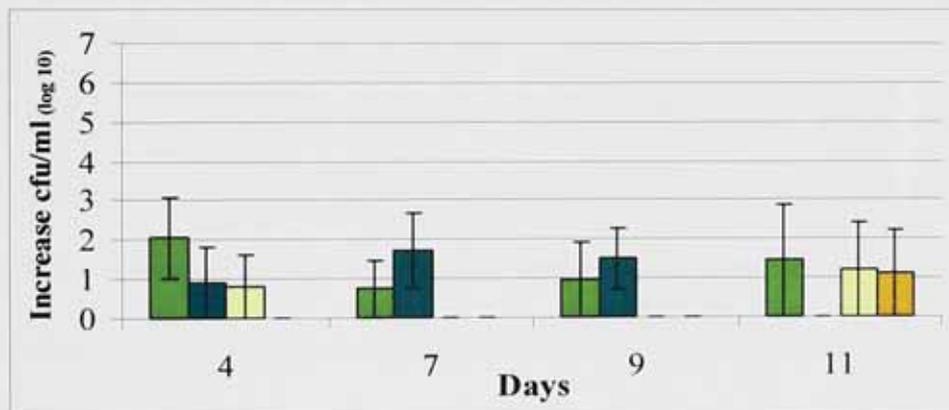


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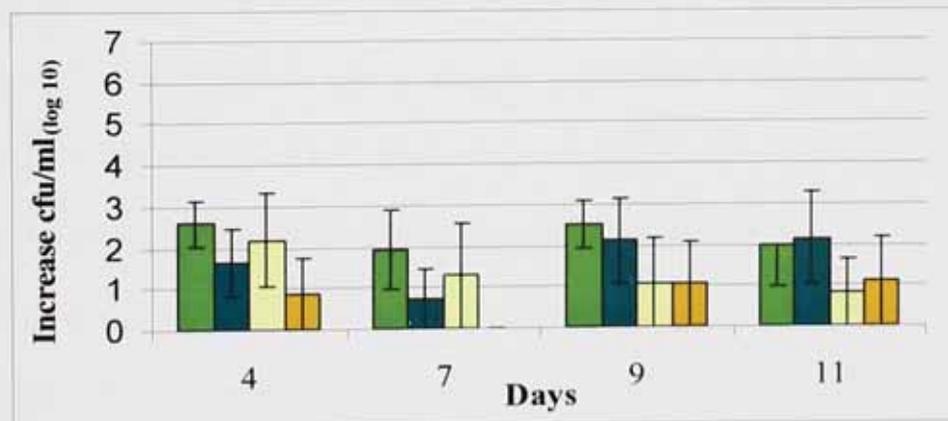
Figure 6.3 Growth of *A. acidoterrestris* vegetative cells in apple juice (pH3.5) at 30°C mean \pm SEM ($n = 6$) (a) shaken sample; (b) unshaken top sample; (c) unshaken bottom sample -■- 0% headspace; -■- 25% headspace; -■- 50% headspace; -■- 75% headspace.



a



b

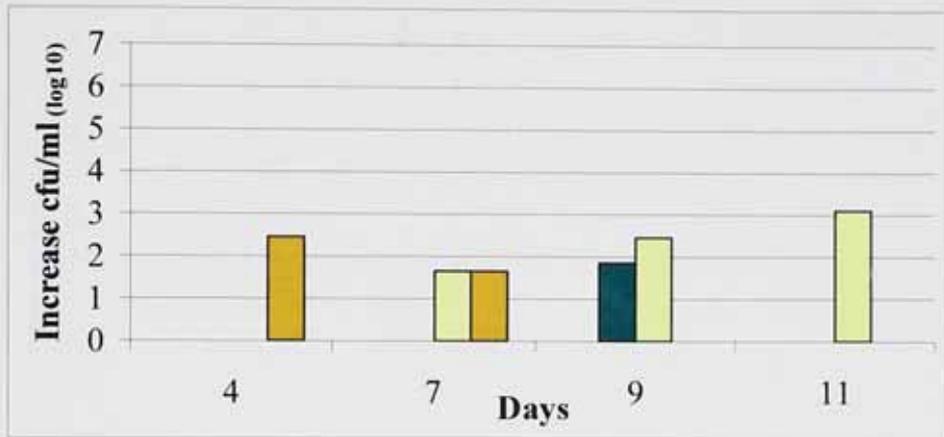


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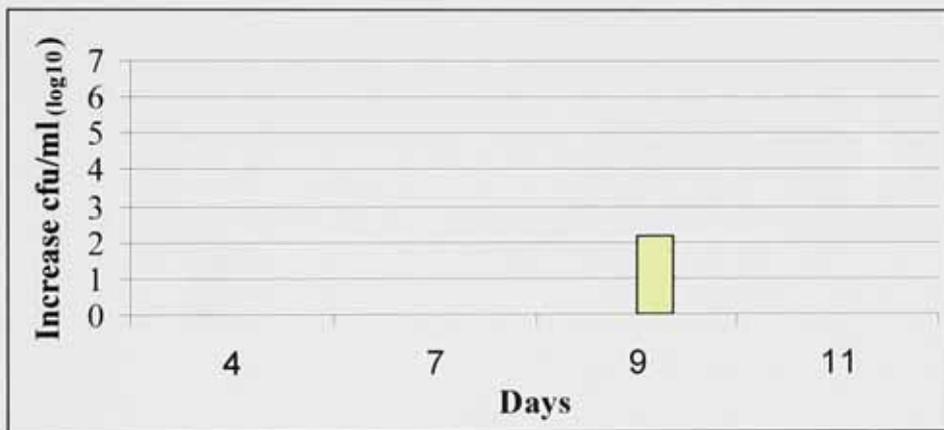
Figure 6.4 Growth of *A. acidoterrestris* spores in apple juice (pH3.5) at 30°C mean \pm SEM ($n = 6$) (a) shaken sample; (b) unshaken top sample; (c) unshaken bottom sample -■- 0% headspace; -■- 25% headspace; -■- 50% headspace; -■- 75% headspace

At 30°C (Figure 6.3) vegetative cell growth in all bottles was lower than at 35°C (Figure 6.1). The shaken samples showed higher growth of *A. acidoterrestris* than the unshaken samples at all times and all conditions, although this was not always statistically significant. Sporulation was also lower compared with that at 35°C, except at day 4 when it was higher, especially in the 0% headspace bottles (Figure 6.4). This suggests that, in conditions of little or no available oxygen, *A. acidoterrestris* grows more slowly at 30°C than at 35°C after four days incubation.

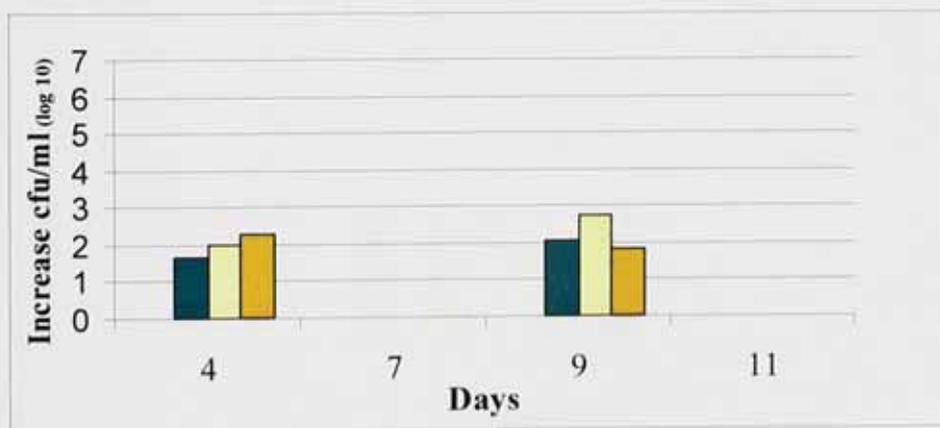
At 27°C (Figures 6.5 and 6.6) only very low numbers of viable vegetative cells or spores were detected in the majority of the samples. No vegetative cell growth (Figure 6.5) occurred at 0% headspace under any conditions. In the unshaken top samples (Figure 6.5a) growth of vegetative cells was detected only on day 9 of the 50% headspace samples and similarly in the unshaken top samples (Figure 6.6b) spores grew only on day 4 at 25% and 50%. Vegetative cell growth (Figure 6.5c) in the unshaken bottom samples occurred at 25%, 50% and 75% on days 4 and 9, but no growth occurred on days 7 and 11. Spores grew at 0% and 50% headspace in the unshaken bottom samples (Figure 6.6c) on day 7 and in the 25% headspace sample on day 9. After day 9 the only growth recorded under any of the conditions was on day 11 (Figures 6.5a and 6.6a) in the 50% headspace shaken samples of spores and vegetative cells. These results confirm previous studies suggesting *A. acidoterrestris* only grows slowly at suboptimal temperatures (Walls and Chuyate, 1998; Jensen, 1999).



a

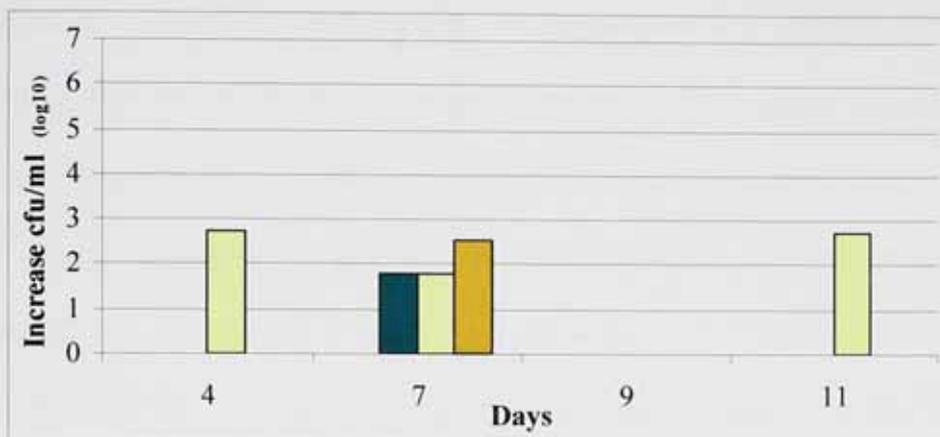


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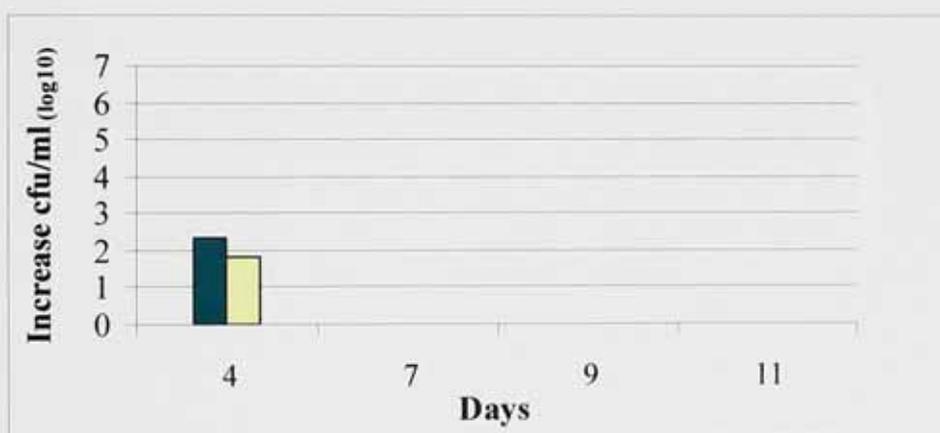


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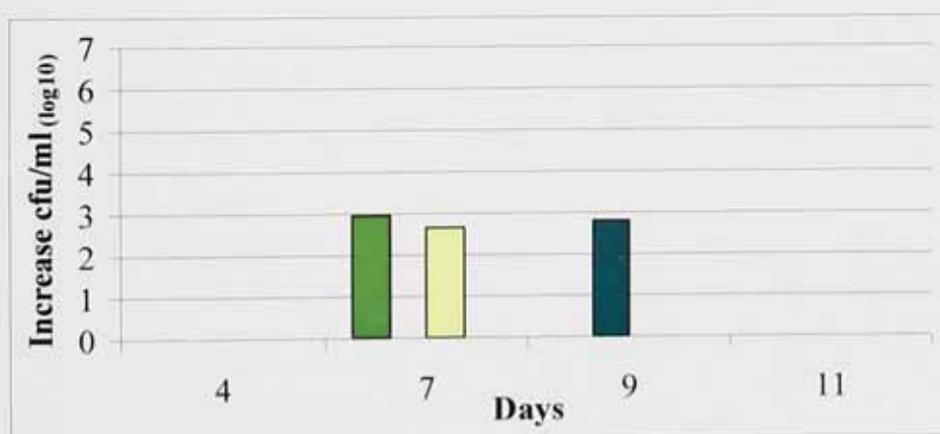
Figure 6.5 Growth of *A. acidoterrestris* vegetative cells in apple juice (pH3.5) at 27°C mean \pm SEM ($n = 4$) (a) shaken sample; (b) unshaken top sample; (c) unshaken bottom sample -■- 0% headspace; -■- 25% headspace; -■- 50% headspace; -■- 75% headspace



a



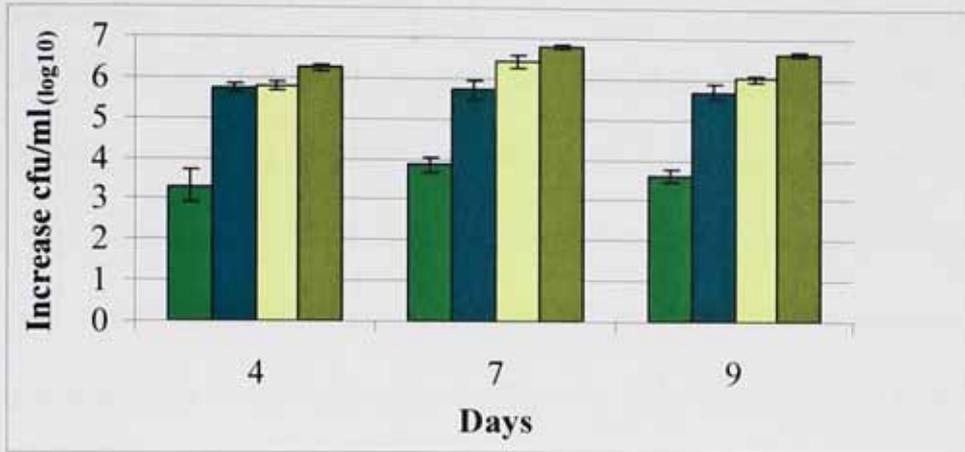
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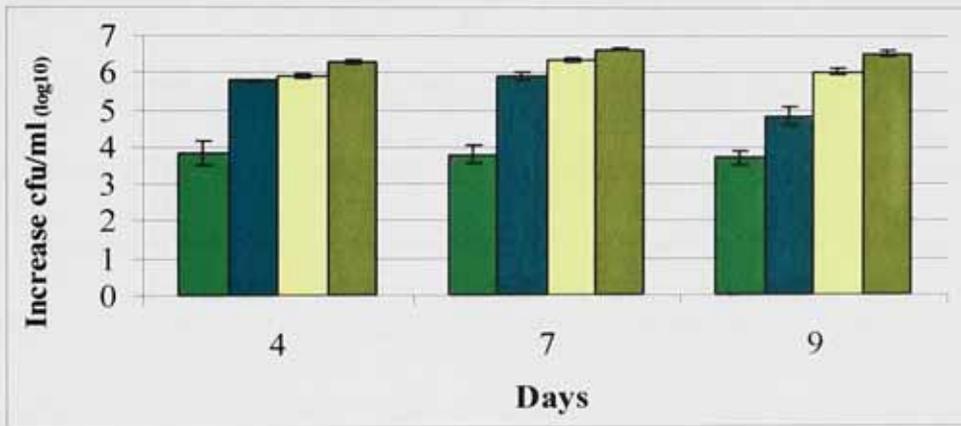
c

Figure 6.6 Growth of *A. acidoterrestris* spores in apple juice (pH3.5) at 27°C mean \pm SEM ($n = 4$) (a) shaken sample; (b) unshaken top sample; (c) unshaken bottom sample -■- 0% headspace; -■- 25% headspace; -■- 50% headspace; -■- 75% headspace

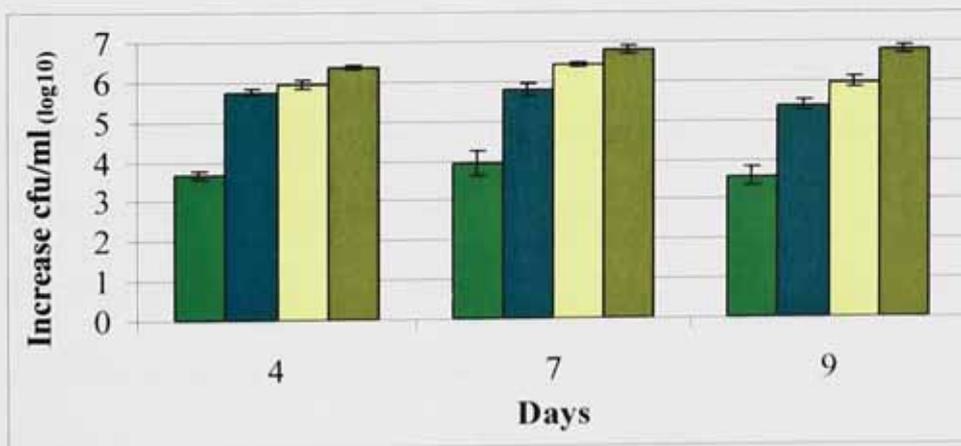
Similar studies were performed at storage temperatures of 44°C over nine days (Figure 6.6) and with no heat shock. The growth of vegetative cells was significantly lower in bottles with 0% headspace compared to other headspaces on all days (Figure 6.6) however growth was not significantly different to the 0% headspace of the 35°C study (Figures 6.1 and 6.2). Cell counts did not differ significantly between the shaken (Figure 6.6a) and unshaken top or bottom samples (Figures 6.6b and c) when comparing similar headspaces. Vegetative cell counts (Figure 6.6) were significantly higher at 25%, 50% and 75% headspaces on all days compared to samples stored at 35°C (Figure 6.1) on days 4, 7 and 9.



a



b



c

Figure 6.7 Growth of *A. acidoterrestris* vegetative cells in apple juice at 44°C mean \pm SEM ($n = 3$) (a) shaken sample; (b) unshaken top sample; (c) unshaken bottom sample -■- 0% headspace; -■- 25% headspace; -■- 50% headspace; -■- 75% headspace

6.8. Discussion

The results of this study suggest that adequate agitation of fruit juice containers before sampling especially at suboptimal growth temperatures (i.e. 27° or 30°C) may be important. If the container is shaken before sampling detection of growth of *A. acidoterrestris* is generally higher (Figures 6.3a and 6.4a) than if the container is unshaken and sampled from either top or bottom (Figures 6.3b and c, 6.4b and c, 6.5b and c and 6.6b and c). It is also important for detection of spores at 35°C in containers with available oxygen. Although shaking a small or medium container or palpating an aseptically packaged bag is fairly easy, agitating a large barrel or intermediate bulk container (IBC) weighing over 1000kg is much more difficult. Openings on the top and lower side of an IBC allow samples to be obtained from the top and near the bottom of the product but this will not allow for the generally higher isolation rates of *A. acidoterrestris* that a shaken sample will provide.

The amount of moving and shaking fruit juice containers are subjected to during transport between processing and manufacture of the final product would allow headspace oxygen to be incorporated into the juice. Temperature fluctuations during transport, particularly throughout summer months, may also affect outgrowth of spores (Orr *et al.*, 2000).

The results of this study suggest that storage temperature, headspace and adequate agitation of fruit juice containers play an important role in the growth and detection of *A. acidoterrestris* in apple juice. Growth rates in partly filled containers are higher than in full containers at temperatures at and in excess of, 35°C emphasizing the aerobic nature of *A. acidoterrestris*.

In trials at 27°C very low numbers of viable vegetative cells (Figure 6.5) or spores (Figure 6.6) were detected in the majority of the samples, confirming previous studies suggesting *A. acidoterrestris* only grows slowly at suboptimal temperatures (Jensen, 1999). When samples were stored at 44°C (Figure 6.6), which is within the optimal reported temperature range of 42°C to 53°C for *A. acidoterrestris* growth (Wisotzkey et al., 1992), the growth of vegetative cells was lower in bottles with 0% headspace on all days (heat shock was not used in this trial) again emphasizing the aerobic nature of *A. acidoterrestris*.

In this study, it has been shown that the amount of headspace does make a significant difference in growth of vegetative cells and sporulation of *A. acidoterrestris* and hence detection rates when apple juice is stored at 35°C. In the first few months of storage Jensen and Whitfield (2003) report that taint occurs more slowly in containers with low headspace, therefore decanting juices from partially filled to smaller containers may aid in reducing the chance of spoilage occurring. Shaking the bottles before sampling did not make a significant difference to the growth of vegetative cells although as storage time increased spore counts decreased in shaken bottles compared with unshaken bottles and samples from the bottom tended to give higher counts. In a study by Orr *et al.*, (2000) it was suggested that unshaken sample bottles minimized oxygen uptake indicating that only cells near the top of containers received sufficient oxygen to grow. Spores may have settled at the base of containers where germination and outgrowth may have been minimized or even prevented.

When apple juice is stored at temperatures that represent a hot summer day in the UK, or an average temperature in many countries, but are suboptimal for *A. acidoterrestris* (27° to 30°C in this study), intermittent shaking i.e. before each sampling, increases growth rate, because available oxygen is distributed throughout the juice, and hence probable detection of the presence of *A. acidoterrestris*. Importantly this may be the case if the original numbers of *A. acidoterrestris* are low. This study showed that detection of growth of both vegetative cells and spores is more erratic when juices are incubated at 27°C and 30° than at 35°C, especially in the unshaken bottles, also emphasizing that, at suboptimal temperatures, in order to detect *A. acidoterrestris* care must be taken to ensure samples are removed from several areas of containers particularly those containers too large to be shaken.

The results of this study suggest that juices and juice products should be kept at low temperatures, containers must, wherever possible, be shaken before sampling and various sampling areas should be tested within each container to ensure detection of *A. acidoterrestris*. Storage of partly filled containers cannot always be avoided but filling the empty headspace of containers with an inert gas such as nitrogen may slow the growth of *A. acidoterrestris*; although further investigations are required in an industrial setting to determine the cost effectiveness of such protocols.

Chapter 7

Co-culture and isolation of

A. acidoterrestris and

P. cyclohexanicum

7.1. Introduction

Although *A. acidoterrestris* and *P. cyclohexanicum* have similar ω -cyclohexyl fatty acids as the major lipids of their cell membranes and both are spoilage micro-organisms isolated from contaminated fruit juices they also have many different characteristics (Table 7.1).

<i>A. acidoterrestris</i>	<i>P. cyclohexanicum</i>
ω -cyclohexyl lipids as major lipids in the cell membrane	ω -cyclohexyl lipids as major lipids in the cell membrane
Cells club shaped or bent	Cells club shaped or bent
Spore former	non sporeformer
Aerobic	Aerotolerant
Thermophilic	Heat resistant
pH range 2.5 to 6.0	pH range 3.2 to 7.5
Gram positive to variable	Gram positive
Catalase positive	Catalase negative
No growth on neutral agar	Grows on neutral agar
No fermentation	Ferments glucose

Table 7.1. Characteristics of *A. acidoterrestris* and *P. cyclohexanicum* showing similarities and differences between the two bacilli.

Propionibacteria are known producers of bacteriocins (Glatz, 1992; Faye *et al.*, 2000) however, most bacteriocins are effective to only a narrow group of micro-organisms mainly those closely related to the bacteriocin producer. *Pediococcus acidilactici* PAC1.0 (Gonzalez and Kunka 1987) produces the

bacteriocin PA1 which is active against the human pathogen *Listeria monocytogenes* (Pucci *et al.*, 1988).

Other bacteriocins have been documented from propionibacteria; *P. theonii* P127 produces a bacteriocin PLG-1 that is moderately heat stable and also stable at a pH range of pH3-pH9 (Lyon and Glatz, 1993). PLG-1 is active against a range of micro-organisms which include propionibacteria and fungi. The heat stable bacteriocin Jensenin G is produced by *P. theonii* P126 (formerly *P. jensenii*) and inhibits some propionibacteria and lactic acid bacteria for example *Lactobacillus delbruecki* subsp *bulgaricus* (Ratnam *et al.*, 1999). This bacteriocin is, however, pH sensitive and most active at pH6.4 (Ekinici and Barefoot 1999).

Propionicin T1 has been isolated from *P. theonii* 419 and *P. theonii* LMG 2792, however in the former the bacteriocin is produced in late logarithmic stage and in the latter it is produced in stationary phase, as in earlier stages of exponential growth the organism is sensitive to its own bacteriocin. Propionicin T1 however, seems to be only active against a small number of propionibacteria, those in the dairy propionibacteria cluster not containing *P. freudenreichii* or *P. cyclohexanicum* (Faye *et al.*, 2000).

An investigation into the frequency of propionicin T1 together with protease-activated antimicrobial peptide (PAMP) and the associated genes in classical propionibacteria demonstrated that in propionibacteria these genes are extensive in *P. jensenii* and *P. theonii* but were not present in the remaining classical propionibacteria included in the investigation (Faye 2004). This investigation did not include *P. cyclohexanicum*; therefore a study was designed

to investigate the possibility of bacteriocin production by *P. cyclohexanicum* and included monitoring *A. acidoterrestris* and *P. cyclohexanicum* in the same sample medium.

Contamination by *A. acidoterrestris* has occurred worldwide and from being a micro-organism of interest only to researchers, *A. acidoterrestris* has become acknowledged as a major quality control bacterium (Chang and Kang, 2004).

7.2. Aims

The aims of this study are:

- To test for the presence of each micro-organism in a variety of raw materials and soft drinks to monitor the amount of spoilage occurring within the industry.
- To monitor the growth in co-cultures of *A. acidoterrestris* and *P. cyclohexanicum* in the same medium.

7.3. Materials and methods

Methods and materials were as described in section 3.17.

7.4. Statistical tests

Statistical was performed by using SPSS version 10 (SPSS Inc., Chicago, IL, USA) using an independent *t*-test analysis. The results are shown as mean \pm SEM. Significance was set at $P < 0.05$.

7.5. Results

7.5.1 Co-cultures of *A. acidoterrestris* and *P. cyclohexanicum*

Each organism was grown on OSA supplemented with 0.5% (w/v) sucrose and PYA both aerobically and anaerobically. The results demonstrated that *A. acidoterrestris* would not grow anaerobically or on PYG although *P. cyclohexanicum* would grow on enriched OSA but colonies were very small, translucent and difficult to see when grown aerobically. *A. acidoterrestris* and *P. cyclohexanicum* both grew in orange juice at temperatures of 30°C and 35°C (Figures 4.1 and 4.3). *A. acidoterrestris* numbers were higher at 35°C than at 30°C even though this was below its reported optimum temperature of 43°C (Walls and Chuyate, 2000). *A. acidoterrestris* however has reported temperature ranges of 42°C to 53°C (Pontius *et al.*, 1998; Walls and Chuyate, 1998), 26°C to 50°C (Borlinghaus and Engel, 1997) and 25°C to 60°C (Yamazaki *et al.*, 1997), and *P. cyclohexanicum* also increased in numbers at this temperature (Figure 4.3) for up to 22 days. Growing cultures of both organisms in the same medium would therefore be possible if orange juice was used as the growth medium with a storage temperature of 35°C.

Therefore this study used orange juice as the growth medium, 35°C for storage of samples and, for *A. acidoterrestris*, enriched OSA for surface plating, acidified peptone broth for dilutions and aerobic incubation at 44°C for 4 days. For *P. cyclohexanicum* PYG was used for surface plating, PYG broth for dilutions and incubation was anaerobic at 30°C.

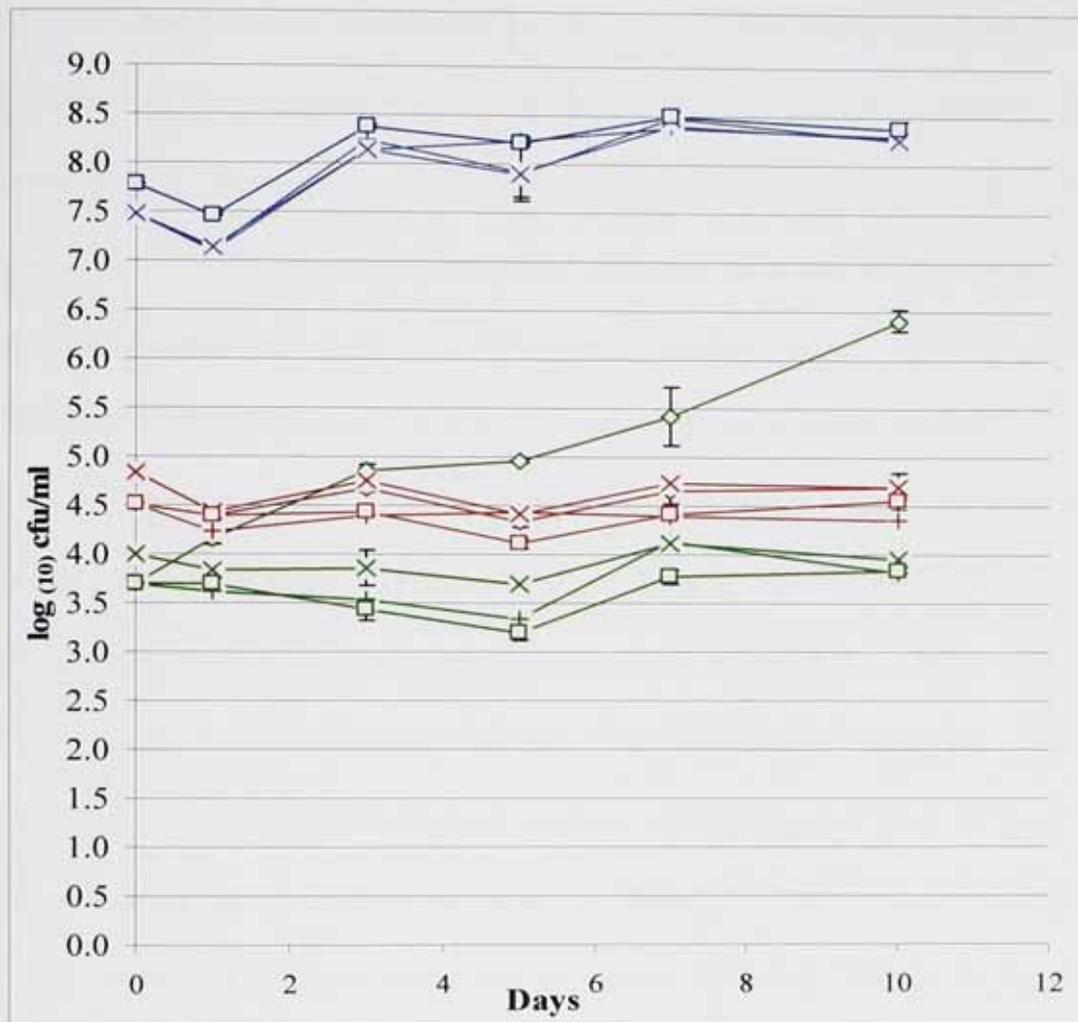


Figure 7.1. Survival of *P. cyclohexanicum* and *A. acidoterrestris* co-cultured in orange juice (pH3.8) at 35°C. Values are means \pm SEM ($n = 6$).

-◇- *A. acidoterrestris* cells (initial inoculum log 3.70cfu/ml) and -◇- *A. acidoterrestris* spores (initial inoculum log 4.53cfu/ml) alone in the juice

-◇- *P. cyclohexanicum* (initial inoculum log 7.48) alone in the juice

-+- *A. acidoterrestris* cells, -+- *A. acidoterrestris* spores and -+- *P. cyclohexanicum* (initial inoculum log 3.7, log 4.53, log 7.48 respectively) when both bacilli are present in the juice.

-x- *A. acidoterrestris* cells, -x- *A. acidoterrestris* spores -x- *P. cyclohexanicum* (initial inoculum log 4.0, log 4.84, log 7.48 respectively) when both bacilli are present in the juice.

-□- *A. acidoterrestris* cells and -□- *A. acidoterrestris* spores and -□- *P. cyclohexanicum* (initial inoculum log 3.7, log 4.53, log 7.78 respectively) when both bacilli are present in the juice.

Numbers of *A. acidoterrestris* vegetative cells were significantly higher ($P<0.05$) in the samples containing no *P. cyclohexanicum* compared to any containing *P. cyclohexanicum* in any concentration on any day (Figure 7.1). By day 7, and also on day 10, all vegetative cells had increased to above the level of the initial inoculum with the samples containing similar amounts of each organism being significantly higher ($P<0.05$) than the initial inoculum.

The numbers of *A. acidoterrestris* spores did not reach the level of the initial inoculum (Figure 7.1) until day 7 and the only other samples to do so were the samples containing the highest initial concentrations of *P. cyclohexanicum*. By day 3 all *P. cyclohexanicum* samples were significantly higher than the initial inoculum ($P<0.05$) with the highest numbers recorded on the same day and in the same samples that *A. acidoterrestris* demonstrated the highest number of spores

Brix and pH were monitored throughout the study (Tables 7.2 and 3). No variation in pH (Table 7.2) was demonstrated in the samples containing only *A. acidoterrestris* in the samples containing (log 3.70) vegetative cells and (log 4.53) spores of *A. acidoterrestris* and (log 7.48) *P. cyclohexanicum*. Very little variation in pH was observed in the samples containing only *P. cyclohexanicum*. However by day 29 the other mixed cultures containing larger variations of each organism the pH was significantly ($P<0.05$) lower.

No change was observed in the Brix values *P. cyclohexanicum* samples but all other samples had Brix values that were significantly lower ($P<0.05$) by day 29. This suggests that the change in Brix values may be caused by a reaction

from *A. acidoterrestris* as the only samples where the Brix remained stable were in the samples containing *P. cyclohexanicum* only.

	Initial Inoculum cfu/ml	day 0	day 1	day 3	day 6	day 8	day 10
<i>P. cyclohexanicum</i> pH of juice Brix of juice	7.48	3.7 10.9	3.6 10.9	3.7 10.9	3.7 10.9	3.7 10.9	3.7 10.9
<i>A. acidoterrestris</i> cells spores pH of juice Brix of juice	3.70 4.53	3.6 10.9	3.6 10.9	3.6 10.8	3.6 10.7	3.6 10.6	3.6 10.5
<i>P. cyclohexanicum</i> with <i>A. acidoterrestris</i> cells spores pH of juice Brix of juice	7.48 3.70 4.53	3.7 10.5	3.7 10.5	3.7 10.4	3.7 10.3	3.7 10.1	3.7 10.1
<i>P. cyclohexanicum</i> with <i>A. acidoterrestris</i> cells spores pH of juice Brix of juice	7.48 4.00 4.87	3.7 9.6	3.7 9.7	3.6 9.5	3.6 9.4	3.6 9.3	3.6 9.3
<i>P. cyclohexanicum</i> with <i>A. acidoterrestris</i> cells spores pH of juice Brix of juice	7.78 3.70 4.53	3.7 10.3	3.7 10.2	3.7 10.1	3.7 10.0	3.6 9.9	3.6 9.9

Table 7.2. pH and Brix values and initial inoculum of orange juice (pH3.8) inoculated with both *A. acidoterrestris* and *P. cyclohexanicum* and incubated at 35°C over 10 days. Values are means \pm SEM ($n = 6$).

Although viable counts were recorded at each time point (Figure 7.1), on day 1 all vegetative cell plates showed distinctive 'zones' around *A. acidoterrestris* colonies (Figure 7.2).

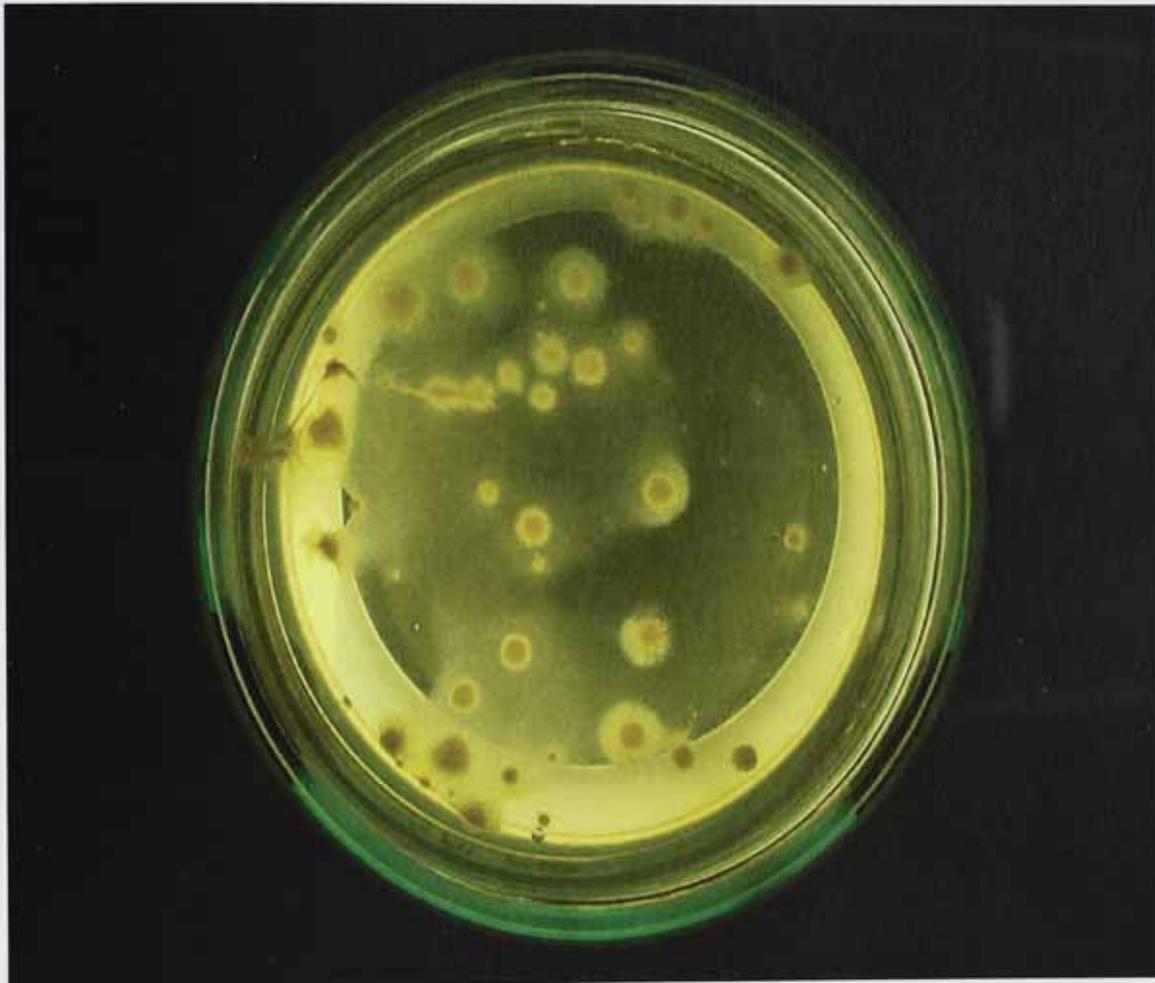


Figure 7.2. Colonies of *A. acidoterrestris* on OSA supplemented with 0.5% (w/v) sucrose (vegetative cells).

By day 3 the zones had spread (Figure 7.2) and *A. acidoterrestris* colonies were smaller and less well developed. Control samples containing only *A. acidoterrestris*, spore plates (Figure 7.3) and *P. cyclohexanicum* plates were not

affected in this way. Colonies of *P. cyclohexanicum* were observed growing on top of *A. acidoterrestris* colonies. The latter were, however, different to the usual growth that is observed on mature colonies of *A. acidoterrestris* or the rough colonies that are sometimes seen (Jensen, 1999).



Figure 7.3. Colonies of *A. acidoterrestris* on OSA supplemented with 0.5% (w/v) sucrose (spore counts).

Samples from each area were carefully picked off and streaked onto PYA and OSA and anaerobically incubated at 30°C and aerobically incubated at 44° respectively. When plates were examined the anaerobic plates had colonies similar to those of *P. cyclohexanicum*, confirmed by spore staining, testing for

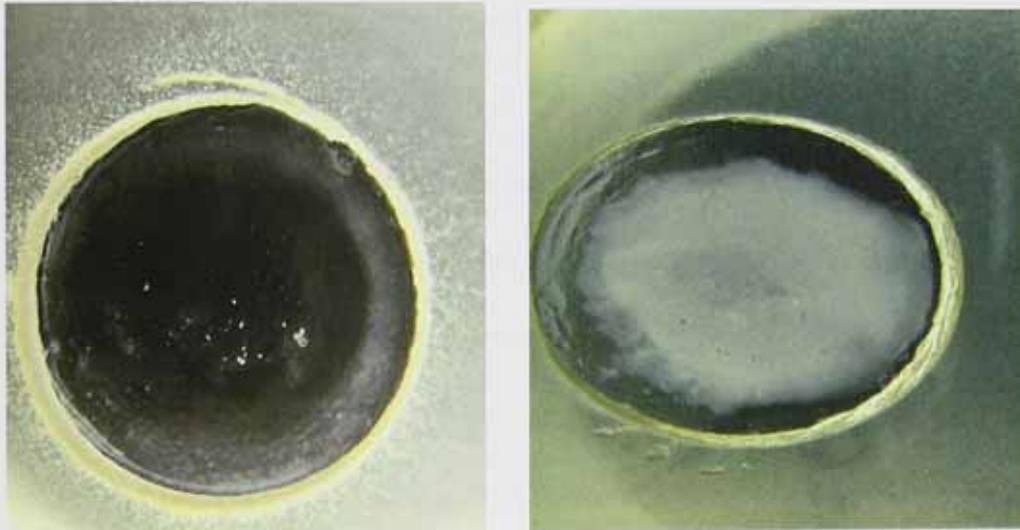
catalase reaction and later genetic identification. The aerobic plates demonstrated *Alicyclobacilli* type colonies and this was confirmed again by spore staining when oval shaped spores were observed, Gram staining and testing for growth on a neutral agar.

By day 7 all samples of orange juice were a fresh orange colour with the exception of those inoculated with *A. acidoterrestris* which had become discoloured. Colonies of *A. acidoterrestris* vegetative cells appeared normal by day 10 with no zones and better defined colonies. This suggests that *A. acidoterrestris* and *P. cyclohexanicum* can grow, and be identified from, the same medium and that one, or both, may have an adaptive mechanism which protects it from the effect of the other.

This suggests that *A. acidoterrestris* and *P. cyclohexanicum* can grow, and be identified from, the same medium and that one, or both, may have an adaptive mechanism which protects it from the effect of the other. *P. cyclohexanicum* is a dairy propionibacterium and produced a reaction (Figure 7.2) when co-cultured in orange juice with *A. acidoterrestris*. When the colonies formed by these two bacteria were examined one particular area was demonstrated to be a mixture of the two micro-organisms.

A further part of this study was designed as a preliminary investigation into the reaction produced with *P. cyclohexanicum* and *A. acidoterrestris* and to investigate the possible production of a bacteriocin by co-culturing on solid media using a (agar/disc) diffusion method.

All plates were examined and no unusual colonies or zones of inhibition were demonstrated after 5 days. However after 8 days clear zones (Figure 7.4b) were observed around wells containing *P. cyclohexanicum* in pre-poured agar plates impregnated with *A. acidoterrestris* and hazy zones (Figure 7.4a) around wells containing *A. acidoterrestris* in pre-poured plates impregnated with *P. cyclohexanicum*.



(a)

(b)

Figure 7.4 Zones around wells containing (a) *A. acidoterrestris* in agar impregnated with *P. cyclohexanicum*. (b) Zones around wells containing (a) *P. cyclohexanicum* in agar impregnated with *A. acidoterrestris*.

The hazy zones were very similar in appearance to the zones in the study involving orange juice containing a combination of the two micro-organisms which were later identified as a mixture of *A. acidoterrestris* and *P. cyclohexanicum*.

When the area of growth around the wells was spore stained and microscopically examined oval shaped spores of *A. acidoterrestris* were detected. It is interesting to note that the reactions were distinctive and opposite with no growth occurring around the wells containing *P. cyclohexanicum* and agar impregnated (Figure 7.4b) with *A. acidoterrestris* and growth around the wells containing *A. acidoterrestris* and agar impregnated (Figure 7.4a) with *P. cyclohexanicum*. This suggests that *A. acidoterrestris* formed spores under stress to counteract the presence of *P. cyclohexanicum* in these plates. However in the plates with clear zones around the wells *P. cyclohexanicum* may have prevented outgrowth of *A. acidoterrestris* spores suggesting that *P. cyclohexanicum* had an inhibitory effect on *A. acidoterrestris*.

No changes were observed on plates containing wells of supernatant of either micro-organism, however these plates were observed only up to the 6th day.

7.5.2. Incidence of *A. acidoterrestris* or *P. cyclohexanicum* in raw materials and shelf stable products

Presence of *A. acidoterrestris* and *P. cyclohexanicum* was monitored in a variety of different raw materials and products (Table 7.4 and Table 7.5) to ascertain the level of spoilage.

Material Tested	Number Tested	Positive <i>A. acidoterrestris</i>	Negative <i>A. acidoterrestris</i>
Juice Concentrate			
Apple juice	11	10	1
Pineapple juice	15	0	15
Orange juice	17	11	6
Peach juice	8	1	7
Lime juice	1	0	1
Strawberry juice	2	0	2
Blackcurrant juice	2	0	2
Raspberry juice	2	0	2
Juice esters			
Blackcurrant esters	10	1	9
Cherry esters	13	0	13
Pear esters	3	0	3
Apple esters	10	2	8
Strawberry esters	1	0	1
Blueberry esters	1	0	1
Apricot aroma	2	2	0
Pineapple aroma	2	0	2
Peach aroma	5	0	5
Juice drinks			
Lemon/lime drink	2	1	1
Orange juice drink	2	0	2
Grapefruit drink	8	0	8
Strawberry drink	1	0	1
Flavoured water	2	2	0
Grapes			
Grapes (green) Ortega	3	0	3
Grapes (green) (Madelaine Angeline)	3	0	3
Grapes (rose) Schonberger	3	0	3
Total number tested	129	30	99

Table 7.4. Incidence of *A. acidoterrestris* in different raw materials and shelf stable products.

Approximately 17% of samples tested were positive for *A. acidoterrestris* however no positive samples (Table 7.5) were observed in samples tested for the presence of *P. cyclohexanicum*.

Material Tested	Number Tested	Positive <i>P. cyclohexanicum</i>	Negative <i>P. cyclohexanicum</i>
Juice Concentrate			
Apple juice	3	0	3
Pineapple juice	13	0	13
Orange juice	17	0	17
Peach juice	6	0	6
Lime juice	1	0	1
Juice esters			
Blackcurrant esters	10	0	10
Cherry esters	13	0	13
Pear esters	2	0	2
Apple esters	7	0	7
Strawberry esters	1	0	1
Pineapple aroma	1	0	1
Peach aroma	4	0	4
Juice drinks			
Lemon/lime drink	2	0	2
Orange juice drink	2	0	2
Grapefruit drink	8	0	8
Strawberry drink	1	0	1
Flavoured water	2	0	2
Grapes			
Grapes (green) Ortega	3	0	3
Grapes (green) (Madelaine Angeline)	3	0	3
Grapes (rose) Schonberger	3	0	3
Total number tested	102	0	102

Table 7.5. Incidence of *P. cyclohexanicum* in different raw materials and shelf stable products.

The majority of *A. acidoterrestris* positive samples were isolated from apple juice. Cultures of the micro-organism isolated from apple juice were later genetically (Appendix) identified as *Alicyclobacilli*.

7.6. Discussion

The rate of spoilage for most companies has been reported as 5% but the results of one survey suggested that 35% of companies had experienced spoilage associated with *A. acidoterrestris* (Walls and Chuyate, (1998). It is difficult to assess a rate of spoilage as some raw materials are preserved with low concentrations of sulphur dioxide (orange juices) or ethanol contained naturally in the raw material such as fruit esters. Ethanol concentrations of 6% have been reported to inhibit the growth of *A. acidoterrestris* (Splittstoesser *et al.*, 1998) however fruit esters may contain below this concentration and therefore would still require monitoring for *A. acidoterrestris*.

No samples tested positive for *P. cyclohexanicum* however, as this micro-organism is a gas producer (Kusano *et al.*, 1997) its presence may be more easily detected. *P. cyclohexanicum* has been demonstrated to survive in four of the juices tested (Figure 4.3) therefore monitoring fruit products for the presence of this organism may be necessary in the future.

The haze formed on plates (Figure 7.2) and the growth around the wells (Figure 7.4a) may have been a stress reaction from either organism. Spores are formed by *A. acidoterrestris* under adverse conditions (Deinhard *et al.*, 1987;

Wisotzkey, 1992; Walls and Chuyate, 1998; Jensen, 1999) which may be why higher numbers of spores formed in the samples containing the highest numbers of *P. cyclohexanicum*.

The numbers of *A. acidoterrestris* inoculated into the orange juice (Figure 7.1) were 10^6 each of vegetative cells and spores while the *P. cyclohexanicum* inoculum contained 10^8 this may have caused competition for nutrients as the plates were over 8 days old when the reaction around the wells (Figure 7.4) in the agar was observed.

Changes in environmental conditions are potential stressors to micro-organisms, a sub-lethal stress such as a temperature or pH change can occasion an adaptive tolerance response (ATR) in bacteria. An ATR has been demonstrated in *P. freudenreichii* involving *de novo* polypeptides in response to acid or bile salts (Jan *et al.*, 2000, 2001; Leverrier *et al.*, 2003). Environmental changes within samples may have caused a stress reaction from one or both organisms. Brix values did not change in the *P. cyclohexanicum* which is interesting as significant changes were observed in all other samples. As *P. cyclohexanicum* ferments glucose to propionic, lactic acid and acetic acid the Brix value of these samples might be expected to decrease and the pH to possibly rise, however both pH and Brix values remained fairly stable.

However another reason for this reaction may have been lower oxygen levels as *P. cyclohexanicum* is anaerobic and ferments glucose with lactic acid and propionic being end products of this process (Kusano *et al.*, 1997) and *A.*

acidoterrestris is aerobic (Table 7.1). Further research is required in this area to investigate the possibility of *P. cyclohexanicum* producing a bacteriocin.

A cross reacting protein was detected from a fermentation of *P. theonii* P127 within 24 hours, however bacteriocin activity failed to be detected until 217 hours (Leversee and Glatz, 2001). In this bacteriocin study changes in and around wells on the test plates (Figure 7.4) did not occur until the 9th day after approximately 211 hours. Further investigations may require longer incubation times to properly assess the effects of any environmental changes on *P. cyclohexanicum*.

Chapter 8

Discussion

8.1. Effect of heat treatment on *A. acidoterrestris* and *P. cyclohexanicum*

Pasteurisation has long been an acknowledged preventative measure against the spoilage of fruit juices and fruit juice products. However, since the emergence of *A. acidoterrestris* as a spoilage micro-organism different pasteurisation regimes have been suggested for high acidic fruit products based on the complete inhibition of outgrowth of spores of *A. acidoterrestris* (Silva *et al.*, 2000).

Another spoilage micro-organism isolated, this time from pasteurised orange juice, is the non spore forming *P. cyclohexanicum*. In our laboratories (Chapter 5) this bacterium was subjected to pasteurisation regimes used in the soft drinks industry. The investigation was performed three times in duplicate at 80°C, 85°C, 87°C, 90°C, 93°C, 97°C, 98°C and 102°C for 16 seconds at each temperature. Juices were also pre-heated at 60°C for thirty minutes before pasteurisation and each time *P. cyclohexanicum* failed to survive. Pre heating the inoculated juice samples and varying the numbers of cells in the inoculum had no effect and even when plates were incubated for up to 8 days no survivors were isolated.

P. cyclohexanicum grows best in anaerobic conditions (Kusano *et al.*, 1997) but is aero-tolerant. Therefore exposure to the rigours of pasteurisation may have stressed the organism as the juice appeared to be aerated immediately after treatment. The inoculated juice was held at ambient temperature and re-sampled and tested weekly for five weeks with no evidence of survivors, by which time the juice had become discoloured and was discarded. In the original study by (Kusano

et al 1997) the length of time after pasteurisation the original contamination was reported is not recorded and this may have influenced the growth of this micro-organism. If pasteurisation had occurred some time before detection of spoilage it would give *P. cyclohexanicum* time to recover and grow as in the case of the nisin experiments (Chapter 5). No reference has been made as to whether the initial spoilage caused by *P. cyclohexanicum* transpired pre or post pasteurisation so a series of heat treatment studies (Figures 4.4 - 4.7) were performed. These were designed to ascertain whether an adaptive tolerance response (ATR) similar to that exhibited by other bacteria such as *Escherichia coli* and *Salmonella* spp. (Bearson *et al.*, 1998); Brudzinski and Harrison, 1998; Foster, 1995; Garren *et al.*, 1998; Lee *et al.*, 2002; O'Driscoll *et al.*, 1996) was responsible for the survival of *P. cyclohexanicum* during pasteurisation in the original study.

The results of these investigations (Chapter 4) not only confirmed the heat resistance of *P. cyclohexanicum* but demonstrated its survival at 95°C for 10 minutes, 5°C higher than the previously reported survival temperature (Kusano *et al.*, 1997). The organism had been subjected to incremental and non-incremental heat treatments during the study, although the incremental treatments were only for approximately five minutes, probably not long enough for an adaptive response to occur (Anastasiou *et al.*, 2006). Therefore, as *P. cyclohexanicum* has, as in the case of *A. acidoterrestris*, ω -cyclohexyl undecanoic acid as its major membrane lipids this probably protects the cell from high temperature and acidity in a similar way to *A. acidoterrestris* (Pontius *et al.*, 1998), although *A. acidoterrestris* also forms spores. This suggests a thermotolerance mechanism

may be involved rather than an ATR as *P. cyclohexanicum* survived after non-incremental heat treatment where if an adaptive mechanism was involved the cells would be more likely to survive after incremental and not non-incremental treatment.

8.2. Effect of preservatives sodium benzoate and potassium sorbate and the bacteriocin nisin on *A. acidoterrestris* and *P. cyclohexanicum*

Both *A. acidoterrestris* and *P. cyclohexanicum* were monitored for the effects of two preservatives used in the fruit juice industry, sodium benzoate and potassium sorbate and the bacteriocin nisin (Chapter 5). Nisin has already been demonstrated to be effective against *A. acidoterrestris* (Komitopoulou *et al.*, 1999; Yamazaki, *et al.*, 2000); however this is dependent on the strain of *A. acidoterrestris* and the pH and type of juice used in the study (Yamazaki *et al.*, 2000).

Investigations in our laboratories (Chapter 5) demonstrated that, while the two preservatives and nisin will prevent growth of *A. acidoterrestris*, viable cells and spores remain that may cause spoilage. The nisin study demonstrated that in clear apple juice outgrowth of *A. acidoterrestris* spores was prevented by nisin at concentrations as low as 5 IU/ml which was also demonstrated by Komitopoulou *et al.*, (1999), although whether the juice in this study was clarified or not is not known. However Yamazaki *et al.*, (2000) observed that in clear apple juice higher concentrations of nisin exceeding 600 IU/ml would be required to prevent

outgrowth of spores. Further investigations are required to identify *A. acidoterrestris* strains already observed to react differently within the species (Splittstoesser *et al.*, 1994; Splittstoesser *et al.*, 1998; Yamazaki *et al.*, 2000; Silva and Gibbs 2001) and to determine their nisin sensitivity.

The reaction of *P. cyclohexanicum* to the two preservatives and nisin was very different to that of *A. acidoterrestris*. Concentrations of 1000 IU/ml of nisin (Figure 5.10) failed to prevent cell growth and the time for growth to occur was related to the concentration of nisin initially used (Figures 5.9 and 5.10). A gradual increase in the numbers of *P. cyclohexanicum* was observed on each day of testing suggesting a tolerance to nisin in a similar way to this organism's heat tolerance. However, Gram positive bacteria possess a permeable cell membrane not usually restrictive to antimicrobial penetration which is vital to the growth and survival of the micro-organism in hostile environments (Lambert 2002). Genomic sequencing may allow identification of genes conferring resistance to antimicrobials (Rosamond and Allsop 2000) in *P. cyclohexanicum*.

8.3. Co-culture of *A. acidoterrestris* and *P. cyclohexanicum*

P. cyclohexanicum belongs to the classical or dairy group of propionibacteria which produce inhibitory compounds such as propionate, acetate and diacetyl. It was diacetyl that produced the 'off odour' in the original spoiled orange juice (Kusano *et al.*, 1997). Some strains of this group of propionibacteria such as *P. theonii* (Lyon and Glatz, 1993) and *P. jensenii* (Faye 2004) produce

bacteriocins. A study was designed to investigate the possibility that *P. cyclohexanicum* might be the producer of bacteriocins (Chapter 7). During this study plates containing *A. acidoterrestris* produced hazy growth around vegetative cells which, when cultured, were identified as spores of *A. acidoterrestris* and cells of *P. cyclohexanicum*. This occurred again in agar diffusion studies with *P. cyclohexanicum* in the wells and the agar containing *A. acidoterrestris*. The haze like growth on the plates was not observed at any other time in any other investigations, however in all other studies each organism was used independently. The changes on the plates were only observed after 8 or 9 days which is longer than the time usually used to incubate these organisms. This suggests that further studies may require longer incubation times and possibly samples should be retained and monitored after the length of a study for any further changes.

8.4. Effect of headspace on *A. acidoterrestris*

During the investigations in our laboratories (Chapter 5), challenging *A. acidoterrestris* and *P. cyclohexanicum* with potassium sorbate and sodium benzoate and nisin, high increases in numbers in some control samples were observed in the *A. acidoterrestris* study. This only occurred in single bottles and at various times during the study. The reason for this was not clear and an investigation into whether the amount of headspace and agitation samples were subjected to affected the growth of vegetative cells or spores of *A. acidoterrestris*.

A delivery of juice may not always be used immediately and may have come from overseas by road being shaken during loading, transport and again during unloading with consequent temperature changes. This shaking allows mixing of any available oxygen and thus, if present, growth of the aerobic *A. acidoterrestris*. Containers are usually refrigerated until required and, as demonstrated in this study, if containers were shaken before sampling particularly at suboptimal growth temperatures (<30°C) isolation of *A. acidoterrestris* is generally higher.

8.5. Isolation of *A. acidoterrestris*

Methods of detecting these novel spoilage organisms have been changing since the genus *Alicyclobacillus* was first recognised (Wisotzkey *et al.*, 1992). Agar that was initially made from many ingredients with two separate components that were combined only after autoclaving and cooling to 50°C has now been superseded by a specific agar for isolating *A. acidoterrestris* (Chang and Kang, 2005).

Calculating the percentage of overall positive samples from those tested probably gives an inaccurate picture of contamination rates as probability of isolating the organism depends wholly on the materials being tested. Some by their nature would be unlikely to support growth of *A. acidoterrestris* due to the presence of naturally occurring ethanol. However the possibility of the presence of *A. acidoterrestris* must always be considered when sampling raw materials or products.

Routine quality control measures can include testing for *A. acidoterrestris* as, even if confirmatory tests are required, these can be performed during quarantine periods when raw materials will not be used. It is in the interests of the soft drinks manufacturer to locate spoilage organisms before they enter production areas as once this has occurred *A. acidoterrestris* can contaminate other products as well as the factory environment and other raw materials and can prove difficult to eliminate (Jensen, 1999).

P. cyclohexanicum ferments lactic, propionic and acetic acids from glucose and during all studies involving this micro-organism after shaking sample bottles prior to testing the contents 'fizzed' and occasionally overflowed explosively. Possibly this may have been part of the initial complaint about the original spoilage incident concerning *P. cyclohexanicum* as well as the 'off' odour (Kusano *et al.*, 1997). Therefore *P. cyclohexanicum* should be easier than *A. acidoterrestris* to detect unless it is in a carbonated product. However, to date, no other spoilage incidents have been reported involving this bacterium.

8.6. Conclusions

These studies have demonstrated that the growth of *A. acidoterrestris* and *P. cyclohexanicum* may be inhibited by the use of chemical preservatives. However consumers are requesting the exclusion of many chemically synthesised preservatives and demanding minimally processed food and drinks together with safety, quality and long shelf life (Roller, 1995). Nisin a natural non-toxic

antibacterial food preservative approved for use in 1969 (Delves-Broughton, 1990) was found to inhibit the growth of *A. acidoterrestris*. Nisin is a polypeptide produced by *Lactococcus lactis* a food grade micro-organism and is regarded as a natural food grade bacteriocin (Delves-Broughton, 1990). Therefore as nisin is regarded as natural and has been demonstrated as inhibitory to *A. acidoterrestris* this may, if approved, fulfil the requirements of the soft drinks industry of the future.

The discovery and isolation of *A. acidoterrestris* from fruit juice has been demonstrated to be one of the most significant challenges to the fruit beverage industry in the past few decades. Other novel micro-organisms such as *L. perolens* a contaminant of red lemonade (Back *et al.*, 1999) or *P. cyclohexanicum* (Kusano *et al.*, 1997) isolated from pasteurised orange juice have failed to have the same impact as *A. acidoterrestris*. Pasteurisation alone will not inhibit the growth of these organisms and particularly in the case of *A. acidoterrestris* may enhance the growth as heat shock at pasteurisation temperatures is used to encourage growth in the laboratory.

The development of a substantial well validated isolation protocol is required to assess the degree of contamination in the processing arena by *A. acidoterrestris*, other species of this genus and novel micro-organisms that may prove important in terms of spoilage in the future. The use of standard isolation media such as SK agar recently developed by Chang and Kang (2005) which is more sensitive to *Alicyclobacilli* spores may provide truer estimates of the level of contamination by this micro-organism. SK agar has been designed to isolate low

numbers of *Alicyclobacillus* spp. which is required to reduce the number of false negative results; this newly designed media may be useful in the fruit juice industry (Chang and Kang (2005).

Alicyclobacilli ssp have been isolated from soils in different areas of the world (Darland and Brock 1971; Hippchen *et al.*, 1981; Norris *et al.*, 1996) and *Alicyclobacilli* have been isolated from fruit surfaces (Wisse and Parish 1998). The presence of these micro-organisms in soil, on the surface of fruits and their isolation from shelf stable products suggests that their complete elimination from final products may not be possible (Wisse and Parish, 1998). However, contamination by *A. acidoterrestris* or *P. cyclohexanicum* may be controlled by the use of good monitoring procedures and adherence to the principles of HACCP with particular emphasis on the control of *A. acidoterrestris* spores as they are more resistant to the pasteurisation process and are therefore more likely to survive (Chang and Kang, 2005)

The designs of reliable rapid methods that may be used in an industrial setting and are inexpensive and reduce the time from sampling to identification are required. New preservation regimes must be developed by the soft drinks industry to keep pace with changes in consumer requirements and to monitor and control novel and emerging species of spoilage organisms.

Chapter nine

Future Work

9.0. *Future work*

- Investigate other members within the *Alicyclobacillus* genus isolated from beverages, to assess their importance to the beverage industry
- Monitor pasteurisation temperatures used within the soft drinks industry to assess their impact on heat resistant novel micro-organisms
- Investigate phenolic compounds within fruit juices to monitor their effects on novel micro-organisms

Chapter 10

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Appendix

1

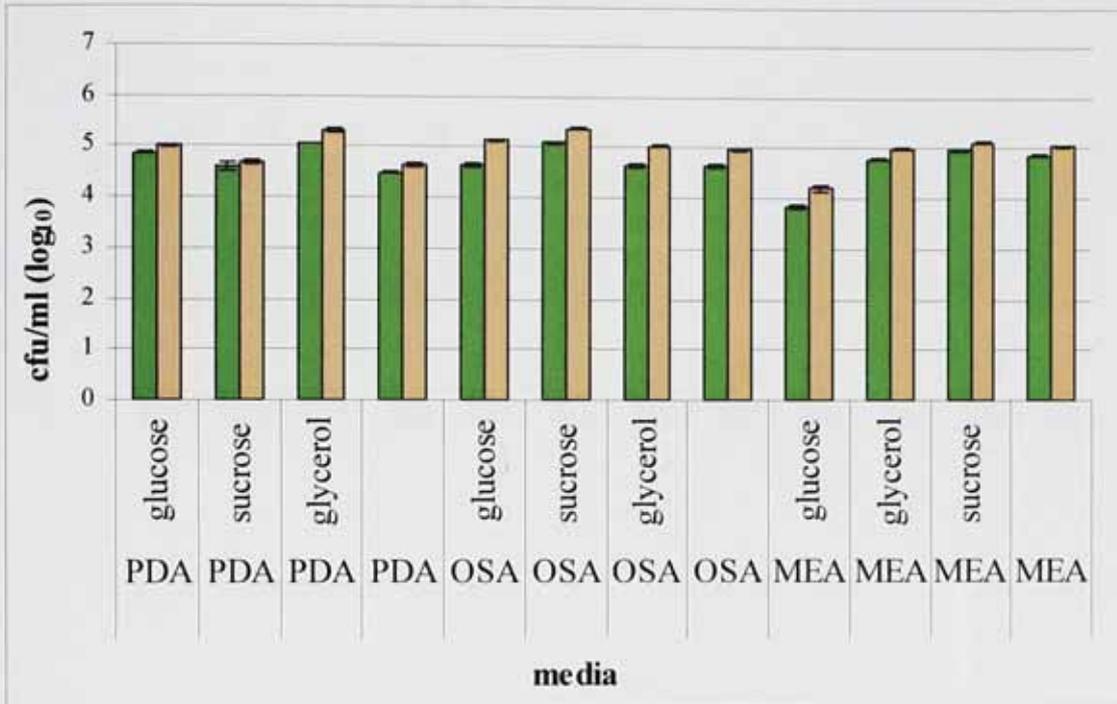


Fig a. Vegetative cell counts of *A. acidoterrestris* in apple juice (pH3.5) -■- and orange juice (pH3.8) -■- using different isolation media incubated at 44°C for 5 days before enumeration. Values are means ± SEM (*n* = 6).

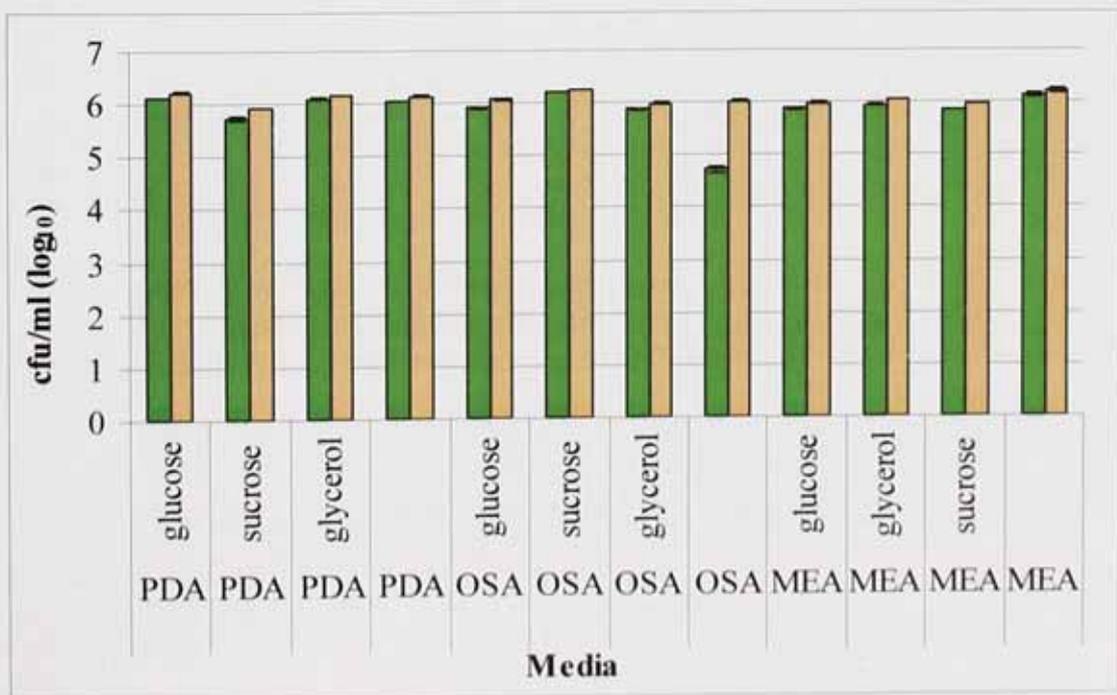


Fig b Spore counts of *A. acidoterrestris* in apple juice (pH3.5) -■- and orange juice (pH3.8) -■- using different isolation media incubated at 44°C for 5 days before enumeration. Values are means ± SEM (*n* = 6).

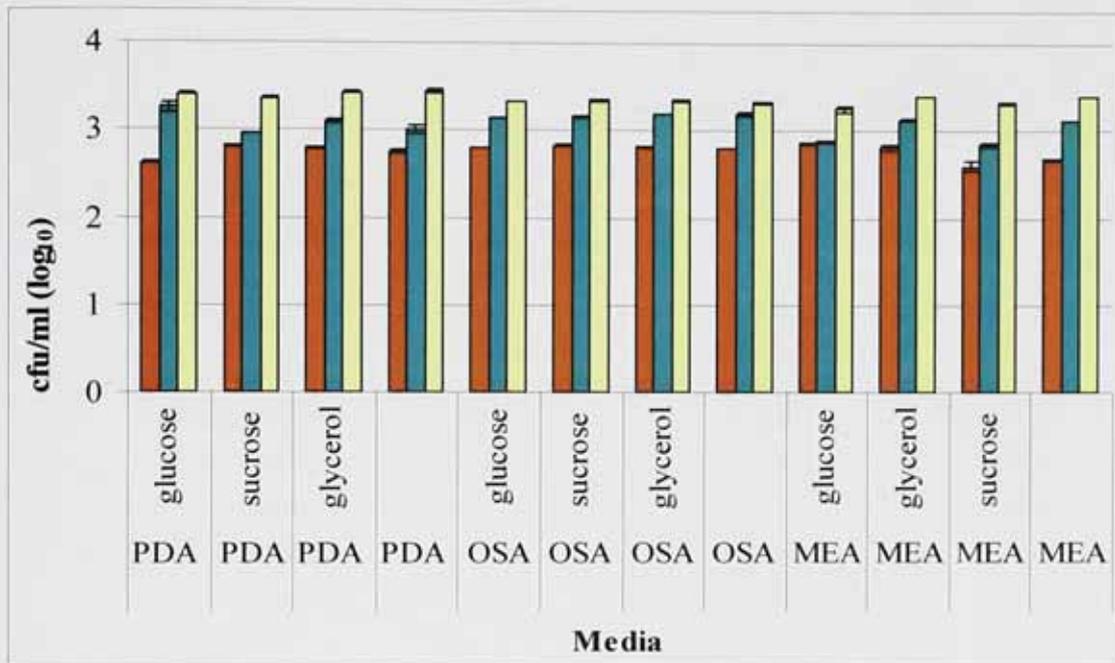


Fig c Vegetative cell counts of *A. acidoterrestis* in acidified peptone broth (pH4.0) using different isolation media inoculated with low concentrations of *A. acidoterrestis* -■- 0.1ml; -■- 0.2ml; -■- 0.4ml (all per 10ml of broth) incubated at 44°C for 5 days before enumeration. Values are means ± SEM ($n = 6$).

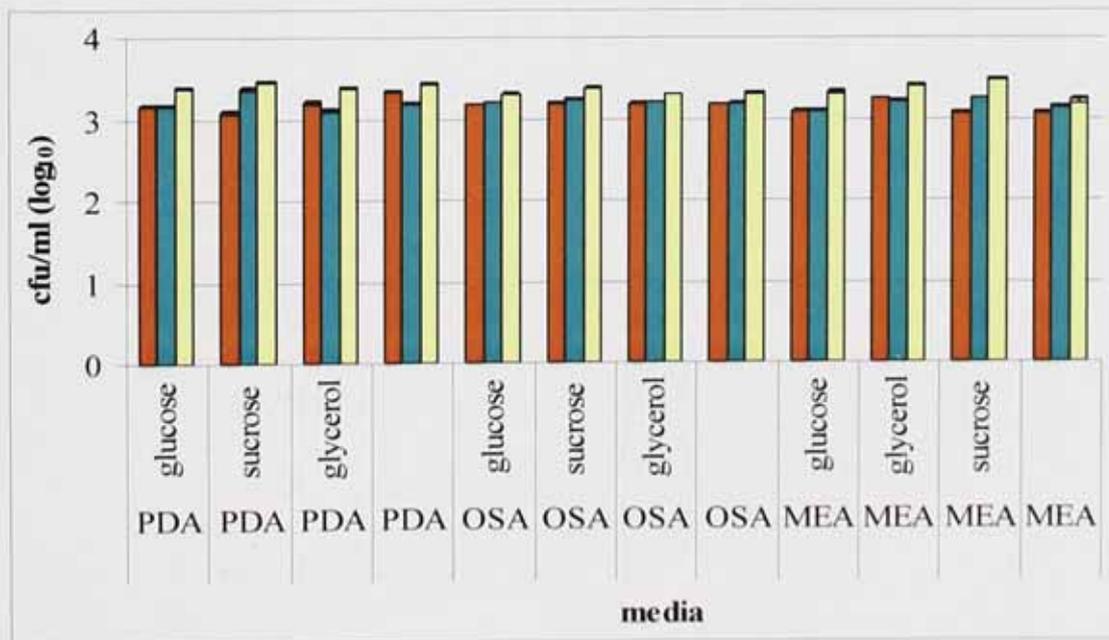


Fig d Spore counts of *A. acidoterrestis* in acidified peptone broth (pH4.0) using different isolation media inoculated with low concentrations of *A. acidoterrestis* -■- 0.1ml; -■- 0.2ml; -■- 0.4ml (all per 10ml of broth) incubated at 44°C for 5 days before enumeration. Values are means ± SEM ($n = 6$).

Carbon-source	<i>B. acidocaldarius</i>	<i>B. acidoterrestris</i>
Glycerol	8	13
Erythritol	0	13
L-arabinose	8	13
Ribose	5	13
D-Xylose	8	13
Adonitol	0	7
Galactose	8	13
D-Glucose	8	13
D-Fructose	8	13
D-Mannose	8	13
Rhamnose	4	13
Inositol	3	6
Mannitol	8	13
Sorbitol	0	12
α -Methyl-D-glucoside	4	12
Amygladine	0	4
Arubtine	8	10
Esculine	8	13
Salicine	7	12
Cellobiose	8	13
Maltose	8	12
Lactose	6	11
Melibiose	5	0
Saccharose	8	3
Trehalose	8	4
Melezitose	5	1
D-Raffinose	7	1
Starch	6	2
Glycogen	8	2
Xylitol	0	12
β -Gentibiose	8	12
D-Turanose	7	2
D-Arabitol	0	7

Deinhard *et al.*, (1987).

Table a. Strains of *B. acidocaldarius* and *B. acidoterrestris* which form acid from different carbon sources.

PDA	0.5% (w/v) glucose	3.31	3.61
PDA	0.5% (w/v) sucrose	3.28	3.36
PDA	0.5% (w/v) glycerol	3.33	3.46
PDA	No supplement	3.24	3.40
OSA	0.5% (w/v) glucose	3.35	3.37
OSA	0.5% (w/v) sucrose	3.42	3.51
OSA	0.5% (w/v) glycerol	3.39	3.44
OSA	No supplement	3.39	3.46
MEA	0.5% (w/v) glucose	3.14	3.43
MEA	0.5% (w/v) sucrose	3.10	3.41
MEA	0.5% (w/v) glycerol	3.09	3.35
MEA	No supplement	3.25	3.37

Table b Growth of *A. acidoterrestris* vegetative cells in acidified peptone broth (pH4.0) using different isolation media 1ml and 2ml (per 10ml of broth) pH4.0 (cfu/ml log₍₁₀₎) incubated at 44°C for 5 days before enumeration. Values are means ± SEM (*n* = 6).

PDA	0.5% (w/v) glucose	cfus merged	cfus merged
PDA	0.5% (w/v) sucrose	cfus merged	cfus merged
PDA	0.5% (w/v) glycerol	cfus merged	cfus merged
PDA	No supplement	cfus merged	cfus merged
OSA	0.5% (w/v) glucose	3.43	3.44
OSA	0.5% (w/v) sucrose	3.45	3.48
OSA	0.5% (w/v) glycerol	3.43	3.45
OSA	No supplement	3.45	3.48
MEA	0.5% (w/v) glucose	cfus merged	cfus merged
MEA	0.5% (w/v) sucrose	cfus merged	cfus merged
MEA	0.5% (w/v) glycerol	cfus merged	cfus merged
MEA	No supplement	cfus merged	cfus merged

Table c Growth of *A. acidoterrestris* spores in acidified peptone broth (pH4.0) using different isolation media 1ml and 2ml (per 10ml of broth) (cfu/ml log₍₁₀₎) incubated at 44°C for 5 days before enumeration. Values are means ± SEM (*n* = 6).

Appendix

2

ANALYSIS REPORT

TO: Michelle Walker
FROM: Tom Capper
DATE: 24/09/04

SUBJECT: BLUE NUMBER 54725
 DAY 15 PRESERVATIVES ANALYSIS

SAMPLES:

5472555	A. acidoterrestris/sodium benzoate 500 ppm SB 1A 500
5472556	A. acidoterrestris/sodium benzoate 1000 ppm SB 1A 1000
5472557	A. acidoterrestris/sodium benzoate 1500 ppm SB 1A 1500
5472558	A. acidoterrestris/sodium benzoate 500 ppm SB 2A 500
5472559	A. acidoterrestris/sodium benzoate 1000 ppm SB 2A 1000
5472560	A. acidoterrestris/sodium benzoate 1500 ppm SB 2A 1500
5472561	A. acidoterrestris/ potassium sorbate 500 ppm PS 1B 500
5472562	A. acidoterrestris/ potassium sorbate 1000 ppm PS 1B 1000
5472563	A. acidoterrestris/ potassium sorbate 1500 ppm PS 1B 1500
5472564	A. acidoterrestris/ potassium sorbate 500 ppm PS 2B 500
5472565	A. acidoterrestris/ potassium sorbate 1000 ppm PS 2B 1000
5472566	A. acidoterrestris/ potassium sorbate 1500 ppm PS 2B 1500

	Sodium Benzoate (mg/l)	Potassium Sorbate (mg/l)
5472555	548	ND
5472556	1079	ND
5472557	1603	ND
5472558	546	ND
5472559	1044	ND
5472560	1577	ND
5472561	ND	506
5472562	ND	1033
5472563	ND	1528
5472564	ND	494
5472565	ND	1036
5472566	ND	1479

ND = Not detected

ANALYST: *Tom Capper*

AUTHORISED: *MW*

ANALYSIS REPORT

TO: Michelle Walker
 FROM: Tom Capper
 DATE: 14/09/04

SUBJECT: **BLUE NUMBER 54725**
DAY 5 PRESERVATIVES ANALYSIS

SAMPLES: 5472531 A. acidoterrestri/sodium benzoate 500 ppm SB 1A 500
 5472532 A. acidoterrestri/sodium benzoate 1000 ppm SB 1A 1000
 5472533 A. acidoterrestri/sodium benzoate 1500 ppm SB 1A 1500
 5472534 A. acidoterrestri/sodium benzoate 500 ppm SB 2A 500
 5472535 A. acidoterrestri/sodium benzoate 1000 ppm SB 2A 1000
 5472536 A. acidoterrestri/sodium benzoate 1500 ppm SB 2A 1500
 5472537 A. acidoterrestri/ potassium sorbate 500 ppm PS 1B 500
 5472538 A. acidoterrestri/ potassium sorbate 1000 ppm PS 1B 1000
 5472539 A. acidoterrestri/ potassium sorbate 1500 ppm PS 1B 1500
 5472540 A. acidoterrestri/ potassium sorbate 500 ppm PS 2B 500
 5472541 A. acidoterrestri/ potassium sorbate 1000 ppm PS 2B 1000
 5472542 A. acidoterrestri/ potassium sorbate 1500 ppm PS 2B 1500

	Sodium Benzoate (mg/l)	Potassium Sorbate (mg/l)
5472531	553	ND
5472532	1081	ND
5472533	1608	ND
5472534	540	ND
5472535	1060	ND
5472536	1594	ND
5472537	ND	518
5472538	ND	1059
5472539	ND	1564
5472540	ND	502
5472541	ND	1048
5472542	ND	1510

ND = Not detected

ANALYST: *Tom Capper*

AUTHORISED: *[Signature]*

ANALYSIS REPORT

TO: Michelle Walker
 FROM: Tom Capper
 DATE: 13/09/04

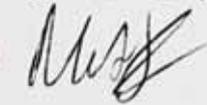
SUBJECT: BLUE NUMBER 54725
 DAY 1 PRESERVATIVES ANALYSIS

SAMPLES: 5472519 A. acidoterrestris/sodium benzoate 500 ppm SB 1A 500
 5472520 A. acidoterrestris/sodium benzoate 1000 ppm SB 1A 1000
 5472521 A. acidoterrestris/sodium benzoate 1500 ppm SB 1A 1500
 5472522 A. acidoterrestris/sodium benzoate 500 ppm SB 2A 500
 5472523 A. acidoterrestris/sodium benzoate 1000 ppm SB 2A 1000
 5472524 A. acidoterrestris/sodium benzoate 1500 ppm SB 2A 1500
 5472525 A. acidoterrestris/ potassium sorbate 500 ppm PS 1B 500
 5472526 A. acidoterrestris/ potassium sorbate 1000 ppm PS 1B 1000
 5472527 A. acidoterrestris/ potassium sorbate 1500 ppm PS 1B 1500
 5472528 A. acidoterrestris/ potassium sorbate 500 ppm PS 2B 500
 5472529 A. acidoterrestris/ potassium sorbate 1000 ppm PS 2B 1000
 5472530 A. acidoterrestris/ potassium sorbate 1500 ppm PS 2B 1500

	Sodium Benzoate (mg/l)	Potassium Sorbate (mg/l)
5472519	563	ND
5472520	1071	ND
5472521	1592	ND
5472522	552	ND
5472523	1055	ND
5472524	1598	ND
5472525	ND	518
5472526	ND	1046
5472527	ND	1543
5472528	ND	506
5472529	ND	1060
5472530	ND	1527

ND = Not detected

ANALYST: Tom Capper

AUTHORISED: 

ANALYSIS REPORT

TO: Michelle Walker
 FROM: Tom Capper
 DATE: 10/09/04

SUBJECT: BLUE NUMBER 54725
 DAY 0 PRESERVATIVES ANALYSIS

SAMPLES: 5472510 Potassium sorbate 10% Solution
 5472511 A. acidoterrestris/potassium sorbate 0 ppm PS 1B 0
 5472512 A. acidoterrestris/ potassium sorbate 500 ppm PS 1B 500
 5472513 A. acidoterrestris/ potassium sorbate 1000 ppm PS 1B 1000
 5472514 A. acidoterrestris/ potassium sorbate 1500 ppm PS 1B 1500
 5472515 A. acidoterrestris/ potassium sorbate 0 ppm PS 2B 0
 5472516 A. acidoterrestris/ potassium sorbate 500 ppm PS 2B 500
 5472517 A. acidoterrestris/ potassium sorbate 1000 ppm PS 2B 1000
 5472518 A. acidoterrestris/ potassium sorbate 1500 ppm PS 2B 1500

	Potassium sorbate
5472510	10.21 % w/v
5472511	ND
5472512	524 mg/l
5472513	1062 mg/l
5472514	1567 mg/l
5472515	ND
5472516	511 mg/l
5472517	1057 mg/l
5472518	1533 mg/l

ND = Not detected

ANALYST: Tom Capper
 AUTHORISED: *[Signature]*

ANALYSIS REPORT

TO: Michelle Walker
 FROM: Tom Capper
 DATE: 20/09/04

SUBJECT: **BLUE NUMBER 54725**
DAY 8 PRESERVATIVES ANALYSIS

SAMPLES: 5472543 A. acidoterrestris/sodium benzoate 500 ppm SB 1A 500
 5472544 A. acidoterrestris/sodium benzoate 1000 ppm SB 1A 1000
 5472545 A. acidoterrestris/sodium benzoate 1500 ppm SB 1A 1500
 5472546 A. acidoterrestris/sodium benzoate 500 ppm SB 2A 500
 5472547 A. acidoterrestris/sodium benzoate 1000 ppm SB 2A 1000
 5472548 A. acidoterrestris/sodium benzoate 1500 ppm SB 2A 1500
 5472549 A. acidoterrestris/ potassium sorbate 500 ppm PS 1B 500
 5472550 A. acidoterrestris/ potassium sorbate 1000 ppm PS 1B 1000
 5472551 A. acidoterrestris/ potassium sorbate 1500 ppm PS 1B 1500
 5472552 A. acidoterrestris/ potassium sorbate 500 ppm PS 2B 500
 5472553 A. acidoterrestris/ potassium sorbate 1000 ppm PS 2B 1000
 5472554 A. acidoterrestris/ potassium sorbate 1500 ppm PS 2B 1500

	Sodium Benzoate (mg/l)	Potassium Sorbate (mg/l)
5472543	557	ND
5472544	1057	ND
5472545	1586	ND
5472546	549	ND
5472547	1019	ND
5472548	1567	ND
5472549	ND	513
5472550	ND	1036
5472551	ND	1490
5472552	ND	489
5472553	ND	1025
5472554	ND	1513

ND = Not detected

ANALYST: *Tom Capper*

AUTHORISED: *Michelle Walker*

ANALYSIS REPORT

TO: Michelle Walker
 FROM: Tom Capper
 DATE: 08/10/04

SUBJECT: BLUE NUMBER 54725
 DAY 29 PRESERVATIVES ANALYSIS

SAMPLES: 5472579 A. acidoterrestris/sodium benzoate 500 ppm SB 1A 500
 5472580 A. acidoterrestris/sodium benzoate 1000 ppm SB 1A 1000
 5472581 A. acidoterrestris/sodium benzoate 1500 ppm SB 1A 1500
 5472582 A. acidoterrestris/sodium benzoate 500 ppm SB 2A 500
 5472583 A. acidoterrestris/sodium benzoate 1000 ppm SB 2A 1000
 5472584 A. acidoterrestris/sodium benzoate 1500 ppm SB 2A 1500
 5472585 A. acidoterrestris/potassium sorbate 500 ppm PS 1B 500
 5472586 A. acidoterrestris/potassium sorbate 1000 ppm PS 1B 1000
 5472587 A. acidoterrestris/potassium sorbate 1500 ppm PS 1B 1500
 5472588 A. acidoterrestris/potassium sorbate 500 ppm PS 2B 500
 5472589 A. acidoterrestris/potassium sorbate 1000 ppm PS 2B 1000
 5472590 A. acidoterrestris/potassium sorbate 1500 ppm PS 2B 1500

	Sodium benzoate (mg/l)	Potassium sorbate (mg/l)
5472579	570	ND
5472580	1048	ND
5472581	1607	ND
5472582	544	ND
5472583	1046	ND
5472584	1573	ND
5472585	ND	496
5472586	ND	1019
5472587	ND	1510
5472588	ND	482
5472589	ND	1009
5472590	ND	1459

ND = Not detected

ANALYST: *Tom Capper*
 AUTHORISED: *[Signature]*

ANALYSIS REPORT

TO: Michelle Walker
FROM: Tom Capper
DATE: 10/09/04

SUBJECT: BLUE NUMBER 54725
DAY 0 PRESERVATIVES ANALYSIS

SAMPLES:

5472501	Sodium benzoate 10% Solution
5472502	A. acidoterrestris/sodium benzoate 0 ppm SB 1A 0
5472503	A. acidoterrestris/sodium benzoate 500 ppm SB 1A 500
5472504	A. acidoterrestris/sodium benzoate 1000 ppm SB 1A 1000
5472505	A. acidoterrestris/sodium benzoate 1500 ppm SB 1A 1500
5472506	A. acidoterrestris/sodium benzoate 0 ppm SB 2A 0
5472507	A. acidoterrestris/sodium benzoate 500 ppm SB 2A 500
5472508	A. acidoterrestris/sodium benzoate 1000 ppm SB 2A 1000
5472509	A. acidoterrestris/sodium benzoate 1500 ppm SB 2A 1500

	Sodium Benzoate
5472501	10.59% w/v
5472502	ND
5472503	558 mg/l
5472504	1056 mg/l
5472505	1586 mg/l
5472506	ND
5472507	524 mg/l
5472508	1062 mg/l
5472509	1569 mg/l

ND = Not detected

ANALYST: Tom Capper
AUTHORISED: *MW*

ANALYSIS REPORT

TO: Michelle Walker
 FROM: Tom Capper
 DATE: 30/09/04

SUBJECT: BLUE NUMBER 54725
 DAY 22 PRESERVATIVES ANALYSIS

SAMPLES: 5472567 A. acidoterrestris/sodium benzoate 500 ppm SB 1A 500
 5472568 A. acidoterrestris/sodium benzoate 1000 ppm SB 1A 1000
 5472569 A. acidoterrestris/sodium benzoate 1500 ppm SB 1A 1500
 5472570 A. acidoterrestris/sodium benzoate 500 ppm SB 2A 500
 5472571 A. acidoterrestris/sodium benzoate 1000 ppm SB 2A 1000
 5472572 A. acidoterrestris/sodium benzoate 1500 ppm SB 2A 1500
 5472573 A. acidoterrestris/ potassium sorbate 500 ppm PS 1B 500
 5472574 A. acidoterrestris/ potassium sorbate 1000 ppm PS 1B 1000
 5472575 A. acidoterrestris/ potassium sorbate 1500 ppm PS 1B 1500
 5472576 A. acidoterrestris/ potassium sorbate 500 ppm PS 2B 500
 5472577 A. acidoterrestris/ potassium sorbate 1000 ppm PS 2B 1000
 5472578 A. acidoterrestris/ potassium sorbate 1500 ppm PS 2B 1500

	Sodium Benzoate (mg/l)	Potassium Sorbate (mg/l)
5472567	557	ND
5472568	1063	ND
5472569	1581	ND
5472570	546	ND
5472571	1037	ND
5472572	1571	ND
5472573	ND	503
5472574	ND	1025
5472575	ND	1537
5472576	ND	488
5472577	ND	1020
5472578	ND	1471

ND = Not detected

ANALYST: *Tom Capper*

AUTHORISED: *[Signature]*

ANALYSIS REPORT

TO: Michelle Walker
FROM: Tom Capper
DATE: 11/10/05

SUBJECT: RED NUMBER 59454
PRESERVATIVES INVESTIGATIONS & P. CYCLOHEXANICUM IN FRUIT JUICE,
DAY 15 (INTERNAL)

SAMPLES:

5945435	Fruit Juice Bottle 2
5945436	Fruit Juice Bottle 3
5945437	Fruit Juice Bottle 4
5945438	Fruit Juice Bottle 5
5945439	Fruit Juice Bottle 17
5945440	Fruit Juice Bottle 18
5945441	Fruit Juice Bottle 19
5945442	Fruit Juice Bottle 20
5945443	Fruit Juice Bottle 21
5945444	Fruit Juice Bottle 22
5945445	Fruit Juice Bottle 23
5945446	Fruit Juice Bottle 24
5945447	Fruit Juice Bottle 25
5945448	Fruit Juice Bottle 26
5945449	Fruit Juice Bottle 27
5945450	Fruit Juice Bottle 28

	Sodium Benzoate
5945435	517 mg/l
5945436	553 mg/l
5945437	1015 mg/l
5945438	1110 mg/l
5945439	512 mg/l
5945440	516 mg/l
5945441	521 mg/l
5945442	557 mg/l
5945443	512 mg/l
5945444	553 mg/l
5945445	1102 mg/l
5945446	1052 mg/l
5945447	1063 mg/l
5945448	1098 mg/l
5945449	1131 mg/l
5945450	1072 mg/l

ANALYST:

Tom Capper

AUTHORISED:

MLK

ANALYSIS REPORT

TO: Michelle Walker
 FROM: Tom Capper
 DATE: 26/09/05

SUBJECT: RED NUMBER 59454
 PRESERVATIVES INVESTIGATIONS & P. CYCLOHEXANICUM IN FRUIT JUICE,
 DAY 0 (INTERNAL)

SAMPLES:	5945418	Potassium Sorbate 10% solution
	5945419	Fruit Juice Bottle 7
	5945420	Fruit Juice Bottle 8
	5945421	Fruit Juice Bottle 9
	5945422	Fruit Juice Bottle 10
	5945423	Fruit Juice Bottle 29
	5945424	Fruit Juice Bottle 30
	5945425	Fruit Juice Bottle 31
	5945426	Fruit Juice Bottle 32
	5945427	Fruit Juice Bottle 33
	5945428	Fruit Juice Bottle 34
	5945429	Fruit Juice Bottle 35
	5945430	Fruit Juice Bottle 36
	5945431	Fruit Juice Bottle 37
	5945432	Fruit Juice Bottle 38
	5945433	Fruit Juice Bottle 39
	5945434	Fruit Juice Bottle 40

	Potassium Sorbate
5945418	9.92 % w/w
5945419	527 mg/l
5945420	510 mg/l
5945421	999 mg/l
5945422	1025 mg/l
5945423	535 mg/l
5945424	529 mg/l
5945425	496 mg/l
5945426	504 mg/l
5945427	496 mg/l
5945428	498 mg/l
5945429	1031 mg/l
5945430	1005 mg/l
5945431	1019 mg/l
5945432	993 mg/l
5945433	999 mg/l
5945434	964 mg/l

ANALYST:

Tom Capper

AUTHORISED:

TC

ANALYSIS REPORT

TO: Michelle Walker
FROM: Tom Capper
DATE: 23/09/05

SUBJECT: RED NUMBER 59454
PRESERVATIVES INVESTIGATIONS & P. CYCLOHEXANICUM IN FRUIT JUICE,
DAY 0 (INTERNAL)

SAMPLES: 5945401 Sodium Benzoate 10% solution
5945402 Fruit Juice Bottle 2
5945403 Fruit Juice Bottle 3
5945404 Fruit Juice Bottle 4
5945405 Fruit Juice Bottle 5
5945406 Fruit Juice Bottle 17
5945407 Fruit Juice Bottle 18
5945408 Fruit Juice Bottle 19
5945409 Fruit Juice Bottle 20
5945410 Fruit Juice Bottle 21
5945411 Fruit Juice Bottle 22
5945412 Fruit Juice Bottle 23
5945413 Fruit Juice Bottle 24
5945414 Fruit Juice Bottle 25
5945415 Fruit Juice Bottle 26
5945416 Fruit Juice Bottle 27
5945417 Fruit Juice Bottle 28

	Sodium Benzoate
5945401	10.16% w/w
5945402	530 mg/l
5945403	545 mg/l
5945404	1032 mg/l
5945405	1057 mg/l
5945406	503 mg/l
5945407	523 mg/l
5945408	531 mg/l
5945409	550 mg/l
5945410	516 mg/l
5945411	540 mg/l
5945412	1079 mg/l
5945413	1063 mg/l
5945414	1076 mg/l
5945415	1063 mg/l
5945416	1090 mg/l
5945417	1072 mg/l

ANALYST: *Tom Capper*
AUTHORISED: *[Signature]*

ANALYSIS REPORT

TO: Michelle Walker
FROM: Tom Capper
DATE: 11/10/05

SUBJECT: RED NUMBER 59454
PRESERVATIVES INVESTIGATIONS & P. CYCLOHEXANICUM IN FRUIT JUICE,
DAY 15 (INTERNAL)

SAMPLES:

5945451	Fruit Juice Bottle 7
5945452	Fruit Juice Bottle 8
5945453	Fruit Juice Bottle 9
5945454	Fruit Juice Bottle 10
5945455	Fruit Juice Bottle 29
5945456	Fruit Juice Bottle 30
5945457	Fruit Juice Bottle 31
5945458	Fruit Juice Bottle 32
5945459	Fruit Juice Bottle 33
5945460	Fruit Juice Bottle 34
5945461	Fruit Juice Bottle 35
5945462	Fruit Juice Bottle 36
5945463	Fruit Juice Bottle 37
5945464	Fruit Juice Bottle 38
5945465	Fruit Juice Bottle 39
5945466	Fruit Juice Bottle 40

	Potassium Sorbate
5945451	497 mg/l
5945452	468 mg/l
5945453	937 mg/l
5945454	983 mg/l
5945455	492 mg/l
5945456	490 mg/l
5945457	468 mg/l
5945458	485 mg/l
5945459	474 mg/l
5945460	463 mg/l
5945461	954 mg/l
5945462	956 mg/l
5945463	972 mg/l
5945464	944 mg/l
5945465	903 mg/l
5945466	888 mg/l

ANALYST:

Tom Capper

AUTHORISED:

[Signature]

Company: Belmay LTD

Sample: C173440 - 1AAa

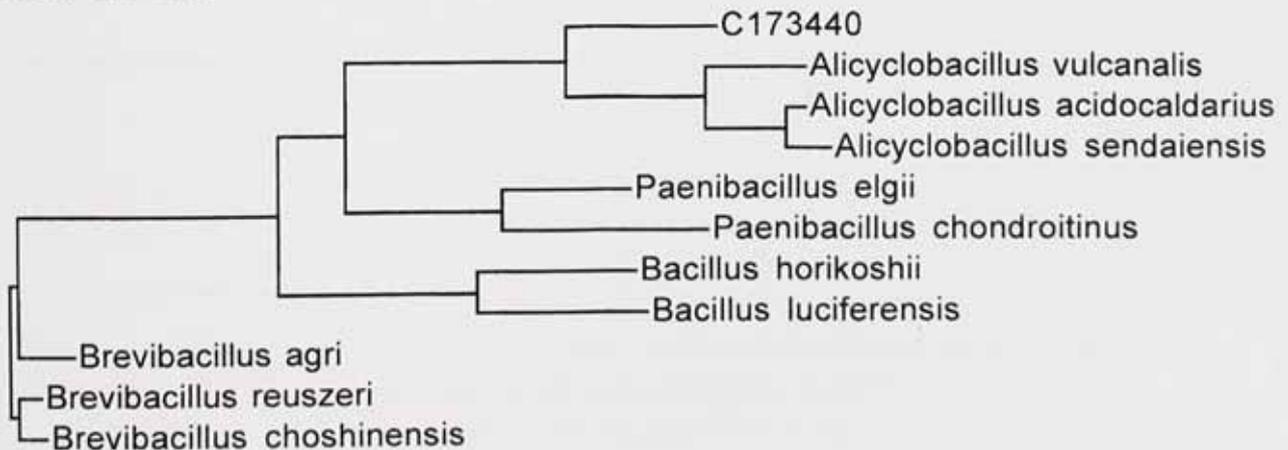
Date: Thursday, July 6, 2006 2:18 PM

Alignment: 519 C173440

- 7.90 % 522 Alicyclobacillus acidocaldarius
- 8.67 % 522 Alicyclobacillus sendaiensis
- 8.86 % 523 Alicyclobacillus vulcanalis
- 14.74 % 520 Paenibacillus elgii
- 15.99 % 524 Brevibacillus agri
- 16.38 % 536 Bacillus horikoshii
- 16.38 % 535 Bacillus luciferensis
- 16.38 % 522 Paenibacillus chondroitinus
- 16.47 % 523 Brevibacillus reuszeri
- 16.76 % 523 Brevibacillus choshinensis

N. Joining Tree

N Join: 5.643 %



Accugenix Database Search Result

Identification: Alicyclobacillus sp.

Generated by: TB 7/6/06

Reviewed by: AM 7/6/06

QA Review: GR 7/6/06

Confidence Level: Genus

Company: Belmay LTD

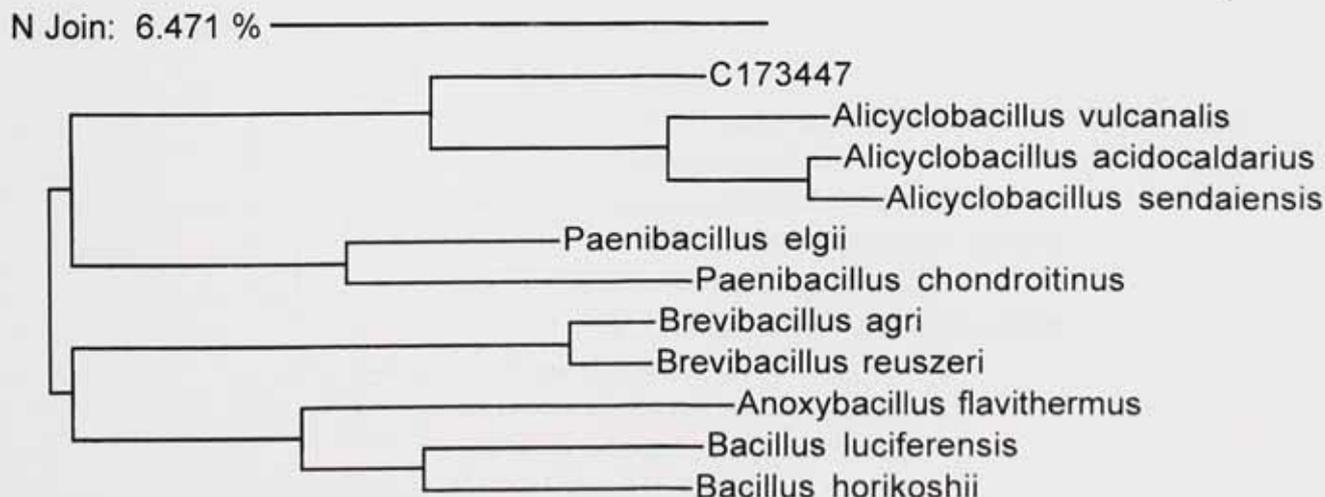
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Date: Thursday, July 6, 2006 2:19 PM

Alignment: 518 C173447

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- 9.27 % 522 Alicyclobacillus sendaiensis
- 15.25 % 520 Paenibacillus elgii
- 16.41 % 524 Brevibacillus agri
- 16.60 % 535 Bacillus luciferensis
- 16.60 % 536 Bacillus horikoshii
- 16.70 % 523 Brevibacillus reuszeri
- 16.80 % 536 Anoxybacillus flavithermus
- 16.80 % 522 Paenibacillus chondroitinus

N. Joining Tree



Accugenix Database Search Result

Identification: Alicyclobacillus sp.

Confidence Level: Genus

Generated by: TB 7/6/06

Reviewed by: JM 7/6/06

QA Review: JM 7/6/06

Company: Belmay LTD

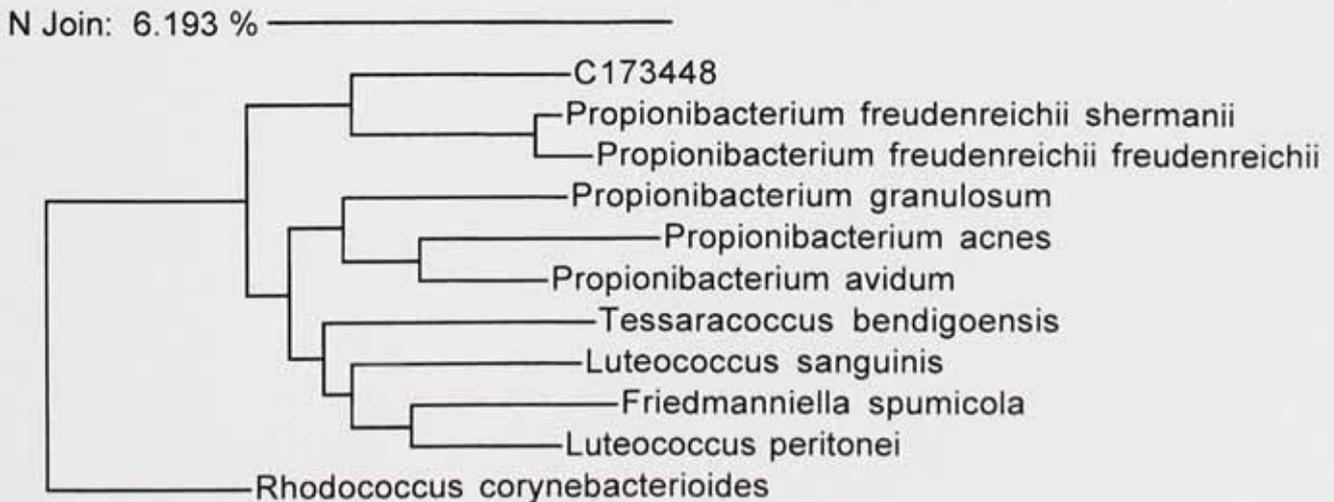
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Date: Friday, July 7, 2006 2:11 PM

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- 7.14 % 505 Propionibacterium freudenreichii freudenreichii
- 8.38 % 501 Tessaracoccus bendigoensis
- 9.72 % 506 Propionibacterium acnes
- 10.34 % 503 Propionibacterium avidum
- 10.56 % 502 Friedmanniella spumicola
- 10.60 % 500 Luteococcus sanguinis
- 11.00 % 500 Luteococcus peritonei
- 11.13 % 503 Propionibacterium granulosum
- 11.20 % 500 Rhodococcus corynebacterioides

N. Joining Tree



Accugenix Database Search Result

Identification: No Match

Confidence Level: No Match

Generated by: JB 7/7/06

Reviewed by: mm 7/7/06

QA Review: Ja 7/17/06

Appendix

3

The growth of *Propionibacterium cyclohexanicum* in fruit juices and its survival following elevated temperature treatments

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Received 19 May 2006; received in revised form 22 August 2006; accepted 23 August 2006
Available online 9 October 2006

Abstract

This study investigated the growth of *Propionibacterium cyclohexanicum* in orange juice over a temperature range from 4 to 40 °C and its ability to multiply in tomato, grapefruit, apple, pineapple and cranberry juices at 30 and 35 °C. Survival after 10 min exposure to 50, 60, 70, 80, 85, 90 and 95 °C in culture medium and in orange juice was also assessed.

In orange juice the organism was able to multiply by 2 logs at temperatures from 4 to 35 °C and survived for up to 52 days. However, at 40 °C viable counts were reduced after 6 days and no viable cells isolated after 17 days. The optimum growth temperature in orange juice over 6 days was 25 °C but over 4 days it was 35 °C.

The growth of *P. cyclohexanicum* was monitored in tomato, grapefruit, cranberry, pineapple and apple juices at 30 and 35 °C over 29 days. Cranberry, grapefruit and apple juice did not support the growth of *P. cyclohexanicum*. At 30 °C no viable cells were detected after 8 days in cranberry juice or after 22 days in grapefruit juice while at 35 °C no viable cells were detected after 5 and 15 days, respectively. However, in apple juice, although a 5 log reduction occurred, viable cells could be detected after 29 days. *P. cyclohexanicum* was able to multiply in both tomato and pineapple juices. In tomato juice, there was a 2 log increase in viable counts after 8 days at 30 °C but no increase at 35 °C, while in pineapple juice there was a 1 log increase in numbers over 29 days with no significant difference between numbers of viable cells present at 30 and 35 °C.

The organism survived at 50 °C for 10 min in culture medium without a significant loss of viability while similar treatment at 60, 70 and 80 °C resulted in approximately a 3–4 log reduction, with no viable cells detected after treatment at 85 or 90 or 95 °C but, when pre-treated at intermediate temperatures before exposure to higher temperatures, some cells survived. However, in orange juice a proportion of cells survived at 95 °C for 10 min without pre-treatment and there was no significant difference between numbers surviving with and without pre-treatment.

The results from this study demonstrate that *P. cyclohexanicum* is able to grow in a number of juices, other than orange juice, and able to survive a number of high temperature procedures. Therefore, if initially present in the raw materials *P. cyclohexanicum* might survive the pasteurization procedures used in the fruit juice industry, contaminate and consequently spoil the final product.

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Keywords: Propionibacteria; *P. cyclohexanicum*; Spoilage; Fruit juices; Temperature; Survival

1. Introduction

During the last two decades several novel spoilage micro-organisms have emerged causing concern within the fruit juice industry. The one receiving most of the scientific and industrial attention, *Alicyclobacillus acidoterrestris*

(Wisotzkey et al., 1992), was first considered to be of academic interest only, but has now become regarded as an industry wide concern (Duong and Jensen, 2000) due to the fact that it survives pasteurization and grows at low pHs commonly found in fruit juices.

In 1993, an acid tolerant, non-spore-forming coryneform bacterium was isolated from spoiled 'off flavour' orange juice. In common with the *Alicyclobacillus* genus, this strain (TA-12^T) had ω -cyclohexyl undecanoic acid which represented 52.7% of the total cellular fatty acid but,

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unlike *A. acidoterrestris*, this organism did not form spores. Growth of the isolate was reported to occur at pH 3.2–7.5, with an optimum range of pH 5.5–6.5 and the heat resistant cells able to withstand a temperature of 90 °C for 10 min (Kusano et al., 1997). The G+C content of this strain was 66.8 mol% and comparative sequence analyses revealed it to be genetically and phenotypically distinct from other propionibacteria and the name *Propionibacterium cyclohexanicum* sp. nov. was proposed (Kusano et al., 1997). This represents, along with *Alicyclobacilli* and *Curtobacterium pusillum*, a third group of organisms with cell membranes that contain ω -cyclohexane fatty acids.

Strains of the genus *Propionibacteria* are classified within the class *Actinobacteria* and are grouped into cutaneous or dairy (classical) propionic acid bacteria. Using a phylogenetic tree constructed from 16S rDNA gene sequences *P. cyclohexanicum* clusters with *P. freudenreichii* but separate from the classical propionibacteria *Propionibacterium theonii*, *Propionibacterium jensenii* and *Propionibacterium acidipropionici* (Meile et al., 1999). *Propionibacteria* ferment sugars and lactic acid to propionic acid, acetic acid and carbon dioxide and are used extensively as propionic acid producers in industry (Ozadali et al., 1996). However, there is increasing evidence of the ability of *Propionibacteria* to act as probiotics in that they produce a number of bacteriocins, produce vitamin B₁₂, stimulate the growth of other beneficial bacteria such as *Bifidiobacteria* (Faye et al., 2000; Grinstead & Barefoot, 1992; Lyon and Glatz, 1993) and have the ability to survive gastric digestion (Huang and Adams, 2004; Leverrier et al., 2003; Leverrier et al., 2005).

P. cyclohexanicum is a Gram-positive, acid tolerant, aero-tolerant, heat resistant, non-motile, pleomorphic, rod shaped bacterium, considered non-pathogenic. The cells are catalase and oxidase negative, 1.5–3.0 μ m in length and 1.1–1.6 μ m in width, with some cells club shaped or bent. It is non-spore forming and no specialized structures have been noted during microscopic examination (Kusano et al., 1997). *P. cyclohexanicum* produces propionic, acetic and lactic acids from glucose, in the ratio of 5:4:2 respectively. This is in comparison with *P. freudenreichii* that ferments glucose to propionic and acetic acids only. *P. propionicus* is the other species of this genus to produce lactic acid.

The aims of this study were to determine the optimal growth temperature of *P. cyclohexanicum* in orange juice, to determine whether the organism was able to grow in other juices and to investigate the survival of *P. cyclohexanicum* after a range of elevated temperature treatments both in culture media and in orange juice.

2. Materials and methods

2.1. Growth conditions and organism

P. cyclohexanicum (NCIMB 13575) was obtained from NCIMB Ltd., Aberdeen, UK. The organism was maintained on peptone yeast extract agar (PYA, pH 5.9),

containing 0.5% (w/v) bacteriological peptone, 0.25% (w/v) yeast extract, 1.5% (w/v) agar, (Oxoid, Basingstoke, UK), supplemented with 0.5% (w/v) glucose (VWR International, Poole, UK), anaerobically using Anaerocult® A (Merck, Darmstadt, Germany) at 35 °C, the optimal temperature suggested by Kusano et al. (1997), sub-culturing at four day intervals. Before inoculation into fruit juices cells were grown in peptone yeast extract broth containing 0.5% (w/v) bacteriological peptone, 0.25% (w/v) yeast extract, 0.5% (w/v) glucose (pH 5.9) and incubated anaerobically at 35 °C for 48 h.

2.2. Determination of temperature growth range of *P. cyclohexanicum* in orange juice

A ready to drink orange juice purchased from a local retail outlet was inoculated with *P. cyclohexanicum* at a final concentration of approximately 5×10^6 cfu/ml. Aliquots were decanted into 250 ml Duran bottles with minimal head space and tightly sealed in order to produce anaerobic conditions. Duplicate bottles were incubated at the following temperatures: 4, 15, 20, 30, 35 and 40 °C. On days 0, 4, 6, 8, 11, 17 and 52 triplicate samples from the bottles were surface plated onto PYA, enriched with 0.5% (w/v) glucose. The plates were incubated anaerobically at 35 °C and counts were carried out after four days. This procedure was carried out on two separate occasions.

2.3. Growth of *P. cyclohexanicum* in fruit juices

Ready to drink pasteurized fruit juices (cranberry, tomato, grapefruit, pineapple, apple and orange) were brought from a local retail outlet and inoculated with *P. cyclohexanicum* at a final concentration of approximately 1×10^7 cfu/ml. Duplicate samples were decanted into 250 ml Duran bottles with minimal head space and tightly sealed in order to produce anaerobic conditions. Gas producers, such as *Propionibacteria*, require some head space in the bottles to allow shaking to mix contents before sampling whilst reducing the possibility of an 'explosive' overflow on opening.

Triplicate samples were taken at various time intervals and surface plated onto peptone, yeast extract agar supplemented with 0.5% (w/v) glucose (PYG agar, pH 5.9). The plates were incubated anaerobically at 35 °C and counts were carried out after four days.

The pH and Brix of the juices were also measured at each sampling time. The pH was measured using a pH meter (Orion model 410A, Denver, Colorado, USA) and the Brix value (expressed in terms of percentage sucrose content) was obtained using direct readings from a refractometer (Bellingham and Stanley RFM80), designed specifically for Brix readings at a temperature of 20 °C where Brix = w/w% soluble solids as sucrose (Batchelor, 1984). The experiment was carried out on two separate occasions.

2.4. Investigation of survival of *P. cyclohexanicum* after heat treatment at 50–95 °C in culture medium or in orange juice

About 500 ml of PYG broth (pH 5.9) or orange juice was inoculated with *P. cyclohexanicum* at 10^8 cfu/ml. After anaerobic incubation for 24 h at 35 °C 10 ml aliquots were pipetted into thin-walled test tubes preheated to 50 °C. A tube containing 10 ml of broth in which was placed a thermometer acted as the temperature control. Once the thermometer in the control tube read the temperature required timing began.

At 1, 5 and 10 min, two tubes were removed and the contents surface plated in triplicate onto PYG and incubated anaerobically at 35 °C for 4 days. The procedure was repeated at 60, 70, 80, 90 and 95 °C.

In a second set of experiments an alternative protocol was adopted. In this case all tubes containing *P. cyclohexanicum* cultures were held at 35 °C for 2 min prior to any heat treatment. For trials at 50, 60 and 70 °C, tubes were placed into a water-bath at 45 °C, until the temperature in the control tube had reached the desired temperature and then for a further 2 min before being heat treated. After removal of the tubes from the test temperature they were placed at 45 °C for 2 min and then

at held at 35 °C. Samples were then surface plated onto PYG agar and incubated anaerobically at 35 °C for 4 days.

For trials at 80 and 85 or 90 and 95 °C the cultures were treated as above but replacing the 45 °C 'holding temperature' with 55 and 60 °C, respectively. Survivors were determined after surface plating onto PYG agar and incubation anaerobically at 35 °C for 4 days.

All experiments were carried out on at least two separate occasions.

2.5. Statistical analysis

All statistical analysis was carried out using SPSS version 11.5 (SPSS Inc. Chicago, IL, USA) using independent *t*-test analysis for parametric data or repeated measures ANOVA for differences between pH and Brix at different temperatures in the various juices tested. Significance was set at $P < 0.05$.

3. Results

In orange juice, *P. cyclohexanicum* was able to survive at all temperatures tested for at least 11 days. By day 6, survival at 40 °C was declining but survival at lower temperatures was sustained until day 17 (Fig. 1). After 52 days no viable cells were detected at 35 °C but at all other temperatures at least 6 log cells/ml remained viable.

P. cyclohexanicum was also able to survive in a number of different juices as well as orange juice. At 30 °C no viable cells could be detected after 8 days in cranberry juice and 22 days in grapefruit juice (Fig. 2a) whereas at 35 °C none could be detected after 5 and 15 days, respectively (Fig. 2b). The organism multiplied in tomato juice by approximately 1 log over 8 days at 30 °C whereas at 35 °C there was no significant increase in numbers. In apple juice viable counts declined over 29 days at 30 or 35 °C with no significant difference between the counts. In pineapple juice there was no significant difference ($P < 0.05$) between numbers of viable cells present at 30 and 35 °C at day 29 and the increase was approximately 1 log overall. Over 15 days in orange juice, the juice from which the organism was first isolated, *P. cyclohexanicum* multiplied to significantly ($P > 0.05$) higher numbers at 30 °C compared with 35 °C

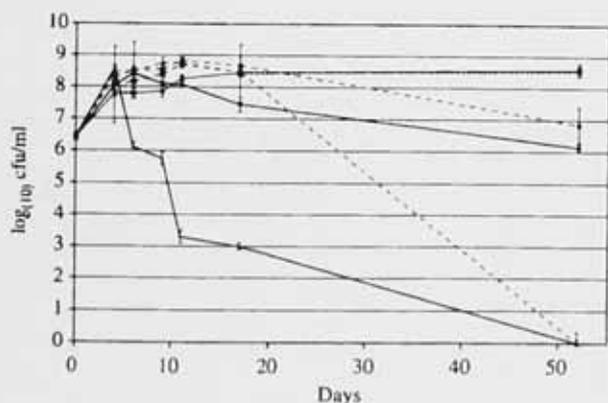


Fig. 1. Growth of *P. cyclohexanicum* in orange juice at different temperatures. Values are means \pm SEM ($n = 6$). —◆— 4 °C; —■— 15 °C; —▲— 20 °C; —×— 25 °C; —●— 30 °C; —▲— 35 °C; — 40 °C.

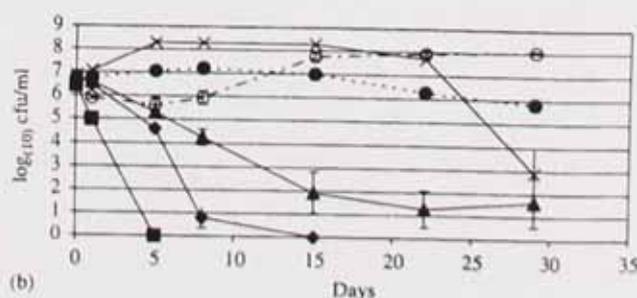
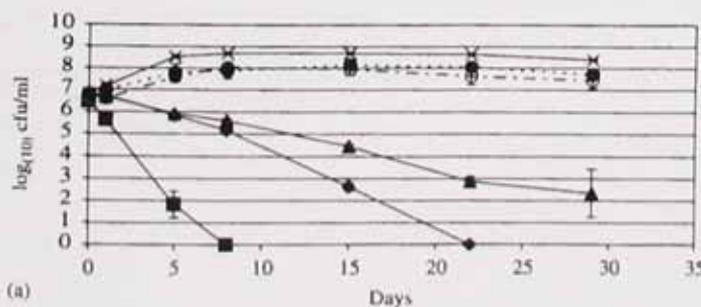


Fig. 2. Survival of *P. cyclohexanicum* in various juices (a) at 30 °C, (b) at 35 °C. Values are means \pm SEM ($n = 6$). (—◆— grapefruit; —■— cranberry; —●— tomato; —×— orange; —○— pineapple; —▲— apple).

increasing by 2 log compared with 1.5 log (Fig. 2) and this trend continued at 29 days. Table 1 shows the changes in Brix values and pH over the incubation times. The Brix values all reduced over 29 days, although this was not significant, except in the case of orange juice ($P < 0.05$), while pH did not significantly change over the same time. Although there is significant differences between the initial pH and Brix of the juices tested ($F = 51$, $P = 0.00$), for each juice there are no significant differences between the initial pH or Brix and those at 29 days at either 30 or 35 °C or between the different temperatures ($F = 1$, $P = 0.337$), with the only exception being Brix in the case of orange juice.

A proportion of the population was able to survive when subjected to heat treatment for 10 min at various

Table 1
Brix and pH values of fruit juices inoculated with *P. cyclohexanicum*

Juice	pH at day 0	pH at day 29		Brix at day 0	Brix at day 29	
		30°	35 °C		30°	35 °C
Grapefruit	3.3	3.3	3.3	10.6	10.3	10.3
Cranberry	2.7	2.8	2.8	11.9	11.5	11.5
Tomato	4.1	3.9	4.0	5.7	5.2	5.4
Orange	3.9	3.9	3.8	11.1	9.9	10.2
Pineapple	3.6	3.7	3.7	12.5	12.0	11.7
Apple	3.5	3.5	3.5	11.0	10.6	10.3

temperatures, with this survival dependant on the time-temperature combination. Heat treatment in culture medium at 50 °C for 10 min had very little effect on viable counts. However, there was a ~3–4 log reduction after 10 min at 60, 70 or 80 °C, with no statistically significant difference ($P > 0.05$) between the responses. Following an initial decline in survival at 1 min, there was no further statistically significant reduction in survivor numbers after 5 or 10 min (Fig. 3a). No survivors were detected after treatment at 90 or 95 °C, even after 1 min (results not shown).

When *P. cyclohexanicum* was treated to the incremental heat treatment in culture medium there was no significant difference in numbers of survivors at 50, 60, 70 or 80 °C compared with the non-incremental regime but after treatment at 85, 90 and 95 °C for 10 min some cells remained viable (Fig. 3b) with no significant difference between the effect of these three temperature treatments ($P > 0.05$).

In orange juice (Fig. 4) some cells survive after both treatment regimes at 95 °C for 10 min with approximately 1 log viable counts detected. Generally there was no significant difference between the log reductions demonstrated in the non-incremental treatment in orange juice compared with the incremental treatment regime at any of the temperatures tested. There was also no significant difference in the effect of heat treatments in culture medium or orange juice at 50–80 °C, although at 85, 90

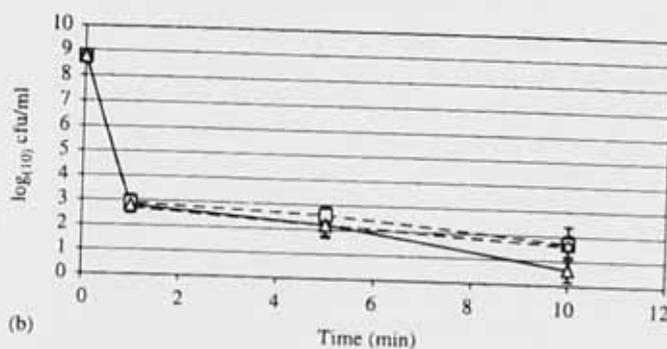
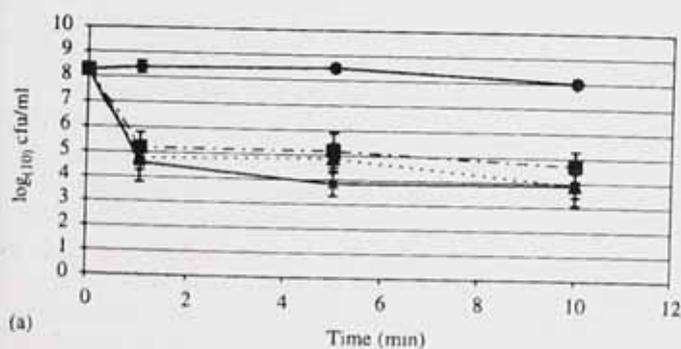


Fig. 3. Survival of *P. cyclohexanicum* exposed to heat treatment for 10 min in PYG broth (pH 5.9) (a) non-incremental treatment, (b) incremental treatment. Values are means \pm SEM ($n = 6$). —◆— 50 °C; —■— 60 °C; —▲— 70 °C; —×— 80 °C; —— 85 °C; —○— 90 °C; —△— 95 °C.

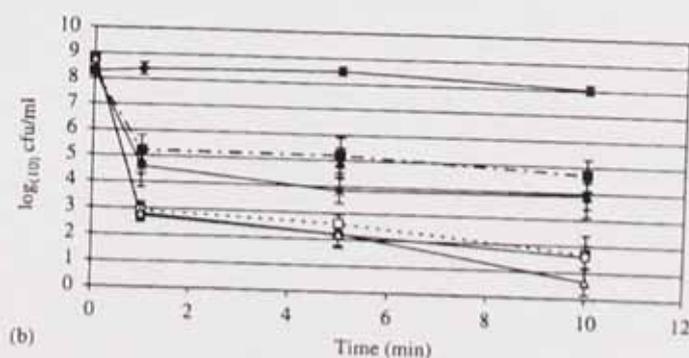
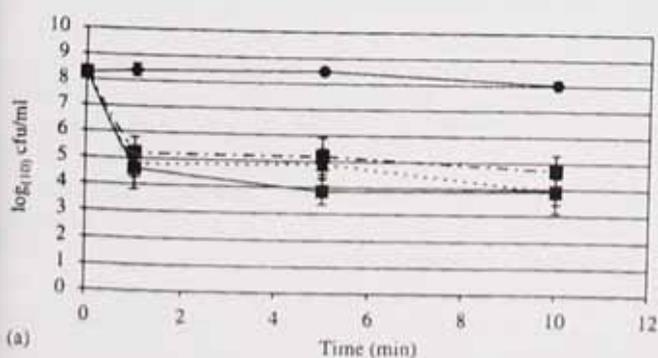


Fig. 4. Survival of *P. cyclohexanicum* exposed to heat treatment for 10 min in orange juice (a) non-incremental treatment, (b) incremental treatment. Values are means \pm SEM ($n = 6$). —◆— 50 °C; —■— 60 °C; —▲— 70 °C; —×— 80 °C; —— 85 °C; —○— 90 °C; —△— 95 °C.

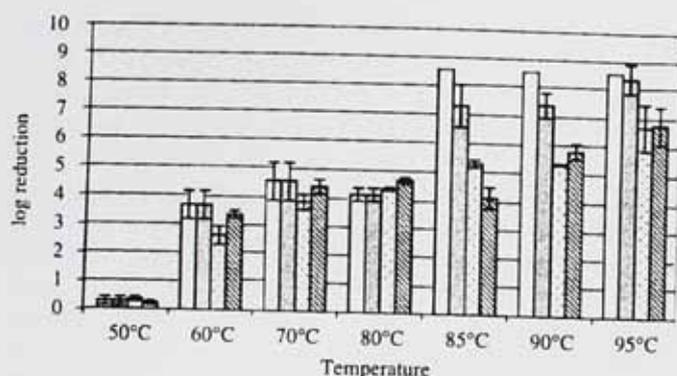


Fig. 5. Log reductions in viable counts of *P. cyclohexanicum* after 10 min exposure to various temperatures regimes. [] non-incremental treatment in PYG broth (pH5.9), [■] incremental treatment in PYG broth (pH5.9), [▨] non-incremental treatment in orange juice, [▩] incremental treatment in orange juice.

and 95 °C there was a significantly greater ($P > 0.05$) log reduction in culture medium compared with orange juice (Fig. 5).

4. Discussion

The results of this study confirm the fact that *P. cyclohexanicum* is a very heat resistant bacterium, surviving 10 min exposure at 95 °C in orange juice which corresponds to the original description of the species in which it was reported as surviving 10 min at 90 °C (Kusano et al., 1997). This makes it the most heat resistant of the propionibacteria, surviving higher temperatures than *P. freudenreichii*, previously considered the most heat resistant of this group of bacteria. Since no spores have been reported in *P. cyclohexanicum* heat resistance is probably due to another mechanism rather than spore formation.

Changes in environmental conditions including those of temperature or pH are potential stressors to organisms. It is well-established that a sub-lethal stress can induce an adaptive tolerance response (ATR) in bacteria and may provide protection towards subsequent exposure to lethal stress. An ATR mechanism has been identified in a number of bacteria such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* spp. and survival studies of *E. coli* and *Salmonella* spp. have shown that they have a range of interlinked genetic regulatory networks that allow adaptation of cells to oxidative, osmotic, acid and thermal stress (Bearson et al., 1998; Brudzinski and Harrison, 1998; Foster, 1995; Garren et al., 1998; Lee et al., 2002; O'Driscoll et al., 1996).

An ATR involving the synthesis of de novo polypeptides such as the universal chaperonins GroEL and GroES, has been demonstrated in *P. freudenreichii* in response to acid or bile salts (Jan et al., 2000, 2001; Leverrier et al., 2003) and also in response to heat (Anastasiou et al., 2006). In the latter case both constitutive thermotolerance and adaptive thermal thermotolerance were evidenced in natural isolates from cheese and two separate but

overlapping mechanisms were suggested. Thermotolerance involving molecular chaperones, antioxidant thiol compounds and molecular actors of the stringent response and thermal adaptation involving signal transduction, biosynthetic pathways and cell wall maintenance in addition to protein turn-over.

The fact that *P. cyclohexanicum* survives for 10 min with non-incremental heat treatment at 95 °C in orange juice suggests that the induction of an ATR is not the mechanism. If this were the case then cells would be more likely to survive after an intermediate heat treatment although the intermediate treatment used in this study (approximately 5 min) is relatively short for an adaptive response to take place (Anastasiou et al., 2006). The cell membrane of *P. cyclohexanicum* has ω -cyclohexyl undecanoic acid as its major fatty acid and, as in the case of *A. acidoterrestris*, this probably protects the cell from high temperatures and high acidity (Pontius et al., 1998), although *A. acidoterrestris* also produces spores. A mechanism of thermotolerance would seem to be the most likely explanation for the heat resistance of *P. cyclohexanicum* and further investigations are required in order to elucidate the molecular actors involved as with *P. freudenreichii* (Anastasiou et al., 2006).

P. cyclohexanicum was able to survive and multiply in orange juice at low temperatures such as 4 and 15 °C commonly considered to reduce growth of food spoilage bacteria such as *A. acidoterrestris* (Walker and Phillips, 2005), suggesting that if the organism is present after pasteurization, either because it survived the pasteurization process or due to post-pasteurization contamination, it will be able to survive for up to 52 days. A common heat treatment for fruit juices in an industrial situation is 90–95 °C for 30 s to 1 min. The fact that our results demonstrate the ability of *P. cyclohexanicum* to survive 10 min at 95 °C suggest that the original isolation was as likely to be due to pre-heat-treatment contamination as post-processing contamination. Although there is no significant difference at 50, 60, 70 and 80 °C between the log reductions in PYG broth and orange juice the log reductions are less in orange juice at the higher temperatures tested. This could be due to a number of factors intrinsic to the juice, including pH, and reflects the fact that orange juice, the juice from which the organism was first isolated, provides a more suitable growth environment for recovery after heat treatment at these higher temperatures.

Kusano et al. (1997) reported the optimum growth temperature in culture medium as 35 °C. In orange juice (Fig. 1) over four days growth is fastest at 35 °C but over longer time periods the optimum temperature appears to be 25 °C. In the same study the pH growth range was suggested as 3.2–7.5. However, the results of this present study demonstrates that in cranberry juice (pH 2.7) a proportion of the *P. cyclohexanicum* population is able to survive for five days at 30 °C and at least one day at 35 °C.

Although the organism was first isolated from orange juice the results of this study demonstrate that it is able to

survive and, in some cases, multiply in a number of other juices with pHs varying from 2.7 (grapefruit) to 4.0 (tomato) and Brix values from 5.7° (tomato) to 12.5° (pineapple), with the length of time of survival varying. In cranberry (pH 2.7; Brix 11.9), grapefruit (pH 3.3; Brix 10.6) and apple (pH 3.5; Brix 11.0) juices, although the organism survives, viable counts decrease while in tomato (pH 4.1; Brix 5.2), orange (pH 3.9; Brix 9.9) and pineapple (pH 3.6; Brix 12.0) juices, the organism multiplies. The results suggest therefore that Brix values do not have as much influence as pH on the growth of *P. cyclohexanicum* since it is able to grow in juices with Brix values of 5.2 (tomato), 9.9 (orange) and 12.0 (pineapple) while it does not multiply in apple juice (pH 3.5) but does in pineapple juice (pH 3.6) with similar initial Brix values, suggesting the lowest pH sustaining growth is 3.6.

The fact that a proportion of *P. cyclohexanicum* cells survived a 10 min treatment at 95 °C in orange juice suggests that the organism would survive treatments commonly used in the pasteurization process of fruit juices (88–96 °C for approximately 2 min) and this has implications for the fruit juice industry. Also, because it is able not only to survive but, at some temperatures and in some juices, multiply during storage, if initially present it could become a source of spoilage. Most storage regimes suggest a low temperature and our own previous studies (Walker and Phillips, 2005) have indicated that juices and juice products should be kept at low temperatures to prevent bacterial growth and spoilage by other organisms such as *A. acidoterrestris*. However, in the case of *P. cyclohexanicum* this would probably not be sufficient to prevent spoilage, which may result in a variety of undesirable reactions within the product and ultimately prove very costly to the manufacturer (Jensen, 1999).

In terms of heat resistance, *P. freudenreichii* has been generally considered the most heat resistant of the propionibacteria, surviving 62.8 °C for 30 min, 80 °C for 10 min, but not 90 °C for 10 min (Kusano et al., 1997). In the case of the original isolation of *P. cyclohexanicum* from pasteurized orange juice, there are two possibilities. Firstly, that it survived the pasteurization process and secondly, that contamination was post-pasteurization. If *P. cyclohexanicum*, as suggested by the results of this study, has the ability to survive the acidic environment of fruit juices and the capacity to tolerate temperatures such as those used in the pasteurization process applied to fruit juices *P. cyclohexanicum* could prove to be a significant challenge to the fruit juice industry.

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Short communication

The effect of intermittent shaking, headspace and temperature on the growth of *Alicyclobacillus acidoterrestris* in stored apple juice

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(Received 1 January 2004; Accepted in revised form 7 April 2004)

Summary The presence of *Alicyclobacillus acidoterrestris* in stored juices can be difficult to detect. In this study the effects of storage temperature, headspace and agitation of juice containers was investigated. The results indicate that the amount of headspace has a significant effect on growth of vegetative cells and spores of *A. acidoterrestris* at 35 °C. Intermittent shaking before sampling increased growth and therefore probable detection rates at 30 °C. Agitating containers and sampling from several areas within containers is therefore recommended for determining whether *A. acidoterrestris* is present or absent from stored juice, especially in large containers.

Keywords Aerobic, spoilage, storage.

Introduction

Spoilage of fruit juices and soft drinks by bacterial contamination may result in a variety of undesirable reactions within a product. In the competitive world of soft drinks manufacturing, taste, odour and appearance are of prime importance. Spoilage, which can occur at any point during the processing, may alter any one or all of these organoleptic properties, rendering the product unusable.

Pasteurization in the temperature range of 85–95 °C should inactivate all nonspore-forming spoilage micro-organisms, with any remaining bacterial spores unable to germinate because of the acidic (pH < 4.6) nature of fruit juices (Splittstoesser *et al.*, 1994; Walls & Chuyate, 1998; Silva *et al.*, 1999). However, during the last two decades several novel deleterious micro-organisms have emerged and spoilage of acidified vegetables, fruits and wines by *Alicyclobacillus acidoterrestris* has occurred (Splittstoesser *et al.*, 1994). *Alicyclo-*

bacillus acidoterrestris is a Gram-positive, acidophilic, thermophilic, spore-forming bacterium (Wisotzkey *et al.*, 1992) that can survive the pasteurization process used in fruit juice processing and packaging. This is of particular significance to the retail outlet juice industry that uses 100% pasteurized juice and no preservatives.

Spoilage by *A. acidoterrestris* is difficult to detect. It causes some clarified fruit juices to have a light sediment, cloudiness or haze. However, the main spoilage characteristic is the 'medicinal' or 'phenolic' off-flavour or odour (Walls & Chuyate, 1998), caused by guaiacol (Yamazaki *et al.*, 1996), 2,6-dibromophenol (Borlinghaus & Engel, 1997) and 2,6-dichlorophenol (Jensen & Whitfield, 2003).

Since 1982, when a large-scale spoilage of aseptically packed apple juice occurred in Germany, the fruit juice industry has recognized the potential of *A. acidoterrestris* to cause occasional contamination. However, more recently, spoilage incidents concerning alicyclobacilli have increased and more diverse types of products, for example shelf-stable iced tea, have become contaminated. *Alicyclobacillus acidoterrestris* is more widespread

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doi:10.1111/j.1365-2621.2005.00960.x

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at the present time than originally observed and is becoming an industry-wide problem (Duong & Jensen, 2000).

During previous trials in our laboratory testing the effectiveness of preservatives against *A. acidoterrestris*, growth of high numbers of vegetative cells were observed in unpreserved control samples. The amount of headspace available, agitation of the container and whether sampling occurred from nearer the top or bottom of the juice were all considered as possible contributing factors and this study reports on the effects of these on the growth and hence the subsequent detection of *A. acidoterrestris* in apple juice.

Materials and methods

Agar and agar components used in this study were obtained from Merck KGaA 64271 Darmstadt, Germany or Oxoid, Ltd, Basingstoke, UK. A culture of *A. acidoterrestris* was donated by Aplin and Barrett, Beaminster, Dorset, UK.

Preparation of *A. acidoterrestris* cultures

Cultures of *A. acidoterrestris* were stored on slopes of potato dextrose agar. When required, colonies were streaked onto orange serum agar (OSA) enriched with 0.5% sucrose and plates incubated at 44 °C for 5 days. After this two colonies of approximately 2 mm diameter were aseptically picked from the surface of the plate, suspended in acidified peptone water (0.1% peptone, 0.5% sucrose, adjusted to pH 4 with sulphuric acid) and incubated at 44 °C for approximately 48 h (Jensen, 1999). Vegetative cell counts and spore counts were done before this suspension was used to inoculate the juice samples.

Determination of vegetative cell and spore counts

To determine the number of vegetative cells, samples were serially diluted with acidified peptone water. Duplicate samples, at a minimum of two dilutions, were surface plated onto OSA enriched with 0.5% sucrose.

In order to determine the number of spores present, the culture or sample was heat shocked for 10 min as follows: test tubes, each containing

an aliquot of the culture or sample, were lowered into a water bath at 80 °C. A thermometer was placed into a test tube containing a similar amount of apple juice and 10 min timed from when the temperature reached 80 °C in this tube. The tubes were then removed from the water bath, cooled to 30 °C and the contents surface plated in duplicate onto enriched OSA as before. All plates were incubated at 44 °C for 5 days.

Inoculation of the apple juice

A clear ready-to-drink apple juice was decanted into sterile 1-L Duran bottles and inoculated with *A. acidoterrestris*, prepared as described in the previous section, at the final concentrations of approximately 3×10^1 vegetative cells per mL and 7.8×10^1 spores per mL (35 °C) or approximately 10^2 vegetative cells per mL and 10^3 spores per mL (30 °C). The bottles were shaken and incubated at 44 °C for 4 h to allow dispersal of the inoculum within the apple juice. The inoculated juice was then dispensed into sterile 250-mL glass bottles with headspaces of 0, 25, 50 and 75% and stored at 35 or 30 °C for the 11-day trials, with sampling occurring on days 4, 7, 9 and 11.

On each sampling day, duplicate bottles were removed from the incubator. One was shaken by upending twenty times and sampled from the middle of the bottle. The other was handled carefully to minimize mixing of the contents and sampled first from the top and then from the bottom of the bottle. Each sample was serially diluted in acidified 0.1% peptone water containing 0.5% sucrose and surface plated in duplicate, using at least three separate dilutions, on to enriched OSA as before. The remainder of each sample was heat shocked as described above and also surface plated onto enriched OSA. All plates were incubated at 44 °C for 5 days before enumeration. Each experiment was repeated three times.

Statistical tests

Statistical tests were done by using SPSS version 10 (SPSS Inc., Chicago, IL, USA) using an independent *t*-test analysis. The results are shown as mean \pm SEM. Significance was set at $P < 0.05$.

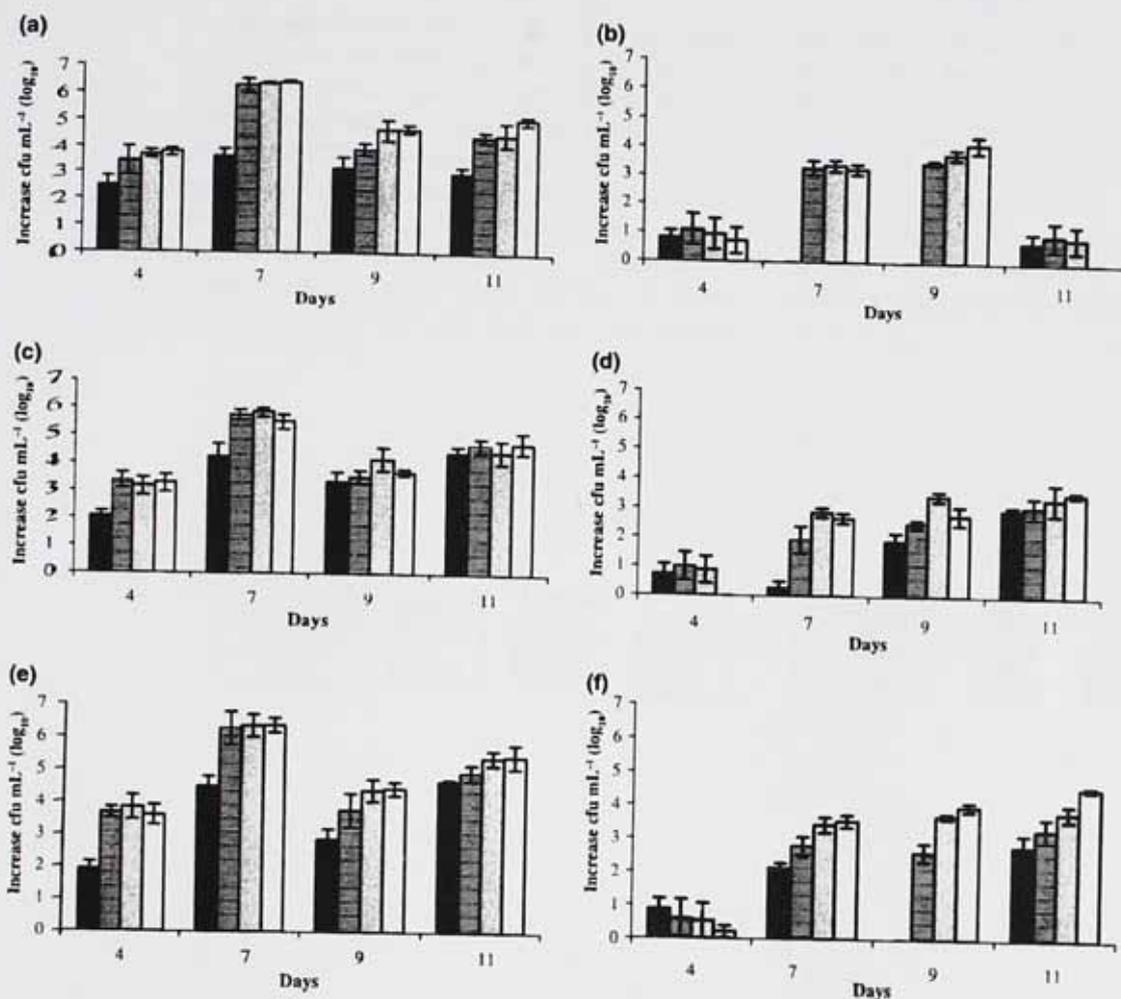


Figure 1 Growth of *Alicyclobacillus acidoterrestris* in apple juice at 35 °C (mean ± SE). (a) Un-heat and (b) heat shocked shaken sample; (c) un-heat and (d) heat shocked unshaken top sample; (e) un-heat and (f) heat shocked unshaken bottom sample. ■, 0% Headspace; □, 25% headspace; ▨, 50% headspace; ▩, 75% headspace.

Results

All results are shown as increase in growth compared with initial inoculum. At 35 °C, vegetative cell growth (Fig. 1a, c and e) generally showed a significantly lower level of growth in 0% headspace samples compared with 25, 50 and 75% headspace ($P > 0.05$). This was particularly the case in the shaken samples (Fig. 1a) compared with the unshaken samples (Fig. 1c and d). The rate of isolation and the numbers of *A. acidoterrestris* isolated from the bottles with 25, 50 and 75% headspace were not significantly different from each other.

At day 11 there was no difference in vegetative cell count in any of the headspace bottles in the

unshaken top sample or unshaken bottom sample. Therefore, by day 11 headspace seems to have little effect on growth in the unshaken bottles (Fig. 1c and e) but continues to have an effect in the shaken bottles (Fig. 1a) where 0% headspace produces lower growth and hence lower potential detection rate. There are no significant differences between the isolation rate from the top or bottom unshaken samples at any of the headspace conditions and it seems immaterial whether the sample is taken from top or bottom of the unshaken bottles.

Spore counts (Fig. 1b, d and f) were significantly lower than the vegetative cell counts at all times and with all headspaces. As with vegetative cell growth there was no significant difference

between sporulation and hence isolation rates from 25, 50 or 75% headspace bottles. 0% Headspace bottles again showed the lowest sporulation rates, with no spores being detected on days 7 and 9 in the shaken samples (Fig. 1b). On day 4 there was no significant difference in spore count between any headspace under any of the three conditions. However, on day 11 spore counts in the shaken samples were significantly lower than in either the unshaken top or bottom

samples, with the unshaken bottom samples giving the highest spore counts, suggesting that *A. acidoterrestris* spores tend to settle at the bottom of containers during storage.

At 30 °C (Fig. 1) vegetative cell growth in all bottles was lower than at 35 °C (Fig. 2). The shaken samples showed higher growth than the unshaken at all times and all conditions, although this was not always statistically significant. Sporulation was also lower compared with that at

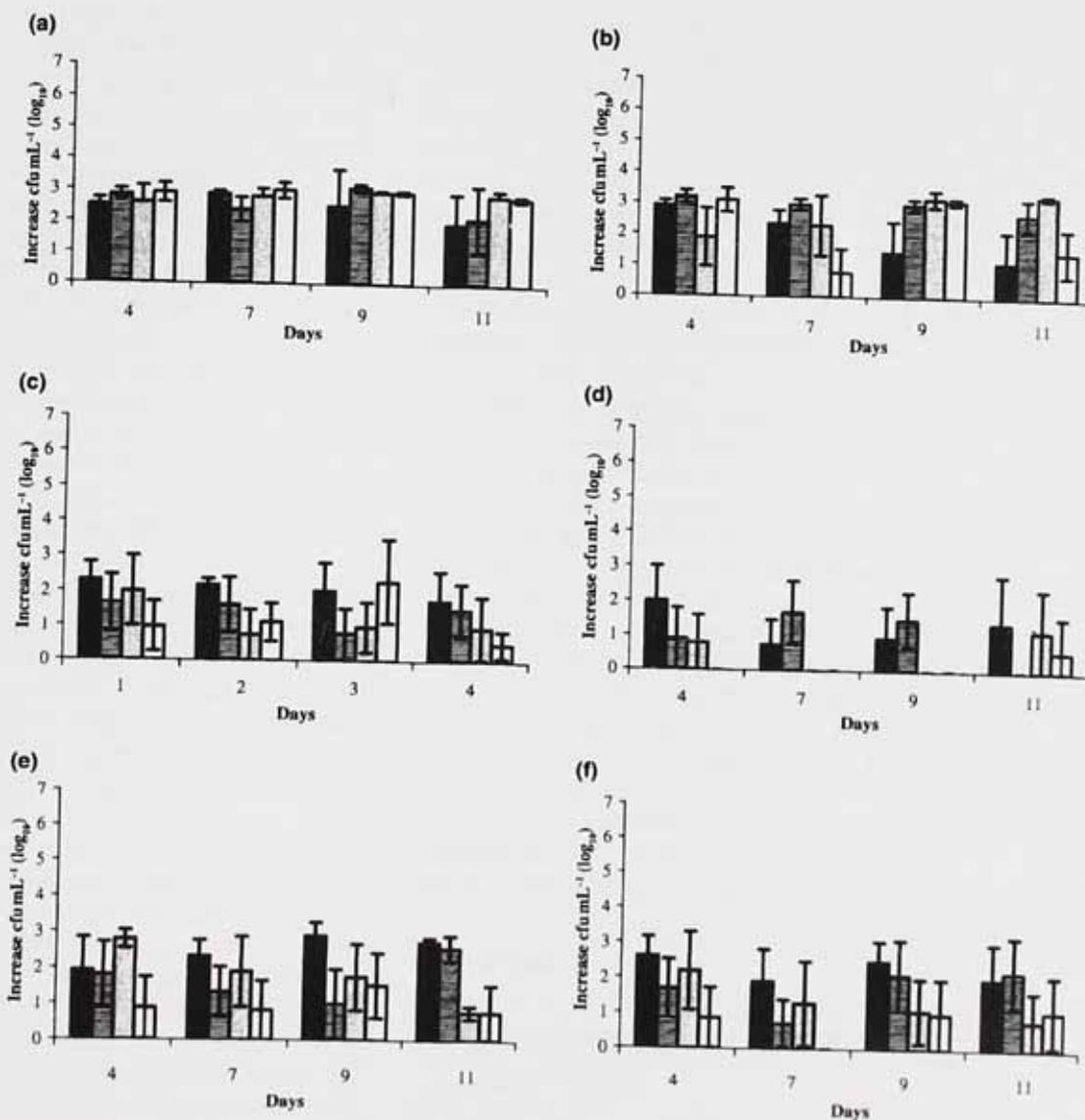


Figure 2 Growth of *Alicyclobacillus acidoterrestris* in apple juice at 30 °C (mean \pm SE). (a) Un-heat and (b) heat shocked shaken sample; (c) un-heat and (d) heat shocked unshaken top sample; (e) un-heat and (f) heat shocked unshaken bottom sample. ■, 0% Headspace; □, 25% headspace; ▒, 50% headspace; ◻, 75% headspace.

35 °C, except at day 4 when it was higher, especially in the 0% headspace bottles (Fig. 2b, d and f). This suggests that, in conditions of little or no available oxygen, *A. acidoterrestris* grows more slowly at 30 °C than at 35 °C after 4 days incubation.

Discussion

The results of this study suggest that adequate agitation of fruit juice containers before sampling especially at suboptimal growth temperatures (i.e. 30 °C) may be important. If the sample is shaken before sampling detection of growth of *A. acidoterrestris* is generally higher (Fig. 2a) than if the container is unshaken and sampled from either top or bottom (Fig. 2c and e). It is also important for detection of spores at 35 °C in containers with available oxygen. Although shaking a small or medium container is fairly easy, agitating a large barrel or intermediate bulk container over 1000 kg in weight is much more difficult.

The amount of moving and shaking a large container of juice is subjected to during transport between processing and manufacture of the final product would allow headspace oxygen to be incorporated into the juice. Temperature fluctuations during transport may also affect outgrowth of spores (Orr *et al.*, 2000).

The results of this study suggest that storage temperature, headspace and adequate agitation of fruit juice containers play an important part in the growth and detection of *A. acidoterrestris* in apple juice. Growth rates in partly filled containers are higher than in full containers at temperatures at, and in excess of, 35 °C emphasizing the aerobic nature of *A. acidoterrestris*.

Similar trials were done at storage temperatures of 27 and 44 °C (data not shown). In the former case very low numbers of viable vegetative cells or spores were detected in the majority of the samples, confirming previous studies suggesting *A. acidoterrestris* only grows slowly at suboptimal temperatures (Jensen, 1999). When samples were stored at 44 °C, that is within the optimal reported temperature range of 42–53 °C for *A. acidoterrestris* growth (Wisotzkey *et al.*, 1992), the growth of vegetative cells was lower in bottles with 0% headspace on all days (heat shock was not used in

this trial), again emphasizing the aerobic nature of *A. acidoterrestris*.

In this study, it has been shown that the amount of headspace does make a significant difference in growth of vegetative cells and sporulation of *A. acidoterrestris* and hence detection rates when apple juice is stored at 35 °C. Shaking the bottles before sampling did not make a significant difference to the growth of vegetative cells although as storage time increases spore counts decreased in shaken bottles compared with unshaken and samples from the bottom tended to give higher counts.

However, when apple juice is stored under temperatures that are suboptimal for growth of *A. acidoterrestris* but represent a hot summer's day in the UK, or an average temperature in many countries (30 °C in this study), intermittent shaking i.e. before each sampling, increases growth and hence probable detection of the presence of *A. acidoterrestris*. This might be the case if the original numbers of *A. acidoterrestris* contaminating the juice are low. In this study, the detection of growth of both vegetative cells and spores is more erratic at 30 °C than at 35 °C, especially in the unshaken bottles, also emphasizing that, at suboptimal temperatures, in order to detect *A. acidoterrestris* care must be taken to sample from more than one area of the container.

Therefore the results of this study suggest that juices and juice products should be kept at low temperatures, containers must wherever possible be shaken before sampling and various sampling areas should be tested within each container to ensure detection of *A. acidoterrestris*. It is also important to treat samples by heat shocking to detect the presence of spores as well as direct plating to detect vegetative cells. Filling the empty headspace of containers with an inert gas such as nitrogen may slow the growth of *A. acidoterrestris*; further investigations are required in this area.

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Appendix

4

Publications and presentations:

Walker, M. and Phillips, C.A. (2002). The effectiveness of different preservative regimes on *Alicyclobacillus acidoterrestris* in ready to drink fruit juices. *Frontiers in Microbial Fermentation and Preservation Conference*, Wageningen, The Netherlands.

Walker, M. and Phillips, C.A. (2004). The effect of temperature on the growth and survival of *Propionibacterium cyclohexanicum*, a novel contaminant of orange juice. *Dairy and Food Microbiology: challenges and opportunities*. Society for Applied Microbiology conference, Cork, Ireland.

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Walker, M and Phillips, C.A. (2006). The growth of *Propionibacterium cyclohexanicum* in fruit juices and its survival following elevated temperature treatments. *Food Microbiology*, **24**, 313-318.

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