International Journal of Food Microbiology 311 (2019) 108314

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Diversity and guaiacol production of *Alicyclobacillus* spp. from fruit juice and fruit-based beverages

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ARTICLE INFO

Keywords:
Bacterial spores
Spoilage microorganisms
Acidic food stability
Guaiacol
RAPD-PCR

ABSTRACT

Alicyclobacillus acidoterrestris is an acido-thermophilic, spore-forming bacterial species that can spoil acidic fruit juice and fruit-based beverages. The metabolism of taint compounds by this bacterial species has led to its status as a targeted microorganism in the fruit juice industry. This study aims to assess the genetic diversity of *Alicyclobacillus* spp. including *A. acidoterrestris* and its correlation to spoilage taint metabolism.

Alicyclobacillus cultures, which were previously isolated from a wide range of domestic and international products including fruit juice, fruit drinks and fruit juice concentrates, were subjected to DNA fingerprint analysis by using randomly amplified polymorphic DNA (RAPD) – polymerase chain reaction. Isolates were classified on the basis of their RAPD profile and the results were used to select representative strains to undergo taint production assessment. The taint guaiacol produced by *Alicyclobacillus* spp. was measured by headspace gas chromatography and mass spectrometry.

From produced RAPD profiles, two genotypic groups and two sub-groups were identified. The groups were independent of product types and geographical origins. A significant number of isolates were clustered in genotypic group I, including *A. acidoterrestris* ATCC 49025. These isolates produced significant levels of guaiacol, 8.7 mg/L on average. A smaller number of isolates was found in genotypic group II including *A. acidocaldarius* and they produced no guaiacol. Primer F-64 was useful to identify *Alicyclobacillus* at the species level, and permitted rapid identification of strains producing fruit juice taint compounds such as guaiacol.

1. Introduction

The genus *Alicyclobacillus* consists of a group of aerobic, thermo-acidophilic and spore-forming bacteria ubiquitous in soil and food environments. Control of *Alicyclobacillus* spp. presents a significant challenge for manufacturers of fruit juices, fruit juice concentrate and other fruit based beverages. Spores may survive pasteurisation processes and vegetative cells can survive and tolerate conditions employed as treatments to maintain microbial safety and organoleptic quality of juices. Under favourable conditions surviving spores can germinate and grow in the acidic conditions of fruit juice, with subsequent production of metabolites resulting in taint flavours and aromas. Of particular note, certain species of *Alicyclobacillus* produce guaiacol (2-methoxyphenol), a taint compound causing medicinal-like flavour in juice products (Jensen, 1999).

Although not associated with foodborne infections or toxaemia the widespread contamination of *Alicyclobacillus* species in fruit juices and fruit juice concentrates represents an economic burden to the global fruit juice beverage industry (Chang and Kang, 2004; Smit et al., 2011). Contamination by spores of *Alicyclobacillus* spp. has been reported across every continent in orchard soil, fruit processing environments including fruit washing water and fruit based beverages (Danyluk et al., 2011; Durak et al., 2010; Félix-Valenzuela et al., 2015; Jensen, 2000; Osopale et al., 2016; Witthuhn et al., 2013).

The two *Alicyclobacillus* species most widely associated with contamination of juice products are *A. acidoterrestris* and *A. acidocaldarius* (Danyluk et al., 2011; Durak et al., 2010), however other species have also been associated with contaminated products. The genetic diversity of *Alicyclobacillus* spp. across differing geographical and ecological niches has been highlighted in previous surveys. For example, two

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https://doi.org/10.1016/j.ijfoodmicro.2019.108314
Received 6 June 2019; Received in revised form 30 July 2019; Accepted 23 August 2019
Available online 24 August 2019
0168-1605/

genotypic groups were found in 16 *A. acidoterrestris* strains isolated from fruit juice products in West Africa (Osopale et al., 2016) while three phylogenetic groups were reported from 36 isolates of *A. acidoterrestris* from the soil of several fruit orchards in Japan (Goto et al., 2008). Similar diversity was observed in European studies, where Dekowska et al. (2018) identified two major genotypes of *A. acidoterrestris* spoilage isolates using RFLP-PCR, while Bevilacqua et al. (2015) identified three grouping of *A. acidoterrestris* isolated from orchard soils and spoiled pear juice in Italy. The genotypic diversity of *A. acidocaldarius* strains was also observed for 19 isolates which were collected from orchard soil samples in Mexico (Félix-Valenzuela et al., 2015).

The presence of guaiacol producing *A. acidoterrestris* is considered an adulterant in fruit concentrates and is often used as a target microorganism in fruit concentrate thermal processes. Due to its significance this species has received attention in the research field (Osopale et al., 2016) more so than other *Alicyclobacillus* species. Methods have been developed to quickly and effectively detect and identify this spoilage bacterium and its production of taint. For example, a randomly amplified polymorphic DNA (RAPD) analysis method has been developed to rapidly identify *A. acidoterrestris* (Yamazaki et al., 1997) while a sensitive analytical method to detect taint compounds by headspace gas chromatography–mass spectrometry (HS GC–MS) has also been reported (Zierler et al., 2004).

Variation in guaiacol metabolism has been observed at both strain (Goto et al., 2008) and species level (Félix-Valenzuela et al., 2015; Groenewald et al., 2009). For instance, significant variation of guaiacol production was observed among *A. acidoterrestris* strains isolated from various commercial fruit crop soils in Japan (Goto et al., 2008). The closely related *A. acidocaldarius*, however is typically considered a non-guaiacol-producing species (Félix-Valenzuela et al., 2015; Witthuhn et al., 2013; Yokota et al., 2007). Despite the reported inability to produce guaiacol, the frequent occurrence of *A. acidocaldarius* in contaminated fruit juice and concentrates has suggested a potential role of this species in incidences of spoilage. On the other hand, *A. cycloheptanicus* has been reported to be capable of producing a range of taint compounds including guaiacol, 2,6-dichlorophenol (DCP) and 2,6-dibromophenol (DBP) (Gocmen et al., 2005). However, detection and identification of this species is difficult as it generally requires cultivation of samples in complex growth media due to its fastidious demand for yeast extract (Deinhard et al., 1987; Jensen, 1999; Yokota et al., 2007). Consequently, *A. cycloheptanicus* has received less attention than *A. acidoterrestris* or *A. acidocaldarius*.

A lack of information on both the genetic diversity of *Alicyclobacillus* species exists and the relationship between genotype and phenotype of *Alicyclobacillus*. This study aims to define the genetic diversity of a broad range of *Alicyclobacillus* spp. isolated from various fruit-based beverages produced domestically in Australia and internationally. In addition, guaiacol production of these isolates will be conducted to investigate the phenotypic and genotypic relationship in order to provide further insight into the spoilage characteristic of the microorganism.

2. Materials and methods

2.1. RAPD-PCR

2.1.1. Isolates

Two hundred and fifteen *Alicyclobacillus* isolates from the Commonwealth Scientific and Industrial Research Organisation (CSIRO), North Ryde, New South Wales, Australia *Alicyclobacillus* culture collection were assessed. The majority of isolates were recovered from a survey of the status of *Alicyclobacillus* in fruit juices and concentrates manufactured and in retail in Australia plus isolates received from testing laboratories (Table 1). Isolates were recovered between 1990 and 2016 from range of apple juice and juice concentrates, orange juice and juice concentrates, and other fruit-based products including banana puree, pear juice, apple and pear juice concentrate, grape juice, apple and cranberry juice, mixed juice, iced tea and diced tomato. Reference strains included in the study were ATCC 49025^T (*A. acidoterrestris*); ATCC 27009^T (*A. acidocaldarius*) and ATCC 49028 (*A. cycloheptanicus*).

Table 1
Sources of *Alicyclobacillus* isolates from Australian and international fruit-based products.

Source	Country of origin	No. of isolates investigated
Apple juice	Australia	91
Apple juice concentrate	Australia	24
	China	30
	New Zealand	4
Orange juice	Australia	17
	Brazil	2
Orange juice concentrate	Australia	7
Mango juice	Australia	3
Mango concentrate	Australia	3
Pear juice	Australia	1
Apple – pear juice concentrate	Australia	1
	United States	1
Apple – cranberry juice	Australia	1
	United States	1
Apple – grape – raspberry blend juice	Australia	1
Grape juice	United States	2
10% fruit drink	Australia	1
Banana puree	Australia	3
Diced tomato	Australia	1
Iced tea	Australia	12
Plate sample	Australia	2
Plate sample	New Zealand	4
Reference strains		3
Total		215

2.1.2. DNA preparation

A loopful of frozen culture in glycerol media was resuscitated in 1 mL of pH 4.0 YSG broth (2 g yeast extract, 2 g soluble starch, 2 g glucose and 1 L distilled water, pH adjusted with 1 M hydrochloric acid) at 45 °C for 24 h. Bacterial DNA was extracted from the 24 h cultured cells using Instagene Matrix (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia) following the manufacturer's instructions. DNA extracts were kept frozen at -20 °C until used.

2.1.3. DNA amplification

DNA amplification was carried out by RAPD-PCR using the primers BA 10 (5′-AACGCGCAAC-3′) and F 64 (5′-GCCGCGCCAGTA-3′) referenced for *A. acidoterrestris* (Yamazaki et al., 1997). The RAPD-PCR reactions were processed in a total volume of 10 µL containing 0.32 µL primer, 5 µL MyTaq mix and 1 µL DNA template (Yamazaki et al., 1997). The amplification process was done in a Mastercycler Ep Gradient S (Eppendorf AG, Hamburg, Germany) with the following program: initial denaturation at 94 °C for 15 s; 50 cycles of 94 °C for 4 s, 45 °C for 8 s, 72 °C for 40 s; and a final extension at 72 °C for 3 min (Yamazaki et al., 1997). All primers were purchased from Life Technologies Australia Pty Ltd (Mulgrave, Victoria, Australia) and stored at -20 °C until use.

2.1.4. Gel electrophoresis

Amplified products were electrophoresed on 2.0% agarose gels at 120 V for 60 min in 1 × TBE buffer (890 mM Tris-borate, 890 mM boric acid, 20 mM EDTA, pH 8.3) containing 0.1 µL mL^{-1} SYBR Safe DNA gel stain. A Hyper Ladder II with 200 lanes purchased from BioLine Pty Ltd (Alexandria, NSW, Australia) was used as a DNA reference marker. Images of band patterns were captured under UV light by Kodak

Molecular Imaging Software, standard edition v.5.0.0.90 (Carestream Health, Inc., Rochester, NY, USA).

2.1.5. Data analysis

Band pattern profiles were analysed by Bio-Numerics Version 7.5 (www.applied-maths.com). Genetic clusters were formed based on the band pattern profiles. Selected representative isolates from each cluster underwent further testing for guaiacol production.

2.2. Guaiacol production

2.2.1. Inoculum preparation

Based on RAPD-PCR band profiles and isolation source, *Alicyclobacillus* isolates ($n = 46$) were selected to undergo guaiacol production experiment.

Frozen cultures of *Alicyclobacillus* genetic group I including sub-group I.1 ($n = 19$) and sub-group I.2 ($n = 23$) and genetic group II (n = 4) were resuscitated in 1 mL of YSG broth and incubated for 3 d at optimum growth temperatures of either 45 °C and 60 °C (Yokota et al., 2007), respectively. Broth cultures (10 µL) were streaked on BAT agar [0.25 g $CaCl_2.2H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.2 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 2 g yeast extract, 5 g glucose, 1 mL trace minerals solution and 15 g/L agar (acidified to pH 4.0 with 1 M HCl)] and incubated for 3 d at the respective optimum growth temperatures.

Depending on the colony size, 2 to 8 colonies from BAT agar plates were aseptically transferred into 10 mL of BAT broth (pH 4.0) and incubated for 5 d at either 45 °C or 60 °C, to ensure similar cell concentrations (Witthuhn et al., 2013). After 5 d, the cell concentration was determined by serial dilution and spread plating on YSG agar.

2.2.2. Inoculating vanillin-BAT medium

Vanillin-BAT (VA-BAT) broth was formulated by addition of vanillin (100 mg/L) to pH 4.0 BAT broth prior to sterilisation (Witthuhn et al., 2013). A 10 mL of VA-BAT broth was inoculated with 5 day old culture from BAT broth as above to a final cell concentration of 10^1–10^3 colony forming units (CFU)/ml. The inoculated broth was incubated for 2 d at either 45 °C or 65 °C. Control samples of VA-BAT broth without culture and BAT broth with culture but without vanillin were also tested. Duplicate samples were analysed after 2 d of incubation to determine the guaiacol concentration and the cell concentration.

All chemicals and media ingredients were purchased from Thermo Fisher Scientific Australia Pty Ltd (Scoresby, Victoria, Australia) and Merck Pty Ltd (Bayswater, Victoria, Australia).

2.2.3. Quantitative analysis of guaiacol

Guaiacol production was measured using headspace solid phase microextraction (HS-SPME) and gas chromatography – mass spectrometry (GC–MS). A C^{13}-guaiacol stable isotope (C^{13}-6 ring 99%) was purchased from Novachem Pty Ltd (Heidelberg West, Victoria, Australia) and used as a quantitative internal standard (IS). The C^{13}-guaiacol IS was diluted in methanol to a concentration of approximately 40 mg/L and stored in a sealed amber vial at -20 °C until use.

After inoculation and incubation, a volume of sample was diluted and transferred into a gas-tight headspace vial (20 mL) for headspace analysis where BAT broth (pH 4.0) was used to dilute samples and as a blank control. The extraction was carried out in a total volume of 4020 µL which comprised 4000 µL of 50-fold diluted sample and 20 µL C^{13}-guaiacol IS. Divinylbenzene-Carboxen-polydimethylsiloxane (50/30 µm, 2 cm, 23 gauge) SPME fibres (Supelco, Bellefonte, PA, USA) were used to extract guaiacol from the headspace.

The SPME method was optimised using different dilutions and different volumes of IS to ensure that the response of the instrument was linear across the concentration range of samples. The headspace extraction was automated using an AOC-5-000 autosampler (Shimadzu, Rydalmere, NSW, Australia). Headspace vials were heated to 40 °C for 20 min during extraction. SPME fibres were desorbed in the hot injection port (250 °C, splitless, 5 min) of the gas chromatograph-mass spectrometer (GC–MS, QP-2010-Plus, Shimadzu). Volatiles were separated on a Zebron-Wax capillary column (30 m, 0.25 mm, 0.5 µm) with helium carrier gas (1.0 mL/m) using a temperature program; 35 °C (held 5 min) increased to 250 °C (5 °C/min). The MS was scanned over the mass/charge (m/z) range 40–200 in electron ionization mode. The integrated areas for C^{13}-guaiacol and guaiacol were calculated using the GC–MS proprietary software (Version 4.20, Shimadzu) and using ion with a mass/charge (m/z) ratio of 115 and m/z 109 respectively as quantification ions and m/z 130 and m/z 124 as qualifier ions. The concentration of guaiacol was determined from the ratio of m/z 109/115.

2.2.4. Data analysis

The replicate guaiacol data were statistically analysed using the Analysis of Variance procedure in GenStat® (VSN International Ltd., Hemel Hempstead, UK) to identify the significance of the effects of genetic groups and strains.

3. Results

3.1. RAPD-PCR analysis of genetic diversity of Alicyclobacillus strains isolated from various fruit juice and fruit-based beverages

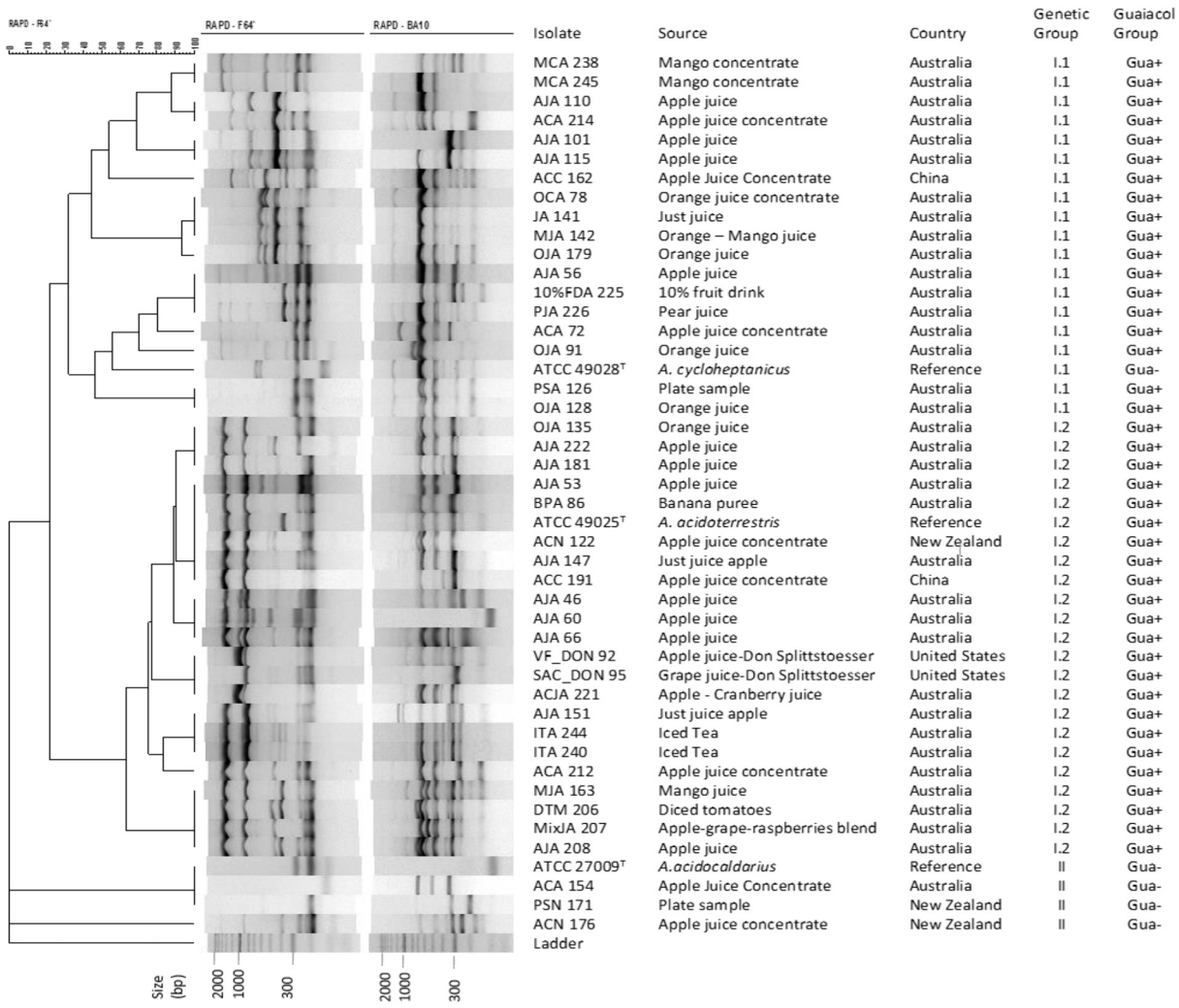
The band profiles of 215 *Alicyclobacillus* isolates were generated by RAPD-PCR using *A. acidoterrestris* referencing primers F-64 and BA-10. Two distinct genotypic groups were clustered by the UPGMA method (Fig. 1) with 93.1% of isolates in Group I and 6.9% of isolates in Group II. Group I isolates were further separated into two sub-groups, sub-group I.1 and sub-group I.2. Sub-group I.1 contained the largest number of isolates (56.9%, $n = 123$) with the remaining isolates in sub-group I.2 (36.1%, $n = 78$ strains). Group II strains consisted of the rest of tested isolates ($n = 15$). The type strains of *A. acidoterrestris* (ATCC 49025^T) and strain *A. cycloheptanicus* (ATCC 49028) were clustered separately into sub-group I.2 and I.1, respectively. The type strain of *A. acidocaldarius* (ATCC 27009^T) was present in group II.

Alicyclobacillus in group I produced RAPD bands of expected sizes with primers BA-10 and F-64 based on Yamazaki and colleagues' method development study (Yamazaki et al., 1997) whereas these fragment sizes were inconsistently presented in genetic group II isolates. With primer BA-10, a DNA band with a size of around 580 bp was amplified from all isolates in both sub-groups of group I, and some isolates of group II. DNA bands of approximately 470 bp and 320 bp in size were also amplified from these groups. Using primer F-64, two unique amplified bands of 850 bp and 1500 bp were observed in sub-group I.2 isolates while these bands were absent from sub-group I.1. There was little or no products observed in group II when amplified with the primer F-64, however one or two major bands were occasionally produced with the primer BA-10.

3.2. Guaiacol production by Alicyclobacillus spp. under the optimum conditions

Guaiacol was produced by *Alicyclobacillus* isolates belonging to genetic group I, however this compound was not detected in analyses from isolates belonging to group II (Fig. 2). The ability to produce guaiacol classified all isolates into two groups in agreement with the RAPD results with the notable exception of *A. cycloheptanicus* reference strain ATCC 49028 in which guaiacol production was not detected.

Guaiacol levels varied in both sub-groups, I.1 and I.2 with the highest value of 16.09 mg/L produced by strain MixJA207 – sub group I.2 which was isolated from mixed fruit juice. Most isolates in the genetic group I were able to produce between 6.06 mg/L and 9.76 mg/L guaiacol, with the exception of strains ACA212, AJA101, DTM206 and ACC162 which produced 2.38, 3.38, 4.07 and 4.19 mg/L, respectively. No guaiacol production was detected from control samples (Fig. 2).



Isolate	Source	Country	Genetic Group	Guaiacol Group
MCA 238	Mango concentrate	Australia	I.1	Gua+
MCA 245	Mango concentrate	Australia	I.1	Gua+
AJA 110	Apple juice	Australia	I.1	Gua+
ACA 214	Apple juice concentrate	Australia	I.1	Gua+
AJA 101	Apple juice	Australia	I.1	Gua+
AJA 115	Apple juice	Australia	I.1	Gua+
ACC 162	Apple Juice Concentrate	China	I.1	Gua+
OCA 78	Orange juice concentrate	Australia	I.1	Gua+
JA 141	Just juice	Australia	I.1	Gua+
MJA 142	Orange – Mango juice	Australia	I.1	Gua+
OJA 179	Orange juice	Australia	I.1	Gua+
AJA 56	Apple juice	Australia	I.1	Gua+
10%FDA 225	10% fruit drink	Australia	I.1	Gua+
PJA 226	Pear juice	Australia	I.1	Gua+
ACA 72	Apple juice concentrate	Australia	I.1	Gua+
OJA 91	Orange juice	Australia	I.1	Gua+
ATCC 49028^{T}	*A. cycloheptanicus*	Reference	I.1	Gua-
PSA 126	Plate sample	Australia	I.1	Gua+
OJA 128	Orange juice	Australia	I.1	Gua+
OJA 135	Orange juice	Australia	I.2	Gua+
AJA 222	Apple juice	Australia	I.2	Gua+
AJA 181	Apple juice	Australia	I.2	Gua+
AJA 53	Apple juice	Australia	I.2	Gua+
BPA 86	Banana puree	Australia	I.2	Gua+
ATCC 49025^{T}	*A. acidoterrestris*	Reference	I.2	Gua+
ACN 122	Apple juice concentrate	New Zealand	I.2	Gua+
AJA 147	Just juice apple	Australia	I.2	Gua+
ACC 191	Apple juice concentrate	China	I.2	Gua+
AJA 46	Apple juice	Australia	I.2	Gua+
AJA 60	Apple juice	Australia	I.2	Gua+
AJA 66	Apple juice	Australia	I.2	Gua+
VF_DON 92	Apple juice-Don Splittstoesser	United States	I.2	Gua+
SAC_DON 95	Grape juice-Don Splittstoesser	United States	I.2	Gua+
ACJA 221	Apple - Cranberry juice	Australia	I.2	Gua+
AJA 151	Just juice apple	Australia	I.2	Gua+
ITA 244	Iced Tea	Australia	I.2	Gua+
ITA 240	Iced Tea	Australia	I.2	Gua+
ACA 212	Apple juice concentrate	Australia	I.2	Gua+
MJA 163	Mango juice	Australia	I.2	Gua+
DTM 206	Diced tomatoes	Australia	I.2	Gua+
MixJA 207	Apple-grape-raspberries blend	Australia	I.2	Gua+
AJA 208	Apple juice	Australia	I.2	Gua+
ATCC 27009^{T}	*A.acidocaldarius*	Reference	II	Gua-
ACA 154	Apple Juice Concentrate	Australia	II	Gua-
PSN 171	Plate sample	New Zealand	II	Gua-
ACN 176	Apple juice concentrate	New Zealand	II	Gua-
Ladder				

Fig. 1. Genetic classification of *Alicyclobacillus* spp. based on RAPD-generated banding profiles and guaiacol productions of representative isolates (n = 46). RAPD band patterns were analysed and clustered by UPGMA method. Gua^{+} (guaiacol positive) and Gua^{-} (guaiacol negative).

Cell concentrations of all three species studied reached 10^{6}–10^{8} CFU/mL after 2 days growth which was above the minimal *Alicyclobacillus* cell concentration of around 10^{5} CFU/mL for detectable guaiacol production (Orr et al., 2000; Pettipher et al., 1997). There is no correlation between number of cells and guaiacol level.

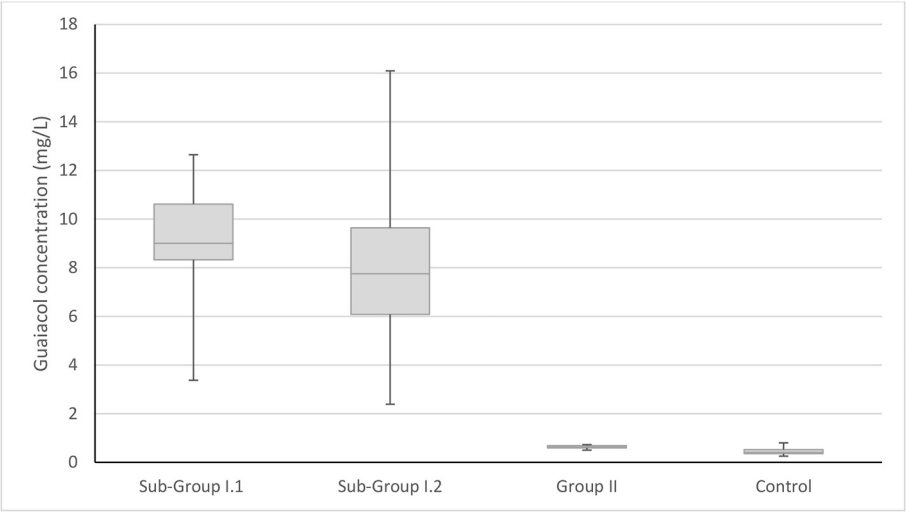


Fig. 2. Boxplots of guaiacol levels produced by representative isolates (n = 46) of genetic Group I including Sub-Group I.1 and Sub-Group I.2 and genetic Group II.

3.3. Relationship between guaiacol production and genetic diversity of *Alicyclobacillus spp.*

RAPD profiles and guaiacol assays separated *Alicyclobacillus* spp. into two distinct groups (Fig. 1). Significant difference ($p < 0.05$) for guaiacol production level was observed between the guaiacol-positive (Gua^+) and guaiacol-negative (Gua^-) groups. Like genetic group I, the Gua^+ group consisted of the majority of isolates (93.0%) including isolates from genetic subgroups I.1 and I.2. The assays also revealed phenotypic similarity between these two genetic sub-groups in which guaiacol levels and variability were observed in the same way. While sub-group I.2 with 56.9% isolates was the most common genotype in this study, sub-group I.1 with 36.1% was the second common genotype and produced the highest levels of guaiacol. Although guaiacol levels produced by isolates from sub-group I.2 were lower than that of sub-group I.1, the average value was still relatively high (8.38 mg/L). Group II isolates including *A. acidocaldarius* were less commonly identified, and guaiacol production was not observed (Fig. 2).

4. Discussion

Contamination of fruit juices and concentrates by *Alicyclobacillus* is widespread in the global fruit juice industry and economically impacts the global fruit juice industry. Taint development can occur in most shelf-stable fruit juices and fruit-based beverages (Danyluk et al., 2011; Durak et al., 2010; Groenewald et al., 2009; Jensen and Whitfield, 2003; Osopale et al., 2016; Witthuhn et al., 2013). Understanding the diverse genotype and phenotype traits among *Alicyclobacillus* allow the early and rapid detection of high-spoilage-risk species/strains to eliminate preventable loss caused by *Alicyclobacillus* spores in the fruit juice industry. This knowledge will also assist the selection of a diverse culture collection to aid in the development of more effective strategies. In this study, the genetic diversity of *Alicyclobacillus* from fruit-based products manufactured and sold in Australia was assessed.

The RAPD-PCR band profiles of 215 *Alicyclobacillus* isolates generated by primers BA-10 and F-64 classified *Alicyclobacillus* spp. strains into two distinct genotypes and two major sub-genotypes. The first genotype consisted of *A. acidoterrestris* strains and produced higher number of significantly amplified DNA bands compared to the second genotype, which included *A. acidocaldarius* strains. Among genotype I, the two classified sub-genotypes also demonstrated diversity in the fingerprint profiles. There were two significant DNA fragments at the sizes of ~850 bp and ~1500 bp produced in sub-genotype I.2, consistent with the previous observation that all *A. acidoterrestris* strains showed these two specific major bands when amplified with primer F-64 (Yamazaki et al., 1997). However, these fragments were insignificant or absent in sub-genotype I.1, and other fragments were significantly amplified instead (~400 bp and ~500 bp), indicating other DNA fragments of *A. acidoterrestris* genomes can be significantly amplified by primer F-64 beside the two reported fragments as seen in Yamazaki and colleagues' study (Yamazaki et al., 1997). In agreement with this finding Bevilacqua et al. (2015) also reported a higher number of significantly amplified fragments with primer F-64 than with primer BA-10. We also observed the amplification of the same DNA fragment throughout isolates in this study including some *A. acidocaldarius* strains when primer BA-10 was used. This indicates that primer BA-10 is suitable at genus level while primer F-64 can be used to identify at species level. The result has demonstrated major genetic diversity among *Alicyclobacillus* at genus and species levels, however there was no evidence of a correlation between the diversity and the isolation source, i.e. fruit type or product geographic origin. This finding is supported by the observation made by Dekowska et al. (2018) in which there was no apparent correlation between diverse 16S rDNA sequences and the sources of the isolates.

Alicyclobacillus spoilage issues are characterised by taint production in which guaiacol is a predominant compound. Identification of guaiacol-producing species has been recognised as a primarily effective strategy in diagnosing *Alicyclobacillus* induced spoilage in fruit-based products (Jensen and Whitfield, 2003; Osopale et al., 2016). Similar to genetic analysis, guaiacol assay classified *Alicyclobacillus* into two distinct groups. All *A. acidoterrestris* strains were able to produce significant levels of guaiacol which were well over the threshold for human detection (0.002 mg/L) in fruit juices and fruit-containing drinks (Gocmen et al., 2005; Orr et al., 2000; Pettipher et al., 1997). However, guaiacol metabolism varied among strains which was consistent with the observation in a previous study (Goto et al., 2008). Moreover, the inability to produce guaiacol by *A. acidocaldarius* strains was confirmed since there was undetectable guaiacol in *A. acidocaldarius* – inoculated samples, and this was in agreement with previous reports (Goto et al., 2008; Osopale et al., 2016; Witthuhn et al., 2013).

Interestingly, while genetic analysis and guaiacol assay classified majority of isolates into group I, the genetic analysis divided this group into two major distinct sub-groups, I.1 and I.2. These sub-groups were similarly capable of guaiacol production, except for a previously labelled ATCC 49028 which was recorded as an *A. cycloheptanicus* strain. The presence of the ATCC 49028 in group I suggests a genetic similarity of this strain to *A. acidoterrestris* species. Therefore, a 16S rRNA analysis was undertaken to clarify the taxonomic identity of this strain. As a result, the BLAST comparison showed 99.5% identity between *A. cycloheptanicus* ATCC 49028 and *A. acidoterrestris* ATCC 49025 (NR 040844.1). This result is not consistent with the identification of ATCC 49028 as *A. cycloheptanicus* but it is rather potentially synonymous with *A. acidoterrestris*. This requires further confirmation by genome-level comparisons. Another 16S rRNA sequence comparison of a different *A. cycloheptanicus* strain, ATCC 49029 (AY573798.1) only showed 94.3% identity to *A. acidoterrestris* (NR 040844.1), indicating that *A. cycloheptanicus* when using this strain is relatively distinct from *A. acidoterrestris*. As a conclusion, the RAPD genetic analysis has revealed the genetic identity of ATCC 49028 as an *A. acidoterrestris* strain and a possible problem with the designated *A. cycloheptanicus* strain.

Contrary to observations among all other isolates within the RAPD group, guaiacol production in *A. cycloheptanicus* ATCC 49028 was not observed. This could be explained in a number of ways. The first possibility could be the short period of incubation prior to measurement which, in some cases, can play an important role in guaiacol metabolism. For instance, *A. cycloheptanicus*, ATCC 49029, was only reported to produce detectable quantities of the taint compound guaiacol and other taints including 2,6-DCP and 2,6-DBP at 45 °C after 28 d (Gocmen et al., 2005) compared to measurement after 2 d in the present study. Other possibilities could be the absence of guaiacol encoding genes, regulatory elements and/or the loss of gene expression in culture in this particular strain. Further investigation of guaiacol encoding genes may provide details related to the guaiacol producing ability of the microorganism.

This study has revealed the diversity of *Alicyclobacillus* in fruit-based products in Australia and neighbouring countries in Asia Pacific region in which two major genotypes and two sub-genotypes were consistently observed regardless of product types and geographic origins. While *A. acidoterrestris* was confirmed as a significant spoilage species in fruit juice and fruit-based beverages, two major sub-genotypes were identified among this species. In this study, the two sub-genotypes presented similar guaiacol production capacity however they may carry other variable phenotypes such as heat tolerance. Our results indicate the rapid detection of *Alicyclobacillus* strains which possess high risk of spoilage taint metabolism in fruit juice and fruit-based beverages. Not only will these results allow effective surveillance of the presence of targeted spoilage *A. acidoterrestris*, they will also assist the selection of a diverse culture set to facilitate the development of innovative treatment processes against *Alicyclobacillus* in the fruit juice industry.

Funding sources

This work was jointly funded by theUniversity of Tasmania and Commonwealth Scientific and Industrial Research Organisation (CSIRO). TSVL was a recipient of University of Tasmania and CSIRO Ph.D. Scholarship.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors would like to thank Dr. Edward Fox for his kind assistance in the use of Bio-Numerics for gel analysis.

References

Bevilacqua, A., Mischitelli, M., Pietropaolo, V., Ciuffreda, E., Sinigaglia, M., Corbo, M.R., 2015. Genotypic and phenotypic heterogeneity in alicyclobacillus acidoterrestris: a contribution to species characterization. PLoS One 10.

Chang, S.-S., Kang, D.-H., 2004. *Alicyclobacillus* spp. in the fruit juice industry: history, characteristics, and current isolation/detection procedures. Crit. Rev. Microbiol. 30, 55–74.

Danyluk, M.D., Friedrich, L.M., Jouquand, C., Goodrich-Schneider, R., Parish, M.E., Rouseff, R., 2011. Prevalence, concentration, spoilage, and mitigation of *Alicyclobacillus* spp. in tropical and subtropical fruit juice concentrates. Food Microbiol. 28, 472–477.

Deinhard, G., Saar, J., Krischke, W., Poralla, K., 1987. *Bacillus cycloheptanicus* sp. nov., a new thermoacidophile containing ω-cycloheptane fatty acids. Syst. Appl. Microbiol. 10, 68–73.

Dekowska, A., Niezgoda, J., Sokolowska, B., 2018. Genetic heterogeneity of Alicyclobacillus strains revealed by RFLP analysis of vdc region and rpoB gene. Biomed. Res. Int. 2018, 12.

Durak, M.Z., Churey, J.J., Danyluk, M.D., Worobo, R.W., 2010. Identification and haplotype distribution of *Alicyclobacillus* spp. from different juices and beverages. Int. J. Food Microbiol. 142, 286–291.

Félix-Valenzuela, L., Guardiola-Avila, I., Burgara-Estrella, A., Ibarra-Zavala, M., Mata-Haro, V., 2015. Genotypic and phenotypic diversity of *Alicyclobacillus acidocaldarius* isolates. Lett. Appl. Microbiol. 61, 367–373.

Gocmen, D., Elston, A., Williams, T., Parish, M., Rouseff, R.L., 2005. Identification of medicinal off-flavours generated by *Alicyclobacillus* species in orange juice using GC-olfactometry and GC-MS. Lett. Appl. Microbiol. 40, 172–177.

Goto, K., Nishibori, A., Wasada, Y., Furuhata, K., Fukuyama, M., Hara, M., 2008. Identification of thermo-acidophilic bacteria isolated from the soil of several Japanese fruit orchards. Lett. Appl. Microbiol. 46, 289–294.

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

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

Jensen, N., 2000. *Alicyclobacillus* in Australia. Food Aust. 52, 282–285.

Jensen, N., Whitfield, F.B., 2003. Role of *Alicyclobacillus acidoterrestris* in the development of a disinfectant taint in shelf-stable fruit juice. Lett. Appl. Microbiol. 36, 9–14.

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

Osopale, B.A., Witthuhn, R.C., Albertyn, J., Oguntoyinbo, F.A., 2016. Culture dependent and independent genomic identification of *Alicyclobacillus* species in contaminated commercial fruit juices. Food Microbiol. 56, 21–28.

Pettipher, G.L., Osmundson, M.E., Murphy, J.M., 1997. Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit juice-containing drinks. Lett. Appl. Microbiol. 24, 185–189.

Smit, Y., Cameron, M., Venter, P., Witthuhn, R.C., 2011. *Alicyclobacillus* spoilage and isolation - a review. Food Microbiol. 28, 331–349.

Witthuhn, R.C., Smit, Y., Cameron, M., Venter, P., 2013. Guaiacol production by *Alicyclobacillus* and comparison of two guaiacol detection methods. Food Control 30, 700–704.

Yamazaki, K., Okubo, T., Inoue, N., Shinano, H., 1997. Randomly amplified polymorphic DNA (RAPD) for rapid identification of the spoilage bacterium *Alicyclobacillus acidoterrestris*. Biosci. Biotechnol. Biochem. 61, 1016–1018.

Yokota, A., Fujii, T., Goto, K., 2007. *Alicyclobacillus*-Thermophilic Acidophilic Bacilli. Springer, Tokyo (1,2,3, 1-48).

Zierler, B., Siegmund, B., Pfannhauser, W., 2004. Determination of off-flavour compounds in apple juice caused by microorganisms using headspace solid phase microextraction–gas chromatography–mass spectrometry. Anal. Chim. Acta 520, 3–11.