Alicyclobacillus spoilage and isolation — A review

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A B S T R A C T

Until recently, acidic products such as fruit juice and fruit based products were generally thought to be susceptible to spoilage by yeasts, mycelia fungi and lactic acid bacteria, as the low pH of these products acts as natural control measures against spoilage by most bacteria. Alicyclobacillus seem to be prevalent in fruit based products as they survive the acidic fruit juice environment, even when they are exposed to pasteurisation temperatures during production. In this review the historical background of the discovery of these bacteria is summarised. The bacterial characteristics and the reported spoilage incidences caused by members of this genus are discussed. As the isolation methods for these bacteria are controversial, this review includes a discussion of the various media that have been reported in the literature for the use in the isolation and enumeration of members of the genus Alicyclobacillus.

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1. Introduction

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

A new spoilage threat for acidic products emerged in 1984, however, with the report of a spoilage incident in Germany involving shelf-stable apple juice (Cerny et al., 1984). The microbe responsible for the incident was ultimately identified as the thermo-acidophilic bacterium Alicyclobacillus acidoterrestris (Cerny et al., 1984; Deinhard et al., 1987a; Wisotzkey et al., 1992). Heat resistance studies revealed the ability of these microbes to survive pasteurisation procedures normally applied to fruit juice and acidic products (Splitstoesser et al., 1998; Eiroa et al., 1999; Vieira et al., 2002) and because of their acidophilic nature (Wisotzkey et al., 1992) the endospores can germinate and increase in products to cell concentrations high enough to produce taint compounds, leading to product spoilage (Pettipher et al., 1997; Orr et al., 2000; Gocmen et al., 2005). Since their implication in the latter and other subsequent spoilage incidents (Splitstoesser et al., 1994; Yamazaki et al., 1996; Walls and Chuyate, 1998; Duong and Jensen, 2000; Jensen, 2000; Matsubara et al., 2002; Gouws et al., 2005), Alicyclobacillus spp., especially A. acidoterrestris, have become the focus of research investigating their involvement in the spoilage of acidic food products, their production of taint compounds, and the development of isolation, detection and control procedures.

Surveys have shown that there is great potential for substantial product and consumer confidence losses, should a spoilage incident occur (Walls and Chuyate, 1998; Howard, 2006). Alicyclobacillus has become a great concern to manufacturers and processors in the fruit industry and it has been suggested as a possible target microbe in the design of pasteurisation processes for acidic products (Silva et al., 1999; Vieira et al., 2002).
2. History and species classification

Uchino and Doi reported the first case of the isolation of thermo-acidophilic microbes in 1967. Three microbial isolates from hot springs in the Tohoku district in Japan were identified as belonging to the genus Bacillus. Even though their endospores were shaped differently and they were more acidophilic and aerobic than Bacillus coagulans and Bacillus stearothermophilus, the two most well known thermophilic species at that time, they were tentatively classified as new strains of B. coagulans based on morphological and cultural characteristics.

Darland and Brock (1971) and De Rosa et al. (1971) isolated similar microbes from aqueous and terrestrial acid thermal environments in Yellowstone National Park in the United States of America (USA), Volcano National Park in Hawaii (USA) and Picarelle in Italy. The precise taxonomic position of the isolates was questioned, as they differed considerably more from B. coagulans, especially in optimum pH and DNA base composition, than from the isolates of Uchino and Doi (1967). They also contained \( \omega \)-cyclohexane fatty acids as the major components (up to 65%) in the saponifiable lipid fraction of their membranes (De Rosa et al., 1971). It was proposed that they be classified into a new species, Bacillus acidocaldarius (Darland and Brock, 1971). Hippchen et al. (1981) set out to identify relatives of B. acidocaldarius and isolated several thermo-acidophiles from a variety of neutral soils. These microbes possessed similar membrane properties to B. acidocaldarius, but their precise relationship to this microbe could not be determined. Although the potential of these microbes to be involved in food spoilage had already been recognised (Uchino and Doi, 1967), it was only confirmed in 1984 when Cerny et al. (1984) reported the isolation of a microbial strain closely related to those of Hippchen et al. (1981) from spoiled apple juice. Subsequently this microbe was classified as a new species, Bacillus acidoterrestris (Deinhard et al., 1987a). A third thermo-acidophilic bacillus, distinct from B. acidocaldarius and B. acidoterrestris was described by Poralla and König (1983). It differed from B. acidocaldarius and B. acidoterrestris, in that it contained primarily \( \omega \)-cycloheptane fatty acids in its membrane and it was subsequently classified into a new species, Bacillus cycloheptanicus (Poralla and König, 1983; Deinhard et al., 1987b). Comparative sequence analyses carried out on the 16S ribosomal RNA (rRNA) genes of the three existing thermo-acidophilic Bacillus strains showed that they were distinct from any other Bacillus species. These findings led to the proposal of a new genus, Alicyclobacillus, to accommodate these unique bacteria (Wisotzkey et al., 1992).

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

3. Characteristics

3.1. General characteristics

The characteristics of all the Alicyclobacillus species identified to date are summarised in Table 1. Alicyclobacillus species are thermo-acidophilic, rod-shaped endospore-formers. All species are Gram-positive, with the exception of Alicyclobacillus sendaiensis which is Gram-negative (Tsuruoka et al., 2003). In many of the species old cultures have a tendency to be Gram variable (Darland and Brock, 1971; Karavaiko et al., 2005; Goto et al., 2007). The classification of a species as Gram variable may be controversial as there are many contributing factors such as culture age, time of decoulourising and cell wall/membrane physiology. All species are aerobic, with Alicyclobacillus pohliae sometimes being facultatively anaerobic (Imperio et al., 2008). Most are motile, with the exception of A. acidocaldarius subsp. rittmannii (Nicolaus et al., 1998), Alicyclobacillus hesperidum, Alicyclobacillus genomic species 1 (Albuquerque et al., 2000), A. sendaiensis (Tsuruoka et al., 2003), Alicyclobacillus tolerans, Alicyclobacillus disulfidoxidans (Karavaiko et al., 2005), Alicyclobacillus fastidiosus (Goto et al., 2007) and Alicyclobacillus ferroxydans (Jiang et al., 2008).

The temperature growth range for all species except A. disulfidoxidans, A. tolerans (Karavaiko et al., 2005) and A. ferroxydans (Jiang et al., 2008) is 20–70 °C (Wisotzkey et al., 1992; Goto et al., 2007; Jiang et al., 2008), with the latter three species also able to grow at temperatures below 20 °C. The optimum growth temperatures for these microbes range from 35 to 65 °C (Albuquerque et al., 2000; Imperio et al., 2008). The pH range for growth is between 2.00 and 6.50 (Wisotzkey et al., 1992; Simbahan et al., 2004; Jiang et al., 2008), again with the exception of A. disulfidoxidans and A. tolerans (Karavaiko et al., 2005). These two species are able to grow at a pH of below 1.50. The optimum pH range is between 3.00 and 5.50 (Walls and Chuyate, 1998; Matsuoka et al., 2002), except for A. disulfidoxidans and A. tolerans, having much lower pH optima ranging from 1.50 to 2.00 (Karavaiko et al., 2005).

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

Since all species of Alicyclobacillus are aerobic (Table 1) the amount of oxygen present in the growth medium influences the growth of these microbes. There is, however, no consistent agreement among studies regarding the effect various concentrations of oxygen may have on cell density and spoilage. Walker and Phillips (2005) found that containers with 0% headspace showed a significantly lower level of growth when compared to containers containing a headspace. In contrast, Cerny et al. (2000) found that the presence or absence of a headspace in the packaging system did not significantly influence the growth of A. acidoterrestris and no spoilage of juices was observed under either condition (Cerny et al., 2000). In apple juice low residual oxygen concentrations (7–3%) resulted in more rapid growth than at atmospheric concentrations (21%), although final cell counts were higher at atmospheric concentrations. Siegmund and Pöllinger-Zierler (2007) also found that a limited oxygen supply slowed the growth rate of A. acidoterrestris, but did not prevent it from reaching high cell concentrations.

3.2. Pathogenicity

When the species of Alicyclobacillus became apparent as spoilage microbes, concerns about pathogenicity arose. Walls and Chuyate (2000a) undertook a study to determine the pathogenicity of several strains of A. acidoterrestris, as well as a strain of
Table 1  
Cultural, morphological and colony characteristics of species belonging to the genus *Alicyclobacillus*.

<table>
<thead>
<tr>
<th><em>Alicyclobacillus</em> species</th>
<th>Source</th>
<th>Cultural characteristics</th>
<th>Morphological characteristics</th>
<th>Colony morphology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH range (optimum)</td>
<td>T-range (°C) Oxygen requirement</td>
<td>Gram stain Shape</td>
<td>Cell size (length × width μm)</td>
</tr>
<tr>
<td><em>A. acidiphilus</em></td>
<td>Acidic beverage</td>
<td>2.50–5.50 (3.00)</td>
<td>20–55 (50)</td>
<td>Aerobic</td>
<td>+</td>
</tr>
<tr>
<td><em>A. acidocaldarius</em></td>
<td>Thermal acid waters</td>
<td>2.00–6.00 (3.50–4.00)</td>
<td>45–71 (53–65)</td>
<td>Aerobic</td>
<td>+ to variable</td>
</tr>
<tr>
<td><em>A. acidocaldarius</em> subsp. acidocaldarius</td>
<td>Soil from crop fields in Fuji city soil</td>
<td>3.50–5.50 (4.00–4.50)</td>
<td>35–60 (50–55)</td>
<td>Aerobic</td>
<td>+ to variable</td>
</tr>
<tr>
<td><em>A. acidoterrestris</em></td>
<td>Geothermal soil of Mount Rittmann, Antarctica soil/apple juice</td>
<td>2.50–5.80 (4.50–5.00)</td>
<td>20–70 (36–53)</td>
<td>Aerobic</td>
<td>+ to variable</td>
</tr>
<tr>
<td><em>A. contaminans</em></td>
<td>Soil from crop fields in Fuji city soil</td>
<td>3.00–5.50 (3.50–4.50)</td>
<td>40–53 (48)</td>
<td>Aerobic</td>
<td>+</td>
</tr>
<tr>
<td><em>A. cycloheptanicus</em></td>
<td>Waste water sludge</td>
<td>0.50–6.00 (1.50–2.50)</td>
<td>4–40 (35)</td>
<td>Aerobic</td>
<td>+ to variable</td>
</tr>
<tr>
<td><em>A. disulfidooxidans</em></td>
<td>Apple juice</td>
<td>2.50–5.00 (4.00–4.50)</td>
<td>20–55 (40–65)</td>
<td>Aerobic</td>
<td>+ to variable</td>
</tr>
<tr>
<td><em>A. fastidiosus</em></td>
<td>Sulfataric soil</td>
<td>2.00–6.00 (3.00)</td>
<td>17–40 (28)</td>
<td>Aerobic</td>
<td>+</td>
</tr>
<tr>
<td><em>Alicyclobacillus</em></td>
<td>Sulfataric soils of São Miguel, Azores</td>
<td>3.50–4.00 (3.00)</td>
<td>40–70 (60–63)</td>
<td>Aerobic</td>
<td>+</td>
</tr>
<tr>
<td>genus species 1 (A. mali)</td>
<td>Soil near a geyser in Kirishima, Japan</td>
<td>2.00–6.50 (4.00–4.50)</td>
<td>35–70 (55–60)</td>
<td>Aerobic</td>
<td>+</td>
</tr>
<tr>
<td>genus species 2</td>
<td>Sulfataric soil</td>
<td>3.50–6.00 (4.50–5.00)</td>
<td>35–65 (55–60)</td>
<td>Aerobic</td>
<td>+</td>
</tr>
<tr>
<td><em>A. herbarius</em></td>
<td>Sulfataric soils of São Miguel, Azores</td>
<td>3.50–4.00 (4.00)</td>
<td>35–60 (50–53)</td>
<td>Aerobic</td>
<td>+</td>
</tr>
</tbody>
</table>

(continued on next page)
<table>
<thead>
<tr>
<th>Alicyclobacillus species</th>
<th>Source</th>
<th>pH range (optimum)</th>
<th>T-range (°C)</th>
<th>Oxygen requirement</th>
<th>Gram stain</th>
<th>Shape</th>
<th>Cell size (length × width μm)</th>
<th>Motility</th>
<th>Endospore characteristics</th>
<th>Sporangia swollen</th>
<th>Colour</th>
<th>Shape</th>
<th>Size (diameter mm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. kakegawensis</em></td>
<td>Soil from crop fields in Kakegawa city</td>
<td>3.50–6.00 (4.00–4.50)</td>
<td>40–60 (50–55)</td>
<td>Aerobic</td>
<td>+ to variable</td>
<td>Rod</td>
<td>4.0–5.0 × 0.5–0.7</td>
<td>Yes</td>
<td>Oval, subterminal</td>
<td>Yes</td>
<td>Non-pigmented (creamy white), opaque</td>
<td>Circular, entire, flat</td>
<td>2.0–3.0</td>
<td>Goto et al., 2007</td>
</tr>
<tr>
<td><em>A. macrosporangiidus</em></td>
<td>Soil from crop fields in Fujieda city</td>
<td>3.50–6.00 (4.00–4.50)</td>
<td>35–60 (50–55)</td>
<td>Aerobic</td>
<td>+ to variable</td>
<td>Rod</td>
<td>5.0–6.0 × 0.7–0.8</td>
<td>Yes</td>
<td>Oval, terminal</td>
<td>Yes</td>
<td>Non-pigmented (creamy white), opaque</td>
<td>Circular, entire, convex</td>
<td>2.0–4.0</td>
<td>Goto et al., 2007</td>
</tr>
<tr>
<td><em>A. pohliae</em></td>
<td>Geothermal soil of Mount Melbourne, Antarctica</td>
<td>4.50–7.50 (5.50)</td>
<td>42–60 (55)</td>
<td>Aerobic, facultatively anaerobic</td>
<td>+ Rod</td>
<td>1.5–2.5 × 0.4–0.6</td>
<td>nr</td>
<td>Round, terminal</td>
<td>Yes</td>
<td>Cream-coloured</td>
<td>Entire, convex</td>
<td>1.5–2.0</td>
<td>Imperio et al., 2008</td>
<td></td>
</tr>
<tr>
<td><em>A. pomorum</em></td>
<td>Mixed fruit juice</td>
<td>3.00–6.00 (4.00–4.50)</td>
<td>30–60 (45–50)</td>
<td>Aerobic</td>
<td>+ to variable</td>
<td>Rod</td>
<td>2.0–4.0 × 0.8–1.0</td>
<td>Yes</td>
<td>Oval, subterminal</td>
<td>Yes</td>
<td>Not pigmented (creamy white), opaque</td>
<td>Circular, entire, umbonate</td>
<td>3.0–4.0</td>
<td>Goto et al., 2003</td>
</tr>
<tr>
<td><em>A. sacchari</em></td>
<td>Liquid sugar</td>
<td>2.50–5.50 (4.00–4.50)</td>
<td>30–55 (45–50)</td>
<td>Aerobic</td>
<td>+ to variable</td>
<td>Rod</td>
<td>4.0–5.0 × 0.6–0.7</td>
<td>Yes</td>
<td>Ellipsoidal, subterminal</td>
<td>Yes</td>
<td>Not pigmented (creamy white), opaque</td>
<td>Circular, entire, umbonate</td>
<td>3.0–5.0</td>
<td>Goto et al., 2007</td>
</tr>
<tr>
<td><em>A. sendaiensis</em></td>
<td>Soil, Japan</td>
<td>2.50–6.50 (5.50)</td>
<td>40–65 (55)</td>
<td>Aerobic</td>
<td>– Rod</td>
<td>2.0–3.0 × 0.8</td>
<td>No</td>
<td>Round or ellipsoidal, terminal</td>
<td>Yes</td>
<td>White and semi-transparent</td>
<td>Circular, convex</td>
<td>1.0</td>
<td>Tsuruoka et al., 2003</td>
<td></td>
</tr>
<tr>
<td><em>A. shizuokensis</em></td>
<td>Soil from crop fields in Shizuoka city</td>
<td>3.50–6.00 (4.00–4.50)</td>
<td>35–60 (45–50)</td>
<td>Aerobic</td>
<td>+ to variable</td>
<td>Rod</td>
<td>4.0–5.0 × 0.7–0.8</td>
<td>Yes</td>
<td>Oval, subterminal</td>
<td>Yes</td>
<td>Non-pigmented (creamy white), opaque</td>
<td>Circular, entire, convex</td>
<td>1.0–2.0</td>
<td>Goto et al., 2007</td>
</tr>
<tr>
<td><em>A. tolerans</em></td>
<td>Oxidizable lead-zinc ores</td>
<td>1.50–5.00 (2.50–2.70)</td>
<td>&lt;20–55 (37–42)</td>
<td>Aerobic</td>
<td>+ Rod</td>
<td>3.0–6.0 × 0.9–1.0</td>
<td>No</td>
<td>Oval, terminal or subterminal</td>
<td>Yes</td>
<td>nr</td>
<td>nr</td>
<td>0.3–0.5</td>
<td>Karavaiko et al., 2005</td>
<td></td>
</tr>
<tr>
<td><em>A. vulcanalis</em></td>
<td>Geothermal pool, Coso hot springs, California</td>
<td>2.00–6.00 (4.00)</td>
<td>35–65 (55)</td>
<td>Aerobic</td>
<td>+ Rod</td>
<td>1.5–2.5 × 0.4–0.7</td>
<td>nr</td>
<td>Terminal or subterminal</td>
<td>nr</td>
<td>Semi-transparent to white</td>
<td>Convex</td>
<td>1.0</td>
<td>Simbahan et al., 2004</td>
<td></td>
</tr>
</tbody>
</table>

nr – not reported
A. acidocaldarius. Mice were injected intraperitoneally with a mixture of cells grown in orange serum broth and observed for one week for signs of illness. Guinea pigs were fed with spoiled apple juice containing 5 × 10⁵ cfu mL⁻¹. A. acidoterrestris and also observed for one week. No adverse symptoms, illnesses or deaths were observed in either the mice or the guinea pigs and it was concluded that Alicyclobacillus was not pathogenic at the levels tested. Although Alicyclobacillus pose an economic threat to the fruit processing industry, consumption of products containing Alicyclobacillus does not pose a health or safety risk (Borlinghaus and Engel, 1997; Walls and Chuyate, 2000b).

3.3. Membrane structure

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

The types of ω-alicyclic fatty acids found in the membranes of Alicyclobacillus are not limited to ω-cyclohexane fatty acids as they were also found to contain ω-cycloheptane fatty acids (Poralla and König, 1983; Deinhard et al., 1987b). Of the 23 species, subspecies and genomic species known to date, 14 possess predominantly ω-cyclohexane fatty acids in their membranes. These are A. acidocaldarius (Uchio and Doi, 1967; Wisotzkey et al., 1992), A. acidocaldarius subsp. acidocaldarius (Goto et al., 2006; Anonymous, 2009), A. acidoterrestris (Hippchen et al., 1981; Wisotzkey et al., 1992; Walls and Chuyate, 1998), A. hesperidum, Alicyclobacillus genomic species 1 (Albuquerque et al., 2000), Alicyclobacillus acidocaldarius species 2 (Goto et al., 2002a), A. acidocaldarius subsp. rittmannii (Nicolau et al., 1998), Alicyclobacillus acidiphilus (Matsubara et al., 2002), A. sendaiensis (Tsuruoka et al., 2003), Alicyclobacillus vulcanalis (Simbahan et al., 2004), A. tolerans (Karavaiko et al., 2005), A. disulfidooxidans (Dufresne et al., 1996; Karavaiko et al., 2005), A. fastidiosus and Alicyclobacillus sacchari (Goto et al., 2007). Four species of Alicyclobacillus, namely A. cycloheptanicus (Poralla and König, 1983; Deinhard et al., 1987b; Wisotzkey et al., 1992), A. herbarius (Goto et al., 2002b), Alicyclobacillus kakegawensis and Alicyclobacillus shizukosensis (Goto et al., 2007) possess predominantly ω-cycloheptane fatty acids.

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

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

3.4. Function of the ω-alicyclic fatty acids in the membrane

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

3.5. Heat resistance

Several studies have been conducted to investigate the heat resistance of Alicyclobacillus endospores under different conditions and in a variety of media. A summary of heat resistance parameters for strains of A. acidoterrestris in fruit products is given in Table 2. D₉₀-values determined for different strains of A. acidoterrestris in apple juice, grape juice, berry juice, orange juice, a fruit drink, a fruit nectar, concord grape juice, cupuaçu extract, grapefruit juice, mango pulp, clarified lemon juice and non-clarified lemon juice range from 1.00 to 9.98 min. The D₉₀-values in apple juice, grape juice, concord grape juice, orange juice, grapefruit juice, a clear apple drink, an orange drink, apple nectar without ascorbic acid, apple nectar with ascorbic acid and mango pulp range from 5.95 to 23.10 min (Table 2). The Z-values range from 6.90 to 21.27 °C in different fruit products (Splitstoesser et al., 1994; McIntyre et al., 1995; Komitopoulou et al., 1999; Bahçeci and Acar, 2007a; De Carvalho et al., 2008) and from 5.90 to 10.00 °C in buffers (Pontius et al., 1998; Murakami et al., 1998; Alpas et al., 2003; Bahçeci and Acar, 2007a).

Heat resistance values obtained in fruit products are higher when compared to those obtained in buffers at the same heating temperature and pH. This could be due to constituents of the fruit products that increase the heat resistance of endospores (Bahçeci and Acar, 2007a). The range of D-values observed between different studies may be attributed to differences in strains, sporulation temperature, nutrient composition and pH of the heating medium, water activity and the presence or absence of divalent cations and antimicrobial compounds (Bahçeci and Acar, 2007a).
Table 2
Heat resistance of *A. acidoterrestris* in various fruit juices and -concentrates.

<table>
<thead>
<tr>
<th>Heating medium</th>
<th>pH</th>
<th>SS (°Brix)</th>
<th>Strain</th>
<th>T (°C)</th>
<th>D-value ± SD/SE (min)</th>
<th>z-value (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple drink (clear)</td>
<td>nr</td>
<td>nr</td>
<td>AB-5</td>
<td>90</td>
<td>20.80</td>
<td>nr</td>
<td>Yamazaki et al., 2000</td>
</tr>
<tr>
<td>Apple juice</td>
<td>3.20</td>
<td>nr</td>
<td>nr</td>
<td>90</td>
<td>15.00</td>
<td>7.70</td>
<td>Cerny et al., 1984</td>
</tr>
<tr>
<td>Apple juice</td>
<td>3.50</td>
<td>11.40</td>
<td>VF</td>
<td>85</td>
<td>56.00 ± 14.00</td>
<td>12.20</td>
<td>Spittstoeisser et al., 1994</td>
</tr>
<tr>
<td>Apple juice</td>
<td>3.51</td>
<td>nr</td>
<td>Z CRA 7182</td>
<td>90</td>
<td>11.10 ± 1.60</td>
<td>8.50</td>
<td>Bahçeci and Acar, 2007b</td>
</tr>
<tr>
<td>Apple juice</td>
<td>3.68</td>
<td>12.20</td>
<td>DSM 2498</td>
<td>90</td>
<td>4.20 ± 0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple nectar without ascorbic acid</td>
<td>2.97</td>
<td>14.00</td>
<td>DSM 2498</td>
<td>90</td>
<td>14.40 ± 0.80</td>
<td>9.20</td>
<td></td>
</tr>
<tr>
<td>Blackcurrant concentrate</td>
<td>2.50</td>
<td>58.50</td>
<td>NCIMB 13137</td>
<td>91</td>
<td>24.10 ± 2.70</td>
<td>nr</td>
<td>Silva et al., 1999</td>
</tr>
<tr>
<td>Blackcurrant light concentrate</td>
<td>2.50</td>
<td>26.10</td>
<td>NCIMB 13137</td>
<td>91</td>
<td>3.84 ± 0.49</td>
<td>nr</td>
<td>Silva et al., 1999</td>
</tr>
<tr>
<td>Concord grape juice</td>
<td>3.50</td>
<td>16.00</td>
<td>WAC</td>
<td>85</td>
<td>53.00</td>
<td>6.90</td>
<td>Splittstoeisser et al., 1998</td>
</tr>
<tr>
<td>Cupuacu extract</td>
<td>3.60</td>
<td>11.30</td>
<td>NCIMB 13137</td>
<td>85</td>
<td>17.50 ± 1.10</td>
<td>9.00</td>
<td>Silva et al., 1999</td>
</tr>
<tr>
<td>Fruit drink</td>
<td>3.50</td>
<td>4.80</td>
<td>nr</td>
<td>95</td>
<td>5.20</td>
<td>10.80</td>
<td>Baumgart et al., 1997</td>
</tr>
<tr>
<td>Fruit nectar</td>
<td>3.50</td>
<td>6.10</td>
<td>nr</td>
<td>95</td>
<td>5.10</td>
<td>9.60</td>
<td>Baumgart et al., 1997</td>
</tr>
<tr>
<td>Grape juice</td>
<td>3.30</td>
<td>15.80</td>
<td>WAC</td>
<td>85</td>
<td>57.00 ± 13.00</td>
<td>7.20</td>
<td>Splittstoeisser et al., 1994</td>
</tr>
<tr>
<td>Grapefruit juice</td>
<td>3.42</td>
<td>nr</td>
<td>Z CRA 7182</td>
<td>80</td>
<td>37.87 ± 0.20</td>
<td>11.60</td>
<td>Kometopoulou et al., 1999</td>
</tr>
<tr>
<td>Lemon juice/ concentrate (clarified)</td>
<td>2.28</td>
<td>50.00</td>
<td>nr</td>
<td>82</td>
<td>17.36</td>
<td></td>
<td>Maldonado et al., 2008</td>
</tr>
<tr>
<td></td>
<td>2.80</td>
<td>50.00</td>
<td>nr</td>
<td>82</td>
<td>25.81</td>
<td></td>
<td>Maldonado et al., 2008</td>
</tr>
<tr>
<td></td>
<td>3.50</td>
<td>50.00</td>
<td>nr</td>
<td>82</td>
<td>33.66</td>
<td></td>
<td>Maldonado et al., 2008</td>
</tr>
<tr>
<td></td>
<td>9.80</td>
<td>nr</td>
<td>82</td>
<td>86</td>
<td>11.23</td>
<td></td>
<td>Maldonado et al., 2008</td>
</tr>
<tr>
<td></td>
<td>6.20</td>
<td>nr</td>
<td>82</td>
<td>86</td>
<td>13.21</td>
<td></td>
<td>Maldonado et al., 2008</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>50.00</td>
<td>nr</td>
<td>82</td>
<td>21.95</td>
<td></td>
<td>Maldonado et al., 2008</td>
</tr>
</tbody>
</table>
Since *A. acidoterrestris* is the *Alicyclobacillus* species mostly associated with spoilage, most studies have focused on the investigation of the heat resistance of this species. However, Palop et al. (2000) investigated the heat resistance of *A. acidocaldarius* in McIlvaine buffer with different pH values, as well as in distilled water and orange juice. No significant differences were observed in the heat resistance of *A. acidocaldarius* between the different heating media, with recorded D-120-values of 0.087–0.11 min. z-values also did not differ significantly and ranged between 6.50 and 7.50 °C. Thus, neither the pH, nor the composition of the heating media, affected the heat resistance at any of the evaluated temperatures. This strain of *A. acidocaldarius* was significantly more heat resistant than *A. acidoterrestris* strains investigated by other researchers (Splittstoesser et al., 1994; McIntyre et al., 1995; Murakami et al., 1998; Pontius et al., 1998; Splittstoesser et al., 1998; Eiroa et al., 1999), but had z-values comparable to those obtained by these authors, indicating a similar thermodependence.

Endospores of a variety of *A. acidoterrestris* strains and a strain of *A. acidocaldarius* are sufficiently heat resistant to enable them to survive the hot-fill-hold pasteurisation process to which fruit juice and similar products are exposed in order to render them commercially sterile (Baumgart et al., 1997; Pontius et al., 1998; Palop et al., 2000; Maldonado et al., 2008). The process parameters may vary between manufacturers, but typically involves heating the product to 90–95 °C for 15–20 s, followed by package filling while the product cools to 82–84 °C. The product is then held at this temperature for approximately 2 min before chilling (Solberg et al., 1990). Due to its high heat resistance and involvement in several spoilage incidents, it has been suggested that *A. acidoterrestris* be designated the target microbe in the design of pasteurisation processes for acidic foods and beverages (Silva et al., 1999; Silva and Gibbs, 2004).

### 3.6. Factors influencing heat resistance

#### 3.6.1. Temperature

Temperature has the greatest influence on D-values, with a nonlinear decrease in D-values (indicating a decreased heat resistance) observed with an increase in temperature (Silva et al., 1999; Bahçeci and Acar, 2007a; Maldonado et al., 2008). The effect of temperature on D-values is greater than that of pH and slight changes in
temperature may have a considerable effect on D-values (Silva et al., 1999; Bahceci and Acar, 2007a). In a study by Silva et al. (1999) a temperature increase of 2 °C (from 95 to 97 °C) caused the D-value to decrease from 2.82 to 0.57 min. Temperature also affects the role that other parameters such as pH and SS play in the overall effect on D-values, as their effects are more pronounced at lower temperatures. Manufacturers need to also take these effects into account when processing at a lower temperature (Pontius et al., 1998; Komitopoulou et al., 1999; Silva et al., 1999).

3.6.2. pH

It has been reported that pH had an effect on heat resistance, with a linear decrease in D-values being observed with a decrease in pH (Silva et al., 1999). This effect seems to be more pronounced at lower processing temperatures of 80—90 °C (Pontius et al., 1998; Komitopoulou et al., 1999, Silva et al., 1999). In contrast to this, Murakami et al. (1998) found that pH did not have a significant influence on heat resistance, as there were no significant differences between D-values of A. acidoterrestris AB-1 endospores in McIlvaine buffer at pH values ranging from 3.00 to 8.00 at a given temperature. Temperature and specific properties of different juices seem to play a bigger role than pH in contributing to heat sensitivity, as A. acidoterrestris still had a lower heat resistance in grapefruit juice than in orange juice, even though grapefruit juice had a slightly higher pH of 4.00 compared to 3.90 of the orange juice (Komitopoulou et al., 1999). Furthermore, the type of acid used to acidify the heating medium does not influence the heat resistance, as the D-values obtained in a model fruit juice system acidified with malic, tartaric or citric acids did not differ significantly from one another in the temperature range (91—100 °C) studied (Pontius et al., 1998).

3.6.3. Soluble solids (SS) content

The total SS also influences the heat resistance of species of Alicyclobacillus. There is a linear relationship (Silva et al., 1999) between SS and D-values, with an increase in SS content leading to an increase in D-values and a higher heat resistance. Therefore, destruction of endospores would be more difficult in juice concentrate than in single strength juice (Silva et al., 1999; Bahceci and Acar, 2007a). The study by Silva et al. (1999) also showed that the effect of SS became less pronounced as temperatures increased from 85 to 97 °C, with no effect being observed at 97 °C. Silva et al. (1999) suggested that water activity, rather than total SS, should be measured, as different sugars and citric acids did not differ significantly from one another in the temperature range (91—100 °C) studied (Pontius et al., 1998).

3.6.4. Alicyclobacillus species/strain

Different strains of A. acidoterrestris differ in their heat resistance. A study on three A. acidoterrestris strains (VF, WAC and IP) showed that in a model fruit juice system acidified with malic acid to pH 3.70, strains VF and WAC had approximately the same heat resistance, while strain IP was less heat resistant (Pontius et al., 1998). In McIlvaine buffer at pH 4.00, strain AB-1 (Murakami et al., 1998) was approximately twice as heat resistant as strain DSM 2498 (Bahceci and Acar, 2007a). In orange juice (pH 3.15, 9.00 °Brix) the heat resistances of four A. acidoterrestris strains (46, 780, 145 and the type strain DSM 2498) were studied (Eiroa et al., 1999). Strains DSM 2498 and 46 had similar D-values, while strains 145 and 70 were more heat resistant. It has been suggested that differences in the heat sensitivity of different Alicyclobacillus species can be correlated with differences in sporulation temperature, pH, nutrient composition of the heating medium, and water activity (Bahceci and Acar, 2007a).

3.6.5. Divalent cations

Divalent cations can also influence the heat resistance of endospores. Mineralisation of endospores with divalent cations, such as calcium or manganese, contributes to the stabilisation of endospores against heat (Bender and Marquis, 1985). Calcium also chelates dipicolinic acid (DPA) to form Ca-DPA, which further stabilises endospores and contributes to heat resistance (Yamazaki et al., 1997). A. acidoterrestris endospores bind Ca2+ and Mn2+ more strongly at a low pH compared to Bacillus species and are also able to keep Ca-DPA levels constant. Thus, stabilisation of Ca-DPA concentrations and the ability to strongly bind divalent cations contribute to the heat resistance of A. acidoterrestris endospores (Yamazaki et al., 1997).

3.6.6. Sporulation temperature

It has been reported that incubation of cultures at a higher temperature could increase the heat resistance of endospores (Jay et al., 2005a; Palop et al. (2000) found this to be true for A. acidocaldarius, as an approximately linear increase in D110-values (and thus heat resistance) was observed when the sporulation temperature was increased from 45 to 65 °C. Even with the decreased heat resistance observed at the lower sporulation temperature, A. acidoterrestris was still approximately 30 times more heat resistant than A. acidoterrestris that had sporulated at the same temperature (Palop et al., 2000), indicating the greater thermophilic properties of this species. As A. acidoterrestris is the main species implicated in spoilage it may be that A. acidoterrestris is more abundant than A. acidocaldarius as both species have the potential to cause spoilage.

3.6.7. Heat resistance prediction models

Models have been developed for predicting the D-values of A. acidoterrestris using response surface methodology. However, the predicted values were consistently lower than those observed in real fruit systems. The models made use of McIlvaine buffer or malt extract broth (MEB) as heating medium. The difference between the predicted and observed D-values could be attributed to other components present in the fruit products that could increase the heat resistance of A. acidoterrestris endospores. Literature indicates that thermal inactivation studies using buffers produce results that are generally not well correlated with studies of the same organism in a food matrix. Further challenge tests and model validation studies are, therefore, recommended by researchers before the prediction models can be used in industry (Silva et al., 1999; Bahceci and Acar, 2007a).

4. Spoilage

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

Alicyclobacillus related problems are relatively widespread, as a survey conducted by the Grocery Manufacturers Association (formerly National Food Processors Association) of the USA in 1998 showed that out of the 60% of companies that responded to the survey (34 out of 57), 35% had experienced spoilage incidents consistent with the presence of acidophilic endospore-formers such as Alicyclobacillus (Walls and Chuyate, 1998). Most companies
had experienced one or two such spoilage incidents in the five years preceding the survey, with apple juice being the product most often involved. Spoilage incidents occurred in spring or summer and spoilage was mainly apparent as an off-flavour or -odour, with or without sediment (Duong and Jensen, 2000) and in some products discolouration or cloudiness occurred. Consumer complaints were often the only reason for companies becoming aware of the problem, since the absence of gas production (Splittstoesser et al., 1994; Duong and Jensen, 2000) made spoilage difficult to detect (Walls and Chuyate, 1998). The European Fruit Juice Association conducted a survey in 2005 amongst a total of 68 participants involved in various areas of the fruit processing industry, including packers, producers andanners (Howard, 2006). Forty five percent of the respondents had experienced Alicyclobacillus related problems in the three years preceding the survey, with 33% of these experiencing more than three incidents. Of those that had experienced spoilage problems, 35% of the incidents were reported as being immediately to majorly severe. Problems occurred primarily in apple raw materials and the type of product involved was primarily concentrates (Howard, 2006).

The off-flavour and -odour caused by Alicyclobacillus, having in most cases been identified as the chemical compound guaiacol (Yamazaki et al., 1996a; Jensen, 2000; Gocmen et al., 2005; Siegmund and Pöllinger-Zierler, 2006), has been described as medicinal, disinfectant-like, antiseptic, phenolic, smoky and hammy (Wasserman, 1966; Duong and Jensen, 2000; Pettipiper and Osmundson, 2000; Gocmen et al., 2005). Although guaiacol seems to be the dominant cause of taint, the halophenols 2,6-dichlorophenol (2,6-DCP) (Jensen and Whitfield, 2003; Gocmen et al., 2005) and 2,6-dibromophenol (2,6-DBP) (Borlinghaus and Engel, 1997; Jensen, 2000; Gocmen et al., 2005; Siegmund and Pöllinger-Zierler, 2006) have also been implicated.

A. acidoterrestris is the species primarily responsible for spoilage incidents (Yamazaki et al., 1996; Walls and Chuyate, 1998; Jensen and Whitfield, 2003) although other species, including A. acidiphilus (Matsubara et al., 2002; Goto et al., 2008), A. pomorum (Goto et al., 2003), A. hesperidum, A. herbarius (Goto et al., 2008), A. cycloheptanicus (Gocmen et al., 2005) and A. acidocaldarius (Gouws et al., 2005) have also been implicated due to their ability to produce taint compounds or because they were isolated from spoiled products. A recent report has identified A. acidocaldarius as the causative microbe in the spoilage of non-concentrated tomato products. Although no guaiacol was detected, 2-methyltetrahydrothiophene-3-one was identified using gas-chromatography mass-spectrometry (GC-MS) as being the compound responsible for the off-flavour (Lottici et al., 2006). Thus, research should not only focus on A. acidoterrestris and guaiacol production, but should be broadened to include other Alicyclobacillus species and taint compounds.

4.1. Guaiacol (2-methoxyphenol)

The predominant metabolite associated with spoilage by Alicyclobacillus is guaiacol (2-methoxyphenol) (Orr et al., 2000). Guaiacol is a well documented flavour compound, as it contributes to the smoky flavour of products such as arabica coffee (Mayer et al., 1999) and smoked salmon (Varlet et al., 2006). However, it is better known for its association with off-flavour spoilage in products such as wine (Simpson et al., 1986; Álvarez-Rodríguez et al., 2003), chocolate milk (Jensen et al., 2001), chocolate ice-cream (Saxby, 1996), vanilla yoghurt (Whitfield, 1958) and fruit juices (Cerny et al., 1984; Splittstoesser et al., 1994; Walls and Chuyate, 1998).

4.1.1. Microbial metabolic production pathway of guaiacol

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

Although the precise metabolic production pathway for guaiacol in Alicyclobacillus has not been completely elucidated, the most common hypothesis is that guaiacol is produced during ferulic acid metabolism. Ferulic acid is ubiquitous in nature and is found in fruits, vegetables, grains, beans, leaves, seeds, nuts, grasses and flowers (Rosza et al., 1995). It is also a component of the structural plant cell wall polymer, lignin, as it cross-links this compound to plant cell wall polysaccharides (Kirk, 1971; Crawford and Crawford, 1980; Provan et al., 1994; Mathew and Abraham, 2004). The ability to metabolise ferulic acid to various products has been observed in yeasts (Huang et al., 1993b; Donaghy et al., 1999; Mathew et al., 2007), fungi (Nazareth and Mavinkurve, 1986; Rahouti et al., 1989; Topakas et al., 2003) and other bacteria (Karmakar et al., 2000). In most microbes the first step of ferulic acid metabolism is its decarboxylation to 4-vinylguaiacol (Rahouti et al., 1989; Topakas et al., 2003; Mathew et al., 2007), although it can also be directly transformed to vanillin (Peleg et al., 1992) or vanillic acid (Huang et al., 1993a) without the production of 4-vinylguaiacol. If it is not metabolised further, 4-vinylguaiacol can cause the unpleasant off-flavour described as “old fruit” or “rotten” (Tatum et al., 1975) in improperly stored citrus products, especially orange juice (Tatum et al., 1975; Naim et al., 1988; Rouseff et al., 1992). Most microbes metabolise 4-vinylguaiacol further to vanillin and subsequently vanillic acid (Nazareth and Mavinkurve, 1986; Rahouti et al., 1989; Karmakar et al., 2000), although it can also be converted directly to vanillin acid (Topakas et al., 2003). Vanillin is normally metabolised rapidly through oxidation or reduction to vanillic acid and vanillyl alcohol, respectively, as it has been shown to have a toxic effect on microbes above certain concentrations (Ander et al., 1980; Cerrutti et al., 1997; Alzamora et al., 2003; Char et al., 2009). The vanillic acid that is formed can then be converted to a number of products, including methoxyhydroquinone, protocatechuic acid and guaiacol. Guaiacol is produced from vanillic acid through a non-oxidative decarboxylation reaction (Crawford and Olson, 1978; Rahouti et al., 1989; Topakas et al., 2003) and can subsequently be transformed to other products, most often catechol (Rahouti et al., 1989; Topakas et al., 2003). A. acidoterrestris is able to produce guaiacol from vanillin (Bahceci et al., 2005a; Bahçeci and Acar, 2007b) and vanillic acid (Niwa and Kuriyama, 2003). The conversion of vanillic acid to guaiacol is more rapid than that of vanillin, which is in agreement with the identification of vanillic acid as the immediate precursor to guaiacol in the metabolic pathway. The ability of Alicyclobacillus species to produce guaiacol from other precursors, such as ferulic acid or lignin, has not been investigated. The metabolism of ferulic acid and subsequent formation of guaiacol and other products in microbes is presented in Fig. 1.
Fig. 1. Microbial production pathways of guaiacol and other products through the metabolism of ferulic acid (Crawford and Olson 1978; Pometto et al., 1981; Rahouti et al., 1989; Huang et al., 1993a; Rosazza et al., 1995; Karmakar et al., 2000; Topakas et al., 2003).
Jensen (2000) suggested that the amino acid tyrosine could be another possible precursor for guaiacol formation. Apple and orange juice contains approximately 4.10 µg mL$^{-1}$ and 3.40–13.50 µg mL$^{-1}$ tyrosine, respectively, which should be sufficient to allow the synthesis of detectable amounts of guaiacol under conditions favourable for growth and taint formation (Jensen, 2000). However, this theory has not been widely investigated and the most widely accepted guaiacol synthetic pathway is that of lignin degradation (Chang and Kang, 2004).

4.1.2. Detection of guaiacol

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

4.1.2.1. Sensory methods. Several studies have determined the sensory odour and taste thresholds for guaiacol in water and apple juice. One of the earliest reports was by Wasserman (1966) who determined taste and odour thresholds of 13.00 ppb and 21.00 ppb, respectively, for guaiacol in water. In more recent reports the threshold values were much lower, with Pettipher et al. (1997) and Orr et al. (2000) reporting best estimated threshold (BET) values of approximately 2.00 ppb for taste in apple, orange and a non-carbonated fruit juice and 2.32 ppb for odour in apple juice. Eisele and Semon (2005) reported even lower values, determining BET values of 0.17 ppb and 0.24 ppb for taste in water and apple juice, respectively and 0.48 ppb and 0.91 ppb for odour in water and apple juice, respectively. Siegmund and Pöllinger-Zierler (2006) reported an odour threshold for guaiacol in apple juice of 0.57 ppb. They also determined the odour recognition threshold for guaiacol in apple juice to be 2.00 ppb. The odour threshold for guaiacol has also been determined in other substances, Jensen et al. (2001) determined an odour threshold of 43.00 ng mL$^{-1}$ for guaiacol in chocolate milk, while the odour threshold for guaiacol in red wine was determined to be 9.50 µg L$^{-1}$ (Ferreira et al., 2000).

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

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

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

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

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

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

Gas-chromatography is coupled to detection systems such as flame ionisation detection (GC-FID) (Bieniek, 2003), diolfactometry (GC-O) (Lee and Noble, 2003; Gocmen et al., 2005) and mass-spectrometry (GC-MS) (Pettipher et al., 1997; López et al., 2002; Lee and Noble, 2003; Gocmen et al., 2005). Mass-spectrometry is most often used as it is a very specific, accurate and sensitive detection method. Gocmen et al. (2005) found that while the guaiacol, 2,6-DCP and 2,6-DBP peaks on a GC-FID chromatogram were almost undetectable, their corresponding aroma peaks on a GC-O chromatogram were relatively large, indicating a considerable impact on the juice aroma and emphasising the value of GC-O for identifying and characterising aroma compounds in a complex food matrix.

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

4.1.2.3. Chemical methods. A third method for the detection of guaiacol in products makes use of a colourimetric assay based on the
oxidation of guaiacol by peroxidase enzymes in the presence of H$_2$O$_2$. During this reaction a brown component is formed which has been identified as 3,3’-dimethoxy-4,4’-biphenolquinone (Doerge et al., 1997) and the change in absorbance can be measured spectrophotometrically at 420 nm (Bahçeci et al., 2005a,b) or 470 nm (Doerge et al., 1997; Niwa and Kawamoto, 2003; Niwa and Kuriyama, 2003). This reaction is widely used in assays testing for peroxidase enzyme activity (Doerge et al., 1997; Bahçeci et al., 2005b) and has also formed the basis of the development of a guaiacol detection kit (Niwa and Kawamoto, 2003; Niwa and Kuriyama, 2003). The kit, manufactured by the Kyokuto Pharmaceutical Industrial Company Ltd. from Japan and distributed by Cosmo Bio Company Ltd. (Anonymous, 2006), makes use of a heat shock treatment. Various heat shock treatments have been applied to determine the presence of guaiacol or guaiacol producing Alicyclobacillus species in a product (Niwa and Kawamoto, 2003; Niwa and Kuriyama, 2003; Niwa, 2004). A similar kit has also been developed by the Döhler Group in Germany (Anonymous, 2006). It differs from the Japanese product in that it makes use of B. acidoterrestris (BAT) medium instead of YSG medium for incubation. The chemical method is both qualitative and quantitative, as the guaiacol concentration in a sample can be quantified by comparing the absorbance of the sample to a standard curve of the absorbance of known guaiacol concentrations.

4.2. Halophenols

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

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

In most cases, production of the halophenols was detected in combination with guaiacol production (Gocmen et al., 2005), but they have also been detected in the absence of guaiacol (Baumgart et al., 1997; Borlinghaus and Engel, 1997; Jensen, 2000). The production of the halophenols also seems to be strain or species specific (Gocmen et al., 2005). Gocmen et al. (2005) found that, along with the production of guaiacol by all three strains, A. cycloheptanicus was also able to produce both 2,6-DCP and 2,6-DBP, while A. acidoterrestris and A. hesperidum produced 2,6-DBP and 2,6-DCP, respectively. In some cases the production of these compounds was time dependent, as A. cycloheptanicus had produced only guaiacol and 2,6-DBP by day 14 of the study, but by the 28th day 2,6-DCP was also present.

4.3. Factors influencing taint production

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

The presence of Alicyclobacillus in acidic products will, however, not necessarily lead to spoilage of the products. Even though cell concentrations as high as 10$^5$ cfu mL$^{-1}$ were recorded in some of the fruit juices and -concentrates analysed by Pinhatti et al. (1997), none of the products were spoiled. Pettipher et al. (1997) also observed cell concentrations higher than 10$^5$ cfu mL$^{-1}$ in some products without spoilage being observed. These results indicate that there are other factors that play a role in the spoilage of acidic products by Alicyclobacillus. The following factors have been identified.

4.3.1. Cell concentration

Several studies have found that there is a critical Alicyclobacillus cell concentration that must be present before taint compounds are produced in detectable concentrations. Pettipher et al. (1997) established this cell concentration to be 10$^5$ cfu mL$^{-1}$, while Bahçeci et al. (2005a) reported that a concentration of 10$^4$ cfu mL$^{-1}$ was necessary for detectable guaiacol production to occur. In apple juice inoculated with 10$^5$ cfu mL$^{-1}$ A. acidoterrestris, guaiacol production started immediately, while in apple juice inoculated with 10$^4$ cfu mL$^{-1}$, guaiacol production only started after approximately 30 h, once a cell concentration of 10$^5$ cfu mL$^{-1}$ had been reached (Bahçeçi et al., 2005a).

4.3.2. Temperature

The rate of taint production seems to increase with an increase in the incubation temperature. Pettipher et al. (1997) found that in juice stored at 25 °C guaiacol production started after 6–10 d, while in juice stored at 44 °C, guaiacol was produced within 3–6 d. Bahçeçi et al. (2005a) found that in apple juice inoculated with 10$^3$–10$^4$ cfu mL$^{-1}$ A. acidoterrestris, maximum guaiacol concentrations were formed after 75 h in cultures stored at 46 °C, while little or no guaiacol was detected in cultures stored at 25 °C. In a juice inoculation study conducted by Jensen (2000), higher concentrations of guaiacol were produced by A. acidoterrestris at 46 °C than at 37 °C. Siegmund and Pöllinger-Zierler (2007) also had similar results, with guaiacol and 2,6-DBP production at concentrations high enough to cause spoilage being observed after 20 d at room temperature (average 21.5 °C) compared to only 15 d at 30 °C.

4.3.3. Heat shock

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

4.3.4. Growth medium/susceptible beverages

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

4.3.5. Headspace/oxygen availability

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

5. Sources of contamination

Soil is one of the primary isolation sources of Alicyclobacillus species (Hippchen et al., 1981; Nicolaus et al., 1998; Albuquerque et al., 2000; Tsuruoka et al., 2003; Goto et al., 2007; Groenewald et al., 2008; Imperio et al., 2008) and thought to be the most important source of contamination of acidic products with these microbes. Soil can cling to fruit that have fallen on the ground and can also be carried into processing facilities by employees. Groenewald et al. (2008) isolated A. acidoterrestis and A. acidocaldarius from the soil of apple and pear orchards in South Africa. Parish and Goodrich (2005) investigated the occurrence of Alicyclobacillus on oranges entering the processing environment at two citrus processing facilities. Although the presence of Alicyclobacillus was widespread, as more than 30% of the sampled oranges were contaminated, they found that the contamination rate was significantly lower at the facility that did not use oranges picked up from the ground.

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

6. Isolation and enumeration

Species of Alicyclobacillus have been isolated from thermal acidic environments (Uchino and Doi, 1967; Darland and Brock, 1971; Simbahan et al., 2004), various types of soil (Hippchen et al., 1981; Nicolaus et al., 1998; Groenewald et al., 2008), herbal tea (Goto et al., 2002b), iced tea and its ingredients (Duong and Jensen, 2000), fruit juices and acidic beverages (Cerny et al., 1984, 1985; Pinhatti et al., 1997; Walls and Chuyate, 1998), fruit juice concentrates (Pinhatti et al., 1997; Gouws et al., 2005), fruit surfaces (Parish and Goodrich, 2005), fruit juice ingredient water (McIntyre et al., 1995), fruit wash water (Wisse and Parish, 1998; Chen et al., 2006), liquid sugar (Goto et al., 2007) and leaves (Goto et al., 2006) using a variety of different media. Not all media are able to support the growth of A. acidoterrestris, including nutrient agar, tryptone soy agar, brain heart infusion agar, standard plate count agar and veal infusion agar, even when these media are acidified to pH 3.50 (Splittstoesser et al., 1994, Pettipher et al., 1997).

Uchino and Doi (1967) used a simple medium consisting of 20 g peptone, 5 g yeast extract and 10 g glucose per litre distilled water at pH 4.00 to isolate thermo-acidophilic microbes, later identified as Alicyclobacillus, from acid hot springs. Since then a variety of media has been used and new media developed for the isolation and enumeration of Alicyclobacillus, the compositions of which are summarised in Table 3.

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

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

B. acidocaldarius medium (BAM) was originally proposed by Deinhard et al. (1987a), who combined the synthetic salt medium used by Darland and Brock (1971) and the trace element solution proposed by Farrand et al. (1983). BAM was used to isolate A. hesperidum, Alicyclobacillus genomic species 1 (in combination with a membrane filtration step) (Albuquerque et al., 2000), Alicyclo- bacillus genomic species 2 (Goto et al., 2002a) and A. cycloheptanicus (on SM agar plates, a modification of BAM plates where 3 g L⁻¹ instead of 1 g L⁻¹ yeast extract is added and the pH is adjusted to 4.30) (Deinhard et al., 1987a,b). It was also used in growth and characterisation experiments during the isolation of A. herbarius.
A standard method developed by the Working Group on Microbiology of the International Federation of Fruit Juice Producers (IFU), the IFU Method No. 12, includes the use of *B. acidoterrestris* medium (BAT), also called *B. acidoterrestris* Producers (IFU), the IFU Method No. 12, includes the use of Microbiology of the International Federation of Fruit Juice (Murray et al., 2007) for the isolation and detection (Murray et al., 2007) found that BAT was more efficient than BAM at recovering species.

### Table 3

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Composition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alicyclobacillus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acidocaldarius medium</td>
<td>AAM</td>
<td>0.25 g CaCl$_2$, 2H$_2$O, 0.50 g MgSO$_4$, 7H$_2$O, 0.20 g (NH$_4$)$_2$SO$_4$, 0.60 g</td>
<td>Yamazaki et al., 1996a</td>
</tr>
<tr>
<td><em>Alicyclobacillus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>medium</td>
<td>ALI</td>
<td>0.25 g CaCl$_2$, 2H$_2$O, 0.50 g MgSO$_4$, 7H$_2$O, 0.20 g (NH$_4$)$_2$SO$_4$, 3.00 g</td>
<td>Wisse and Parish, 1998</td>
</tr>
<tr>
<td><em>Bacillus</em> acidocaldarius</td>
<td>BAM/BAT</td>
<td>Basal medium: 0.25 g CaCl$_2$, 2H$_2$O, 0.50 g MgSO$_4$, 7H$_2$O, 0.20 g (NH$_4$)$_2$SO$_4$, 3.00 g</td>
<td>Deinhard et al., 1987a; IFU, 2007</td>
</tr>
<tr>
<td>medium/Bacillus acidoterrestris</td>
<td></td>
<td>0.25 g CaCl$_2$, 2H$_2$O, 2.0 g yeast extract, 1.0 g glucose and 2.0 g soluble starch per litre distilled water. Acidified to pH 3.50 with 1 N H$_2$SO$_4$ prior to autoclaving. For ALI agar ALI broth is prepared at twice the concentration and mixed with an equal volume of 1.50% (m/v) agar separately. Autosolubilizes the two solutions separately.</td>
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<tr>
<td>Orange serum agar</td>
<td>OSA (modified)</td>
<td>10.00 g yeast extract, 5.00 g peptone, 1.00 g glucose, 1.00 mL Tween 80, 15.00 g agar in 990 mL distilled water. Filter sterilised 25% (m/v) malic acid solution is used to adjust the pH to 3.70 after autoclaving.</td>
<td></td>
</tr>
<tr>
<td>Potato dextrose agar</td>
<td>PDA</td>
<td>4.00 g potato extract, 2.0 g dextrose, 1.0 g glucose, 1.00 mL Tween 80 and 15 g agar in 1 L distilled water. After autoclaving, filter sterilised 10% (m/v) CaCl$_2$ is added to achieve a final concentration of 0.50 g L$^{-1}$. Filter sterilised 10% (m/v) tartaric acid is used to adjust the pH to 4.00.</td>
<td></td>
</tr>
<tr>
<td>SK agar</td>
<td>SK</td>
<td>2.50 g yeast extract, 5.00 g peptone, 1.00 g glucose, 1.00 mL Tween 80 and 15 g agar in 1 L distilled water. After autoclaving, filter sterilised 10% (m/v) CaCl$_2$ is added to achieve a final concentration of 0.50 g L$^{-1}$. Filter sterilised 10% (m/v) tartaric acid is used to adjust the pH to 4.00.</td>
<td></td>
</tr>
<tr>
<td>Yeast starch glucose</td>
<td>YSG</td>
<td>2.00 g yeast extract, 2.0 g soluble starch, 1.00 g glucose and 15.00 g agar (when used as a solid medium) per litre distilled water. Acidified to pH 3.70.</td>
<td>Goto et al., 2002a</td>
</tr>
</tbody>
</table>

A standard method developed by the Working Group on Microbiology of the International Federation of Fruit Juice Producers (IFU), the IFU Method No. 12, includes the use of *B. acidoterrestris* medium (BAT), also called *B. acidoterrestris* thermophilic medium (Murray et al., 2007) for the isolation and enumeration of *Alicyclobacillus* (IFU, 2007). The medium described in this method has the same composition as BAM, with the exception of the amount of yeast extract, which is 2 g L$^{-1}$ instead of 1 g L$^{-1}$ (IFU, 2007). However, some research papers stating the use of BAM uses the formula for BAT, with 2 g L$^{-1}$ yeast extract (Silva et al., 1999, 2000). Even though the compositions of the two media are virtually identical, Pacheco (2002) and Murray et al. (2007) found that BAT was more efficient than BAM at recovering *Alicyclobacillus* endospores. Deinhard et al. (1987a), who first proposed BAM, were also quoted by Pacheco (2002) as the original developers of BAT. Thus, the reason for the distinction between the two media is unclear.

Yamazaki et al. (1996) made use of *Alicyclobacillus acidocaldarius* medium (AAM) to isolate *A. acidoterrestris* from spoiled acidic juices, isotonic water, lemonade, a fruit juice blend and a fruit-carrot juice blend. It was also used for the cultivation of *A. acidoterrestris* in studies investigating the effect of the bacteriocins enterocin AS-48 (Grande et al., 2005) and bovicin HC5 (De Carvalho et al., 2008) on the survival of *A. acidoterrestris* in fruit products. This medium only differs from BAM in that it contains no trace element solution and 0.6 g L$^{-1}$ H$_2$PO$_4$ and 1 g L$^{-1}$ glucose instead of 3 g L$^{-1}$ and 5 g L$^{-1}$, respectively. Murakami et al. (1998) used solidified (by adding 1.50% (m/v) agar) AAM containing 0.05% (m/v) MnCl$_2$-4H$_2$O as a sporulation medium for *A. acidoterrestris*.

Wisse and Parish (1998) modified the media used by Darland and Brock (1971) and Cerny et al. (1984) to produce *Alicyclobacillus* (ALI) medium and agar. ALI medium has the same composition as BAM, except that it contains no trace element solution, 2 g L$^{-1}$ yeast extract and 1 g L$^{-1}$ glucose instead of 3 g L$^{-1}$ and 5 g L$^{-1}$, respectively, as well as 2 g L$^{-1}$ soluble starch. ALI medium and agar were used to isolate presumptive *Alicyclobacillus* species from soil, unwashed and washed fruit surfaces, condensate water, fruit juice concentrates and fruit nectars at 10 different citrus processing plants (Wisse and Parish, 1998). ALI agar performed well when compared to other media for its suitability to isolate *Alicyclobacillus* (Parish and Goodrich, 2005; Murray et al., 2007).

6.2. Yeast starch glucose (YSG) agar

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

6.3. Hiraiishi glucose yeast extract (HGYE) agar

This chemically defined medium was used by Japanese researchers for the cultivation of Alicyclobacillus that had been isolated from Japanese hot springs (Hiraishi et al., 1997). However, it performed poorly when compared to other isolation media frequently used for Alicyclobacillus (Murray et al., 2007). The authors suggested that the low pH of the medium could have played a role in the poor recovery rates.

6.4. Potato dextrose agar (PDA)

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

6.5. Orange serum agar (OSA)

OSA has been used as early as the 1950s for the cultivation and enumeration of microbes associated with citrus product spoilage (Hays and Riester, 1952), and is still often used for citrus product spoilage studies (Chang and Kang, 2004). Hays and Riester (1952) used OSA (pH 5.50) to study off-flavour spoilage in frozen concentrated orange juice. Pettipher et al. (1997) found that spread plating onto OSA gave optimum recovery of Alicyclobacillus when compared to PDA and BAM, although the latter two media also strongly supported the growth of Alicyclobacillus. Jensen (1999, 2000) observed improved growth when approximately 0.50% (m/v) sucrose was added to OSA.

6.6. K agar

Walls and Chuyate first proposed the use of K agar in 1998. They found that it was superior in its recovery of Alicyclobacillus species and that it improved the growth rate of the microbes when compared to a variety of other media, including OSA, tomato juice agar special (TJAS), PDA (pH 3.50, 4.00, 4.50 and 5.00) and dextrose tryptone agar (DTA) (pH 7.40). When compared to the minimal salts medium (pH 4.00) of Farrand et al. (1983), the semi-synthetic medium (pH 4.00) of Darland and Brock (1971) (on which the formulation of BAM, BAT, AAM and ALI agar is based) and OSA (pH 3.50, adjusted with HCl) for isolation of A. acidoterrestris from apple juice, orange juice and a fruit juice blend (containing mainly white grape juice), K agar and the semi-synthetic medium had comparable recoveries which were significantly higher than OSA and the minimal salts medium (Walls and Chuyate, 2000a). K agar has also been included in the IFU Method No. 12 for isolating predominantly A. acidoterrestris (IFU, 2007).

6.7. SK agar

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

6.8. Comparisons between different isolation media

Several studies have been done to compare different isolation media for Alicyclobacillus. Some authors (Pettipher et al., 1997; Pettipher and Osmundson, 2000) found that BAM, PDA and OSA all performed well in supporting the growth of A. acidoterrestris, with OSA giving the highest recovery. Spread plating was found to be more effective than pour plating. Orr and Beuchat (2000) found that K agar was most effective at supporting the development of chemically treated A. acidoterrestris endospores when compared to OSA (pH 5.00) and acidified PDA, while Parish and Goodrich (2005) found that ALI agar was more effective than K agar (pH 3.70) and PDA (pH 3.70) at recovering Alicyclobacillus from the surfaces of oranges. According to Jensen (2005b), BAT agar was more effective at recovering A. acidocaldarius, while the use of K agar gave good results when isolating A. acidoterrestris.

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

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

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

### 6.9. Membrane filtration

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

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

### 6.10. Heat shock treatment

Since *Alicyclobacillus* species are endospore-formers, isolation procedures are often combined with a heat shock treatment in order to activate dormant endospores and encourage germination and enumeration. Cell concentrations are often higher after a heat shock treatment if the microbes are mostly present as endospores. Splittstoesser et al. (1998) found that a heat treatment of 60 °C for 30 min doubled the viable counts of a sample containing *A. acidoterrestris*, indicating that about 50% of the cells had been present in the form of endospores. Wittthuhn et al. (2007) also observed higher cell concentrations after subjecting samples to a heat treatment of 80 °C for 10 min.

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

### 7. Conclusion

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

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

### References


