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**Alicyclobacillus spp. in the Fruit Juice Industry: History, Characteristics, and Current Isolation/Detection Procedures**

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The first *Alicyclobacillus* spp. was isolated in 1982, and was originally thought to be strictly limited to thermophilic and acidic environments. Two years later, another *Alicyclobacillus* sp., *A. acidoterrestris*, was identified as the causative agent in spoilage of commercially pasteurized apple juice. Subsequent studies soon found that *Alicyclobacillus* spp. are soilborne bacteria, and do not strictly require thermophilic and acidic environments. *Alicyclobacillus* spp. possess several distinct characteristics; the major one is their ability to survive commercial pasteurization processes and produce off-flavors in fruit juices. The fruit juice industry has acknowledged *Alicyclobacillus* spp. as a major quality control target microorganism.

Guaiacol and halophenols were identified as the offensive smelling agent in many *Alicyclobacillus* spp. related spoilage. Though the exact formation pathway of these off-flavors by *Alicyclobacillus* spp. are not yet identified, studies report that the presence of *Alicyclobacillus* spp. in the medium may be a major contributor to the formation of these off-flavors.

Many identification methods and isolation media were developed in the last two decades. However, most of these methods were developed specifically for *A. acidoterrestris*, which was the first identified off-flavor producing *Alicyclobacillus*. However, recent studies indicate that other species of *Alicyclobacillus* may also produce guaiacol or the halophenols. In this respect, all *Alicyclobacillus* spp. should be monitored as potential spoilage bacteria in fruit juices. The fruit juice industry has acknowledged *Alicyclobacillus* spp. as a major quality control target microorganism.

This article includes an overall review of the history of *Alicyclobacillus* spp., characteristics, suggested off-flavor production pathways, and commonly used identification methods for the currently identified *Alicyclobacillus* spp.

**Keywords** *Alicyclobacillus* spp.; History; Characteristics; Isolation; Detection

**INTRODUCTION**

*Alicyclobacillus* spp. is currently one of the microorganisms of concern in the fruit juice industry. The thermophilic and acidophilic characteristics of *Alicyclobacillus* spp. allow resistance to current pasteurization processes, and the ability to produce off-flavors in juice poses potential economic losses for the juice industry. Most studies concerning *Alicyclobacillus* spp. related spoilage is focused on *A. acidoterrestris*. However, recent studies have revealed other *Alicyclobacillus* species as equally able to cause off-flavors. Therefore, the purpose of this review is to provide an overall understanding of all identified *Alicyclobacillus* spp.

**HISTORY OF ALCYOCLOBACILLUS SPP.**

*Bacillus acidocaldarius* was first isolated by Darland and Brock (1) from various acid thermal environments in the United States. The bacterium was unique in possessing ω-cyclohexane fatty acids and hopanoid, and being the only known obligately thermophilic and acidophilic member in the *Bacillus* family. Subsequent reports on the isolation of *Bacillus acidocaldarius* were without exception from hot and mostly acidic environments. It was generally believed that *Bacillus* species containing the unusual ω-cyclohexane fatty acid would be strictly limited to these environments.

Cerny et al. (2) reported the first ω-cyclohexane fatty acid *Bacillus* from a non-thermal source. The *Bacillus* was isolated from spoiled apple juice and was implied as the causative agent for the spoilage. Hippchen et al. (3) also isolated bacilli from soil that were neither thermophilic nor acidophilic, and these isolates closely resembled the one isolated by Cerny et al. (2) in apple juice. The genetic relationship of these soil bacilli with the apple juice isolate and *B. acidocaldarius* could not be established because GC rations have not been determined. However, presence of the unique ω-cyclohexane fatty acid and hopanoids suggest a close relationship between *B. acidocaldarius* and the isolates from non-thermal origins.

Relationships among the ω-cyclohexane fatty acid possessing *Bacillus* isolates was finally determined by Deinhard et al. (4). Physiological and biochemical tests were conducted on 13 thermophilic and acidophilic *Bacillus* isolates, including the soil isolates of Hippchen (3) and apple juice isolate of Cerny (2). Results indicated that another *Bacillus* species possessed specific ω-cyclohexane fatty acid and hopanoids once thought to be specific to *B. acidocaldarius* alone. The strains of the new species
included those isolated by Hippchen and Cerny. Since strains of this species were mainly isolated from soil, Deinhard et al. (4) proposed this new species be identified as Bacillus acidocaldarius, with acidoterrestris meaning acid loving and isolated from soil. B. acidocaldarius and B. acidoterrestris differed in the use of carbon sources. The ability to utilize erythritol, sorbitol, and xylitol to produce acid distinguished the new species from B. acidocaldarius.

Deinhard et al. (5) also identified another ω-alicyclic fatty acid possessing microorganism from soil and named it Bacillus cycloheptanicus for its unique possession of ω-cycloheptane fatty acids. B. cycloheptanicus exhibited different characteristics from its other two ω-alicyclic fatty acid possessing relatives by having an obligate nutrient requirement for methionine, isoleucine, and pantothenate. The temperature growth range for this microorganism was also rather narrow, between 40 and 53°C. Results of DNA–DNA hybridization showed low similarity of B. cycloheptanicus with B. acidocaldarius and B. acidoterrestris.

The proper taxonomic classification of these microorganisms as bacilli has been questioned. 16S rRNA catalog data indicate that B. acidocaldarius branches most deeply from the other Bacillus species (6). Wisotzkey et al. (7) focused on the proper taxonomic placement of B. acidocaldarius, B. acidoterrestris, and B. cycloheptanicus using 16S rRNA comparative sequence analysis. Primary sequence comparisons indicated that B. acidocaldarius and B. acidoterrestris were nearly identical (98.8%) and clearly belonged to the same genus. However, B. cycloheptanicus was more distant from its two relatives, scoring 93.2% and 92.7%, respectively. In addition to primary sequence comparisons, the secondary structures of 16S rRNAs were also investigated. Secondary structures do not differ significantly among closely related organisms, thus phylogenetic diversity is suggested if variance among secondary structures is observed. The secondary structures of 16S rRNAs among B. acidocaldarius, B. acidoterrestris, and B. cycloheptanicus were identical or very similar to one another, but differed from other Bacillus. Based on the results of this study, Wisotzkey et al. (7) proposed that B. acidocaldarius, B. acidoterrestris, and B. cycloheptanicus be reclassified into a new genus, Alicyclobacillus, in part due to the unusual ω-alicyclic fatty acids in their cell membrane. As a result of this reclassification, the three species were renamed as Alicyclobacillus acidocaldarius, Alicyclobacillus acidoterrestris, and Alicyclobacillus cycloheptanicus.

Recently, more Alicyclobacillus species were identified. Albuquerque et al. (8) isolated A. hesperidum from volcanic soils. Goto et al. (9) isolated A. herbarius from herbal tea made from dried flowers of hisbicus. A. herbarius is more closely related to A. cycloheptanicus in having predominately ω-cycloheptane fatty acids in the cell membranes. Matsubara et al. (10) isolated A. acidiphilus from an acidic beverage in Japan. This microorganism is able to produce guaiacol and cause spoilage in acidic beverages.

CHARACTERISTICS OF ALICYCLOBACILLUS SPP.

Alicyclobacillus spp. are gram-positive, rod-shaped, thermophilic, and acidophilic spore-forming bacteria. Depending on the different species, growth temperatures range from 20–70°C, with optima from 42–60°C. Alicyclobacillus spp. can also grow over a wide pH range, generally reported between pH 2.5 and 6.0 (11–14). A. acidocaldarius exhibits the most extreme growth characteristics compared with its other genomic relatives, growing over a temperature range of 45–70°C, and in a pH range of 2.0–6.0 (12). Spore formation in Alicyclobacillus spp. is terminal or subterminal, with or without swollen sporangium. Colonies on Bacillus acidocaldarius medium (BAM) are creamy white, non-pigmented, flat, and circular (1, 3–5, 9, 10, 12, 13, 15).

The most distinctive characteristic of Alicyclobacillus spp. is the presence of ω-alicyclic fatty acids as the major membrane component (3, 8, 16). Membranes in A. acidocaldarius, A. acidoterrestris, A. hesperidum, and A. acidiphilus are mainly composed of ω-cyclohexane fatty acids (1, 4, 8, 10, 17, 18) while ω-cycloheptane fatty acids are the major membrane components in A. cycloheptanicus and A. herbarius (5, 9, 16, 19). Researchers suggest that ω-alicyclic fatty acids are associated with the exceptional resistance of Alicyclobacillus spp. to acidic conditions and high temperatures (7). Kannenberg et al. (20) demonstrated that ω-cyclohexane fatty acid-containing lipids pack densely, resulting in low diffusion at high temperatures. Wisotzkey et al. (7) proposed that this property provides an advantage when cultures are grown at high temperatures or low pH. Closely packed rings of the ω-alicyclic fatty acids may form a protective coating for the cell membrane, and contribute to the resistance of Alicyclobacillus spp. to acidic conditions and high temperatures.

The thermal resistance of bacterial spores is influenced by several environmental factors, one of the most important being pH of the heating medium. Spores are most resistant in solutions with pH values slightly higher than the optimum pH of growth, and their thermal resistance was found to diminish markedly along with the pH reduction of the medium (21–23). In a study conducted by Murakami et al. (24), A. acidoterrestris did not exhibit such behavior. According to their observations, A. acidoterrestris spores were not significantly influenced by pH reduction of the heating medium. Z-values remained the same over the pH range tested (pH 3.0–8.0). Kannenberg et al. (20) stated that lipids containing fatty acids with a cyclohexane ring may stabilize the membrane structure and help maintain barrier function of prokaryotic membranes at high temperatures. Thus, ω-cyclohexane lipids may be an aspect of thermo-acidophilic adaptation of bacterial membranes (12, 20, 25–27).

The heat resistance of spores has also been associated with dehydration, dipicolinic acid (DPA) content, presence of heat-stable proteins, and mineralization. Alderton et al. (28) recorded that fully formed spores easily demineralized under different pH values. Many authors agree that demineralized spores have decreased heat resistance, and remineralizing spores with divalent
cations, such as calcium or manganese, can increase heat resistance of the demineralized spores (29). These findings suggest that mineralization of spores is important to heat stability. Yamazaki et al. (30) reported that under low pH conditions, spores of A. acidoterrestris exhibit stronger binding characteristics of Ca\(^{2+}\) and Mn\(^{2+}\) than that of other Bacillus species tested. Little change in Ca-DPA concentration and the strong ability to bind divalent ions in A. acidoterrestris spores are related to their specific heat resistance.

The unique heat and acid resistance of Alicyclobacillus spp. has spawned research in several areas. The role of \(\alpha\)-alicyclic fatty acids and hopenoids on membrane function is being widely studied (20, 31). There is also mounting interest in biotechnological applications of thermostable enzymes derived from A. acidocaldarius-like strains (32–35). Enzymes stable at high temperatures and at extremely low pH values are one of the most attractive targets in connection to potential use in industrial applications. The third field of interest resolving around Alicyclobacillus spp. is the prevention of spoilage due to Alicyclobacillus. Since the first incidence of apple juice spoilage tied to A. acidoterrestris in 1982, this bacteria became a focus of great attention and concern in the juice industry (2). The spoilage impact of Alicyclobacillus spp. on the fruit juice industry will be discussed in the following section.

Being a potential spoilage microorganism, the issue of pathogenicity of Alicyclobacillus spp. was naturally of concern. Walls and Chuyate (36) conducted a study to test the pathogenicity of A. acidoterrestris. Spores were either directly injected into mice or inoculated into fruit juices and fed to guinea pigs. No illness symptoms were reported in the mice injected with A. acidoterrestris spores, indicating the lack of pathogenicity of these strains at the level tested. In the guinea pig study, spoiled juices contained \(5 \times 10^6\) CFU/ml A. acidoterrestris, and were visibly cloudy with a distinct odor. There were no illnesses or deaths in any of the guinea pigs fed in this trial. The researchers concluded that A. acidoterrestris is not pathogenic at the level tested. Also, no reported illnesses in humans have been attributed to consumption of spoiled juice. While spoilage of juices by A. acidoterrestris is a serious economic issue for the juice industry, it is not a safety concern. Table 1 lists specific characteristics among different species of Alicyclobacillus spp.

**Alicyclobacillus spp. as Spoilage Bacteria**

Spoilage in acidic beverages and acidic foods are not originally associated with bacterial spores. Common thermophilic sporeformers can be controlled by the low acid environment (37). Clostridium botulinum spores that can produce the lethal botulinum toxin, are not able to germinate, grow, and/or produce toxins in low pH (pH < 4.6) environments (22). B. steatorrhophilus, a common thermophilic flat sour spoilage microorganism, cannot grow at pH levels below 5.3 (38). Thus, the major concern when designing a suitable pasteurization process to produce shelf stable juices or nectars (pH < 4.5), is focused on yeast, mold, and some non-sporeforming bacteria (39).

Shelf stability of selected 100% fruit juice products stored at room temperature is maintained by the combination of thermal processing and low pH. The naturally low pH of the juices inhibits the growth of many types of bacteria and selects for yeasts, molds and a few groups of aciduric bacteria. Pasteurization, accomplished by a hot-fill and hold process, generally destroys the heat-labile spoilage organisms such as lactic acid bacteria, yeasts, and some types of molds (40). In the hot-fill and hold process, the product is heated for approximately 15–20 s. As the temperature decreases to 82–84°C, the product is filled into the package. Next, the product is held for approximately 2 min before the packages are cooled down in a cooling tunnel (41). Though small numbers of heat-resistant molds may survive the processing steps and cause spoilage, it is assumed that fruit juices adequately processed and handled will remain commercially sterile during the specified shelf life until the container is opened (42, 43).

However, research has indicated that several thermophilic acidophilic sporeformers can survive in low acid foods as spores. Bacillus coagulans was identified as the causative microorganism for flat-sour type spoilage in acidic beverages. It is capable of growing at pH values as low as pH 4.0, and causes spoilage by producing off-flavors and souring of the product (38). The unique survival of this sporeformer drew great attention in the food industry and was listed as one of the important target organisms of acid foods in Japan (44).

For the last two decades, another acidophilic, thermophilic spore-forming bacterium was associated with spoilage of commercially pasteurized fruit juices. A. acidoterrestris was first documented by Cerny et al. (2) as the causative microorganism of apple juice spoilage in Germany in 1982. Since then, Alicyclobacillus spp. were implicated in recent juice spoilage incidents in the United Kingdom, Germany, Australia, Japan, and the United States (2, 11, 12, 44, 45). Though spoilage by Alicyclobacillus spp. was previously regarded as sporadic, the survey by the National Food Processors Association (NFPA) in 1998 (12) shows the large scale of fruit juice spoilage associated with Alicyclobacillus spp. Results of the survey indicated that 35% of the fruit juice manufacturers who responded experienced spoilage unconfirmed but consistent with growth of acidophilic sporeformers. Spoilage occurred in early spring or summer and most commonly in apple juice, though reports of other juices and canned tomatoes were also reported. Spoilage microorganisms were recovered from both products and processing equipment, on a variety of media, with pH ranging from 3.5 to 5.3, and over a temperature range of 25–55°C. Spoilage was mainly apparent as an off flavor or off odor, with or without sediment. Companies often did not realize a spoilage incident until they received consumer complaints.

The resistance of Alicyclobacillus spp. to high temperature and low pH lead many to investigate potentially susceptible products. Splittstoesser et al. (37, 46) investigated various commercial drinks on their ability to support the growth of...
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>A. acidocaldarius</th>
<th>A. acidoterrestris</th>
<th>A. cycloheptanicus</th>
<th>A. hesperidum</th>
<th>A. herbarius</th>
<th>A. acidiphilus</th>
</tr>
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<tbody>
<tr>
<td><strong>Morphological characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gram stain</td>
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<td>+</td>
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<tr>
<td>Shape</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
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<td>Size (width, µm)</td>
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<td>0.6–0.7</td>
<td>0.35–0.55</td>
<td>0.5–0.7</td>
<td>ND</td>
<td>0.9–1.1</td>
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<tr>
<td>(length, µm)</td>
<td>2–3</td>
<td>2.9–4.3</td>
<td>2.5–4.5</td>
<td>2.1–3.9</td>
<td>ND</td>
<td>4.8–6.3</td>
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<td>ND</td>
<td>ND</td>
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<td>+</td>
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<td>Spore formation</td>
<td>Subterminal</td>
<td>Subterminal</td>
<td>Subterminal</td>
<td>Terminal</td>
<td>Subterminal</td>
<td>Subterminal</td>
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<td>Sporangium swollen</td>
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<td>Slight</td>
<td>Slight</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Colony morphology</td>
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<tr>
<td>Color</td>
<td>Not pigmented</td>
<td>Cream white, translucent</td>
<td>Cream white and opaque</td>
<td>Not pigment</td>
<td>Not pigmented</td>
<td>Cream white to opaque</td>
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<td>Round smooth</td>
<td>ND</td>
<td>Circular</td>
<td>Round, smooth</td>
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<td>Size (diameter, mm)</td>
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<td>3–5</td>
<td>0.5</td>
<td>1–2</td>
<td>2–3</td>
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<td>Growth temperature (°C) (optimum temperature)</td>
<td>45–70</td>
<td>35–55 (42–53)</td>
<td>40–53 (48)</td>
<td>35–60 (50–53)</td>
<td>35–65 (55–60)</td>
<td>20–55 (50)</td>
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<td>Growth pH range (optimum)</td>
<td>2–6</td>
<td>2.2–5.8</td>
<td>3–5.5 (3.5–4.5)</td>
<td>2.5–5.5 (3.5–4.0)</td>
<td>3.5–6 (4.5–5)</td>
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<td>Growth factors required</td>
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<td>None</td>
<td>Methionine or Vit, B₁₂, pantothenate, and isoleucine</td>
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<td>Anaerobic growth</td>
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<td>ND</td>
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<td>–</td>
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<td>Liquefaction of gelatin</td>
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<td>Hydrolysis of starch</td>
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<td>Nitrate reduction</td>
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<td>ND</td>
<td>ND</td>
<td>–</td>
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<td>Indole production</td>
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<td>ND</td>
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Acid from carbohydrates

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<td>+</td>
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<td>+</td>
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<tr>
<td>D-Arabinose</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>+</td>
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<td>Cellobiose</td>
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<td>+</td>
<td>ND</td>
<td>+</td>
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<td>D-Fructose</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>D-Glucose</td>
<td>+</td>
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<td>+</td>
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<td>Glycerol</td>
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<td>−</td>
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<td>Inositol</td>
<td>V</td>
<td>V</td>
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<td>Lactose</td>
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<td>D-Mannitol</td>
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<tr>
<td>D-Sorbitol</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>Sucrose</td>
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<td>L-Sorbitol</td>
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Chemical characteristics

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<tr>
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<th>MK-7 (MK-8)</th>
<th>MK-7 (MK-6)</th>
<th>MK-7 (MK-6)</th>
<th>MK-7</th>
<th>MK-7 (MK-3)</th>
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<tbody>
<tr>
<td>ω-alicyclic fatty acid</td>
<td>ω-cyclohexane fatty acid</td>
<td>ω-cyclohexane fatty acid</td>
<td>ω-cyclohexyl fatty acid</td>
<td>ω-cyclohexyl C17:0 and ω-cyclohexyl C19:0</td>
<td>ω-cycloheptane fatty acid</td>
<td>ω-cyclohexylnundecanoic acid and ω-cyclohexyltridecanoic acid</td>
</tr>
</tbody>
</table>

W: weak reaction; ND: not determined; V: variable among isolates.

*Data from Darland and Brock (1); Deinhard et al. (4, 5); Albuquerque et al. (8); Goto et al. (9); and Matsubara et al. (10). Characteristics are listed according to the type strain of each species: A. acidocaldarius ATCC 27009; A. acidoteresstris DSM 3922; A. cycloheptanicus DSM 4006; A. hesperidum DSM12489; A. herbarius DSM 13609; A. acidiphilus DSM 14558.
Alicyclobacillus spp. Apple juice and tomato juice consistently supported growth of the Alicyclobacillus spp. isolates (WAC and VF) used, with tomato juice permitting more growth than apple juice. Apple-orange-pineapple (pH 2.9, 14.8 Brix) supported growth of Alicyclobacillus spp. Growth was inhibited when sugar content in the juice samples exceeded 18 Brix. Phenolic compounds may also influence the growth of Alicyclobacillus spp. since red juice was found to be more inhibitory than white juice. Ethanol prevented growth when concentrations exceeded 6%. This excludes table wines from the susceptible products, but several hard ciders would still be susceptible to spoilage. The expansive list of susceptible products indicates the great potential of Alicyclobacillus spp. to cause spoilage in the fruit juice industry.

The high occurrence of Alicyclobacillus spp. in apple juice is worth noticing. With apple juice consumption approaching approximately 24% of the fruit juice market, the impact of Alicyclobacillus spp. may not only be widespread, but also cause great loss to the manufacturer.

Alicyclobacillus spp. poses a new challenge to the fruit juice industry. Spoilage was often not recognized until consumer complaints were received. The reported spoiled juice products spoiled long before the expiration date, and did not show obvious signs of spoilage, such as swelling of container. Gas was not produced and no substantial change in fruit juice pH was detected. Major complaints describing spoilage were of off flavors described as “medicinal” (12, 13), “antiseptic” (48), or “hammy” (11, 37). Resistance to pasteurization temperatures, low pH, and the ability to produce off flavors led many researchers to acknowledge Alicyclobacillus spp. as a spoilage agent and important target in the quality control of acidic beverages.

Recently, Matsubara et al. (10) isolated A. acidophilus from orange juice that produced taints similar to those associated with the spoilage cause by A. acidoterrestris. This indicates that other species of the genus Alicyclobacillus may also possess the potential to cause spoilage in acidic drinks, and research on Alicyclobacillus spp. spoilage should not be solely focused on A. acidoterrestris.

Several routes of juice contamination by Alicyclobacillus spp. were suggested (47, 54). Fruits used to manufacture juice may be contaminated with either primary or secondary microflora. Primary microflora generally adheres on the surface of the fruit due to interactive forces between the plant surface and the cell wall structure of the microorganism. The secondary microflora is representative of external vectors such as soil, dust, wind, rain, flooding, irrigation, insects, bird, and rodent contamination, which can be deposited on the surface of the fruit. Since A. acidoterrestris is mainly soilborne, they may enter the manufacturing process from the surface of unwashed or poorly washed raw fruit (4, 55–57). Water is also a possible source of contamination. Water used for beverage manufacture was also identified as a source of A. acidoterrestris contamination according to McIntyre et al. (42) and Walls and Chuyate (12).

Spoilage of pasteurized fruit juices by Alicyclobacillus spp. presents a considerable challenge to the food industry. Parameters such as temperature inhibit the growth of Alicyclobacillus spp. or the germination of its spores. Alicyclobacillus spp., a thermophilic microorganism, does not grow below 20 °C (58). Temperature studies indicate that storage of commercial pasteurized fruit juices at temperatures below 20 °C is likely to prevent germination and outgrowth of spores and may provide a potential control measure for the industry to avoid spoilage by Alicyclobacillus spp. However, pasteurized fruit juices are mainly distributed under ambient temperatures. Chilling these products would be a major new cost factor. The other alternative, to raise pasteurization temperature to the sterilization region (i.e. above 100 °C) is not feasible. The extreme heat required to inactivate spores of Alicyclobacillus spp. will also produce unacceptable organoleptic changes in the product.

OFF-FLAVORS CAUSED BY ALICYCLOBACILLUS SPP. SPOILAGE

One of the most common reasons for consumer rejection of a food product is an unacceptable flavor, and every year the food industry receives complaints from consumers concerning off-flavors or taints in fresh, processed or packaged foods. Today’s consumers have become more aware of quality in foods and have an increased expectation of consistency in all quality parameters. With the large scale of the modern food industry and the nationwide distribution of products, a small number of viable organisms have the potential to contaminate large volumes of juice, resulting in severe financial losses for the producers affected.

The major off-flavors associated with the spoilage caused by Alicyclobacillus spp. can be divided into two groups (59): guaiacol (11, 44, 50) and the halophenols, including 2,6-dibromo-phenol (2,6-DBP) and 2,6-dichlorophenol (2,6-DCP) (49, 60). Though guaiacol is generally accepted as the predominant metabolite associated with the smoky taints in fruit juices (58, 61), the importance of 2,6-DBP and 2,6-DCP should not be overlooked.

GUAIACOL (2-METHOXYPHENOL)

Guaiacol has long been recognized as a flavor compound, both good and bad, in various foods. The smoky/phenolic odor of guaiacol is well established (62) and is frequently used as a component of synthetic flavorings in processed foods. Guaiacol contributes to the characteristic odor of some roasted foods including Arabica coffee (63) and barley malt (64), but is probably
better known as an off-odor in wine (65), fruit juices (11, 61), chocolate ice cream (66), and vanilla yogurt (59).

Taint in commercial wine was identified as guaiacol by Simpson and coworkers in 1986 (65). Winery personnel estimated that approximately 20% of the bottling was tainted by the presence of guaiacol and indicated that considerable variation existed in the contaminate concentration of guaiacol. Incidents of guaiacol are also reported in dairy products over the past ten years (67). Guaiacol was implied as causing off-flavors in chocolate ice cream, confectionary products, and vanilla yogurt. In all spoilage cases, guaiacol was identified as a microbial metabolite.

Recent off odors attributed to guaiacol are mainly associated with juice products spoiled by A. acidoterrestris (68). Yamazaki et al. (44) isolated A. acidoterrestris from spoiled fruit juice and determined guaiacol as the offensive odorant. Different studies support this finding that the off-flavor caused by A. acidoterrestris spoilage was guaiacol (11, 69). Splittstoesser et al. (50) mentioned that in commercial apple juice received in 1990, the identified off-odor was due to the presence of guaiacol. Duong and Jensen (69) reported a rare incidence of berry juice–containing iced tea spoilage by Alicyclobacillus spp. The spoilage was manifested as an off-odor of a medicinal or chemical nature. No turbidity, gas production, or sediment development was reported. Doung and Jensen also attributed the off-odor to the presence of guaiacol.

Characteristics of Guaiacol

Guaiacol is frequently used as synthetic flavorings in foods (70). Wasserman (71) and Furia and Bellanca (72) recorded guaiacol as producing a sweet, burnt aroma and smoky taste. The characteristic odor of some roasted foods including Arabica coffee (63) and barley malt (64) are due to guaiacol. Though a desirable smell in smoked or roasted foods, guaiacol produces an undesirable sensory odor in many foods. Adjectives such as “smoky,” “medicinal,” and “phenolic” are frequently used to describe the unpleasant smell of guaiacol (11, 59, 66, 68). Pettipher and Osmundson (73) describe the aroma as a hammy off-odor, much like the smell of smoky bacon crisps.

Microbial Synthetic Pathway of Guaiacol

In roasted products, guaiacol is formed by thermal decomposition of phenolic precursors. Such is also the case with wine oak barrels that contain guaiacol. Guaiacol was detected in oak barrels and is produced by heating processes which occur during manufacture of the barrels (74). Guaiacol in natural cork may also be produced by heat decomposition (65, 75). In fruit juice and dairy foods, guaiacol is a product of microbial metabolism (67). Microorganisms producing guaiacol include Bacillus megaterium (76), Pseudomonas acidovorans (77), Alicyclobacillus acidoterrestris (11), Streptomyces setonii (78), and Rhodotorula rubra (79). A simplified scheme of guaiacol formation with lignin is illustrated in Figure 1.

Guaiacol can be converted from vanillic acid by several strains of B. megaterium (76) and by S. setonii (78). Others report guaiacol production from vanillin by an unidentified species of Streptomyces (75) and from ferulic acid by R. rubra (79). Vanillic acid was the immediate precursor of guaiacol identified in these pathways (67).

The supposed pathway of guaiacol formation from vanillic acid is by nonoxidative decarboxylation. Crawford and Olson (76) demonstrated the conversion of vanillic acid to guaiacol and CO₂ by nonoxidative decarboxylation with Bacillus megaterium and a Streptomyces strain (Figure 2). The ability to decarboxylate vanillic acid to guaiacol is quite common among soil bacilli.
Vanillic acid can be present in fruit juices because of contamination, but is also naturally derived from the plant polymer lignin. Ferulic acid, an abundant and major component of lignin (80), is widely metabolized by both bacteria and fungi (81) and converted to vanillin, vanillic acid, and protocatechuic acid (82–87). Microorganisms further convert vanillic acid to vanillyl alcohol, guaiacol, catechol, and methoxyhydroquinone (79).

Huang et al. (79) conducted an in depth research on the guaiacol production pathway of \textit{R. rubra} from ferulic acid to guaiacol (Figure 3). The first step of this bioconversion reaction from ferulic acid to vanillic acid is dependent on CoA/ATP and NAD$^+$. An acetate moiety is removed from ferulic acid, similar to $\beta$-oxidation of fatty acids, to produce vanillic acid (Figure 4). Similar observations were made with cell-free extracts from \textit{Polyporus hispidus} and \textit{Streptomyces setonii} to convert ferulic acid to vanillic acid (85, 88). The likely involvement of a $\beta$-oxidation mechanism in \textit{R. rubra} transformations of ferulic acid to vanillic acid suggested that the double bond of ferulic acid was hydrated prior to elimination of acetate to give vanillic acid. The mechanism for the conversion of vanillic acid to guaiacol could involve a quinoid intermediate during the course of the reaction (Figure 5). A proton was added from water and attached to the position bearing the carboxyl group in vanillic acid. The conversion of ferulic acid to 4-hydroxy-3-methoxystyrene (89) was demonstrated to also be similar to the decarboxylation of vanillic acid to guaiacol. The conversion of ferulic acid to vanillic acid and subsequently to guaiacol and protocatechuic acid by resting cells of \textit{R. rubra} occurs efficiently and in high yield under standard aerobic conditions.

Another route of ferulic acid degradation is by one carbon cleavage of the side chain to form 4-vinylguaiacol (90–93). Ferulic acid degradation to 4-vinylguaiacol is catalyzed by ferulic acid decarboxylase. Ferulic acid can be released from plant derived tissues through the action of commercial pectic enzymes or by the enzymatic activity of the juice microflora. In the case of apple juice, yeasts present in unpasteurized juice or as pre-processing contaminants could release free phenolic acids into the apple juice through their feruloyl esterase activity (94). Ferulic acid decarboxylase then reacts with ferulic acid resulting in the formation of 4-vinylguaiacol. Objectionable off-flavors resulting from 4-vinylguaiacol have been reported for improperly stored orange juice (95–98). In most cases, conversion of ferulic acid to 4-vinylguaiacol is very slow with low yield of metabolites. However, a recent report stated that \textit{B. coagulans} can rapidly convert ferulic acid to 4-vinylguaiacol by nonoxidative decarboxylation, which is then converted to the high value aromatic compound vanillin (80).

Tyrosine is another possible precursor for guaiacol formation (57). Apple juice contains approximately 4.1 $\mu$g tyrosine/ml juice, and orange juice contains 3.4–13.5 $\mu$g tyrosine/ml juice. The heat shock treatment, coupled with storage temperature and oxygen concentration are important in forming guaiacol by way of tyrosine. This reaction, however, has not been widely investigated, and the guaiacol synthetic pathway mainly recognized is that of lignin degradation.

\textbf{FIG. 3.} Pathways of ferulic acid metabolism to vanillic acid, guaiacol, protocatechuic acid, and 3-methoxy-4-hydroxystyrene by \textit{R. rubra}. (Source: Huang et al. (79).)
Sensory Threshold for Guaiacol

The sensory threshold of guaiacol is low but can be easily detected by olfactory evaluation. The threshold for smelling guaiacol in water and 12% aqueous ethanol are reported as 0.02 mg/l (71) and 0.03 mg/l (99), respectively. The flavor threshold for guaiacol in dry white wine is 0.02 mg/l. Pettipher et al. (11) observed an odor threshold of guaiacol in orange, apple, and noncarbonated fruit juice of approximately 2 ppb. Similar results were reported by Orr et al. (13). They conducted a study on the taste threshold of guaiacol, and estimated the odor threshold at 2.32 ppb.

Compared with their halogenated derivatives, simple phenols substituted by alkyl groups have a much higher taste threshold (66). Such is the case with guaiacol and 2,6-DBP and 2,6-DCP.
2,6-DBP and 2,6-DCP have been reported as having thresholds of 0.5 ng/l juice and 30 ng/l juice, respectively (57). However, guaiacol has been recognized as the major off odor. Saxby (66) suggested several reasons for the predominance of guaiacol over 2,6-DBP and 2,6-DCP: (1) guaiacol is very volatile (2) guaiacol is present in higher concentrations than 2,6-DBP or 2,6-DCP. Jensen (61) reported that guaiacol was detected in juices at concentrations approximately 1,000 times higher than halophenols.

Contamination Route of Guaiacol

Substrates required for the production of guaiacol or 4-vinylguaiacol exist naturally in foods. Therefore taint is easily formed when a microorganism possessing the appropriate catalase is present or if environmental requirements are fulfilled.

Off flavors due to guaiacol was first detected in wine (65). Approximately one out of every five wine products is contaminated by guaiacol. Though the contamination incidence is high, guaiacol in wine is more associated with wine cork than the wine itself. Cork is the bark of cork oak (*Quercus suber*) (100). The main application for cork is in the manufacture of cork stoppers for wine bottles. Cork is used as a closure for its exceptional properties such as compressibility, resilience, impermeability to air and liquids, ability to adhere to a glass surface, and chemical inertness. Volatile compounds such as guaiacol can be formed by lignin degradation, either by heating or microbial reaction (101). Heat degradation of lignin during the processing of corks may lead to the formation of either guaiacol or degradation products that are precursors, such as vanillin or vanillic acid, for guaiacol formation. The formation of guaiacol through microbial reactions can also occur. Microorganisms residing in cork material may slowly breakdown lignin or utilize the precursors produced during heat degradation and produce guaiacol (75, 96). Wine coming in contact with the tainted cork through transportation or storage will eventually be tainted.

Fruit juice tainting by guaiacol is directly associated with the composition and microflora in juice. Apple juice contains a wide range of phenolic components (102–104), including cinnamic acids such as ferulic acid. The phenolic acids may have originally been esterified to plant cell wall components such as xylans and pectins and released as the result of dестerification. With ferulic acid and decoarboxylase in the system, guaiacol or 4-vinylguaiacol could be easily produced.

Factors that Affect Guaiacol Production in *Alicyclobacillus* spp.

1. *Alicyclobacillus* spp. Concentration

The concentration of *Alicyclobacillus* spp. is associated with the formation of guaiacol. According to Pettipher et al. (11), guaiacol was detected in orange juice and apple juice when 10^5 CFU/ml of *A. acidoterrestris* were present. Growth experiments indicated that low numbers of *A. acidoterrestris* readily increased in orange juice, apple juice, and a noncarbonated fruit juice-containing drink to populations at which guaiacol is produced. Komitopoulou et al. (54) also observed similar results. *A. acidoterrestris* grew in apple juice, orange juice, and grapefruit juice stored at 30°C, increasing more than four log cycles to reach a stationary phase population of 10^6–10^7 CFU/ml after 8 days. In all cases taint was subjectively detectable after 4 days when the population had reached 10^5 CFU/ml.

2. Storage Temperature

Pettipher et al. (11) noted significant sensory differences between inoculated and uninoculated apple juice stored at 21°C and 37°C. Significant differences between inoculated and uninoculated samples occurred more frequently at 37°C than at 21°C. Jensen et al. (67) also observed that temperature affected guaiacol production in inoculated UHT chocolate milk. While chocolate milk stored at 4–5°C and 8–9°C both exhibited off-odors of guaiacol after 6 days of incubation, chocolate milk stored at 8–9°C contained higher concentrations of guaiacol. It is hypothesized that the reaction rate of guaiacol production increases as incubation temperature increases.

3. Heat Shock

For metabolism of any kind to occur, a vegetative cell must be present. For *Alicyclobacillus* spp. to produce guaiacol, vegetative cells must be present rather than dormant spores. Activation is the process of conditioning spores to germinate (105). Among the various activation methods available, exposure to sublethal heat is most commonly used. It is also the method that closely resembles the activation process of *Alicyclobacillus* spp. spores in fruit juices. Several heat shock recommendations have been reported for *Alicyclobacillus* spp. (61). Splittstoesser et al. (50) observed a 100% increase when spores were heat shocked at 60°C for 30 min. Eiroa et al. (39) reported greatest recovery at 70°C for 20 min. Walls and Chuyate (106) observed that when *Alicyclobacillus* spp. were inoculated at low levels, heat shocking at 80°C for 10 min gave significantly higher counts than heat shocking at 60°C for 10 min or 100°C for 5 min. When spores were inoculated at higher levels (10^5 spores/ml), no significant differences between the heat shock treatments were observed. The difference between heat treatments is probably arbitrary; however some form of heat shock is essential. On the contrary, Pettipher et al. (11) suggested that heat treatment may not be necessary to induce germination of most of the *A. acidoterrestris* spores in apple juice. Though it is possible that the components of apple juice may be able to induce germination of spores, the germination rate would be relatively slow compared to heat shocking. Therefore heat shocking might accelerate the speed at which guaiacol is formed and the ability to detect the presence of guaiacol in susceptible products.

**HALOPHENOLS (2,6-DIBROMOPHENOL AND 2,6-DICHRROMOPHENOL)**

Contrary to researchers in the United States, Japan, and Germany, Australian workers identify halophenols, mainly
2,6-dibromophenol (2,6-DBP) and 2,6-dichlorophenols (2,6-DCP) as the major cause of taint in fruit juices (57). Both Borlinghaus and Engel (60) and Baumgart et al. (49) have reported that 2,6-DBP was associated with the off flavor caused by *A. acidoterrestris*. Workers at Food Science Australia’s Sydney laboratory detected 2,6-DBP and 2,6-DCP in fruit juice samples spoiled by *A. acidoterrestris* (57). Though halogenated phenolic compounds are one of the most common causes of taints in food, dispute still remains as whether *A. acidoterrestris* plays a role in the formation of 2,6-DBP and 2,6-DCP or that the taints are simply incidents of chemical contamination.

**Characteristics of 2,6-Dibromophenol and 2,6-Dichlorophenol**

Taints caused by 2,6-DBP and 2,6-DCP are described, similarly to guaiacol, as “medicinal” or “disinfectant” (59). Chlorophenols are known to be the major source of disinfectant taints for many years. Many simple chlorophenols impart a disinfectant taste to foodstuffs at levels below 1 ppb (66). Disinfectant taints were experienced in coffee, and has been related to the presence of dichlorophenols. Recently, bromophenols have also been implicated as food taint. A major contributor to the recognition of 2,6-DBP as a taint is Whitfield and coworkers (107). They identified 2,6-DBP as a cause for an isoform-like taint in prawn meat. Anthony et al. (108) also confirmed the results of Whitfield et al. (107), suggesting that iodine or iodine-related taint in shrimps was generally due to the presence of 2,6-DBP. Trace levels of bromophenols have also been identified in tainted fruit juices. At very low concentrations, 2,6-DBP and 2,6-DCP can impart disagreeable odor resulting in an unsatisfactory product.

**Synthetic Pathway of 2,6-Dibromophenol and 2,6-Dichromophenol**

The contamination pathway of 2,6-DBP and 2,6-DCP can be divided into two groups: chemical contamination and microbial synthesis.

Bromophenol and chlorophenol formation in foods are often generated by contact with weak halogen solutions used in cleaning raw materials and food processing lines and during dilution of juice concentrates. When phenol containing water is used to dissolve or dilute the disinfectants, a rapid production of halophenols is likely to occur. If the halophenols are not completely removed, they could be carried over and cause taint in any food product that comes into direct contact with the disinfected area (109).

Another possible pathway of 2,6-DBP and 2,6-DCP is through bacterial biosynthesis rather than external disinfectant contamination. A biosynthetic pathway for synthesis of halophenols has been described in marine algae by Flodin and Whitfield (110). In this process, the key reactants were a phenolic precursor, hydrogen peroxide, halide ions and a haloperoxidase. Haloperoxidases occur widely in nature, including in bacte-ria (111). However, microbial haloperoxidases do not require metal ions or cofactors to catalyse reactions (112). According to this halophenol synthetic pathway by Flodin and Whitfield (110), fruit juice would be highly susceptible to halophenol formation since trace quantities of phenolic compounds, hydrogen peroxide and halide ions could be present and bacteria could be the source of haloperoxidases. Findings of Borlinghaus and Engel (60) and Jensen and Whitfield (58) indicate that strains of *A. acidoterrestris* contain enzyme systems capable of halogenation. Thus it is possible that strains of *A. acidoterrestris* can produce halophenols in shelf-stable juices during storage.

**Factors that Affect 2,6-Dibromophenol and 2,6-Dichromophenol Production in Alicyclobacillus spp.**

1. **Heat Shock Medium**

One study reported by Jensen (57) showed that to induce taint formation in juice, the medium for the heat shock treatment was important. Jensen recorded taint formation in juice, but not in distilled water. No taint formation was evident even if the water was acidified.

2. **Headspace**

Jensen and Whitfield (58) demonstrated that production of disinfectant taint could be affected by the headspace of the packaging. Taint was produced to detectable concentrations in 1–4 d at 44–46°C in bottles with large headspace. In packages with small headspace, taint production occurred more slowly, but was still detectable within the first month of storage. Conclusion of this study indicates that though headspace can affect the speed of halophenol production, as long as *Alicyclobacillus* spp. is present in juice, halophenols could be expected in commercially pasteurized fruit juices.

**Sensory Threshold of 2,6-Dibromophenol and 2,6-Dichlorophenol**

There is no actual agreed sensory threshold for either 2,6-DBP or 2,6-DCP. But all reported numbers are common in the fact that the threshold is very low. In water, 2,6-DCP is reported as having a threshold of 0.2 ppb and 3 ppb by Dietz and Traud (113) and EWender et al. (114), respectively. The latter also listed the taste threshold for 2,6-DBP in water to be 50 ppb. In juice, the reported thresholds for 2,6-DBP and 2,6-DCP are 0.5 ppb and 30 ppb, respectively (57). The low sensory thresholds indicate the high likelihood to cause taint among food products. Bromophenols generally have lower sensory threshold values than chlorophenols and therefore have much higher tainting potential. They have been found to cause disinfectant taints at parts per trillion levels in fish products (107, 115). It has been reported that 2,6-dibromophenol is among one of the compounds to have particularly low thresholds and is very likely to cause taint in food products.
Contamination Route of 2,6-Dibromophenol and 2,6-Dichlorophenol

As listed in the sensory threshold section, 2,6-DBP and 2,6-DCP have extremely low sensory thresholds. With this in mind, it is of utter importance to understand the possible contamination routes. A good understanding would aid in more effective control and prevention of 2,6-DBP and 2,6-DCP formation.

The active ingredients in many fungicides and algicides are chlorophenols. As previously discussed, any residual concentration of these phenolic disinfectants or chemicals will be a potential cause of taint formation. Fiberboard and paper generated from recycled papers and boards also often contain relatively high levels of chlorophenols. Using these products for the direct packaging of raw materials or processed food can also contribute to taint (116).

However, though phenol contamination is regarded as a major factor in halophenol taint formation, it does not appear to be absolutely necessary (117). For example, 2,6-DCP has been identified in carrots treated with sodium hypochlorite and heated at 121°C. This suggests that the phenol was formed naturally or that the hypochlorite reacted with the carrot phenolic compounds. In addition to this finding, Japanese workers also detected the presence of 2,6-DCP in carrots after they had been immersed in sodium hypochlorite (>100 ppm chlorine), even though they could not detect free phenol in the original vegetable (118).

DETECTION OF OFF FLAVORS CAUSED BY ALICYCLOBACILLUS SPP.

The detection of taint can be divided into two categories (119) depending on the purpose of the detection. Instrumental analysis includes use of instruments such as gas-chromatography-mass-spectrum (GC-MS) or high performance liquid chromatography (HPLC). These tests are used for quantitative purposes, if the presence/absence of taint is of interest, sensory analysis is generally used. A simplified overview of the detection methods is discussed in this section.

Instrumental analysis includes three steps: concentration, separation, and identification. Concentration is the step required to obtain a sufficient quantity to allow absolute identification of trace components. To collect taint volatiles in spoiled apple or orange juices, some researchers use headspace analysis. With this method, volatiles are collected on absorbants. Further analysis is possible by heat desorption directly on a gas chromatography column or by extraction with a solvent. However, in most Alicyclobacillus spp. taint related research, taint is evident that no concentration step was performed. Separation involves differentiation of chemical components into individual components according to molecular weight. The identification step uses a known standard to identify those that exhibit identical separation patterns, such as retention time or adsorption peak, as the same compound. To identify the quantity of a specific component, a known concentration of a standard is applied. The concentration would correlate to a known area. The unknown is run through the same procedure and also yields an area. The ratio of area between the known and unknown would reflect the relative concentrations between the standard and unknown. Despite the ability to quantify off-odors, instrumental analysis is expensive and requires professional training to perform, therefore limiting its application in the industry.

Sensory analysis detects the presence of off-flavors by olfactory senses of a trained panel. However, the sensory threshold of guaiacol is low, and the “disinfectant” odor stands out from fruit juices that consumers without formal sensory training are also able to detect the presence of guaiacol.

During initial studies on taint formation by Alicyclobacillus spp., both methods have been used cooperatively. Most use sensory analysis to confirm the presence of taint and then identify the cause of taint using instrumental analysis. Instrumental analysis also quantifies the concentration of taint using instrumental analysis. Instrumental analysis also quantifies the concentration of taint, which assists in determining a correlation between taint concentration and Alicyclobacillus spp. concentrations. Sensory analysis is usually a presumptive method. However, Pettipher et al. (11) reported opposite observation to the low sensitivity of sensory analysis. At three of four sampling times ranging from 13 to 61 days of storage, the sensory panel detected (p ≤ 0.001) guaiacol. The concentration of guaiacol was 8.1 to 11.4 ppb according to the results of chromatographic analysis. The panel detected guaiacol in five samples stored at 21 to 37°C for 8 to 61 days whereas the compound was not detected by the chromatographic analysis. Pettipher and coworkers thus concluded that the sensitivity of the sensory technique is greater than that of the chromatographic technique.

CURRENT ISOLATION AND IDENTIFICATION PROCEDURES OF ALICYCLOBACILLUS SPP.

As the importance of Alicyclobacillus spp. in fruit juices was recognized, so has the need for an accurate isolation and identification procedure. Summarizing the numerous studies regarding Alicyclobacillus spp., there are two major isolation categories: media plating and filtration. Identification includes presence/absence detection, microscopic analysis, taint smell from growth media, or high-tech procedures such as PCR detection or flow cytometry systems (15). Frequently used incubation temperatures range from 37°C to 55°C. Media are usually acidified by malic acid, HCl, or H2SO4 to pH 3.5–4.0.

Isolation Media

Various media have been used in the attempt to isolate Alicyclobacillus spp. Though Alicyclobacillus spp. exhibit extraordinary survival under extreme conditions, they are unable to grow on all media. As reported by several researchers, A. acidoterrestris cells do not grow on nutrient agar, trypticate soy, brain heart infusion, and veal infusion agars and broths, even when the medium is acidified to pH 3.5. Most commonly used
isolation media include *Bacillus acidocaldarius* medium (BAM), orange serum agar (OSA), potato dextrose agar (PDA), Yeast-Starch-Glucose agar (YSG agar), HGYE, and K agar (37, 53, 73, 120).

1. *Bacillus acidocaldarius* medium (BAM)

   *Bacillus acidocaldarius* medium (BAM) was first proposed by Darland and Brock (1). The medium was formulated to isolate *Bacillus acidocaldarius*, hence the name of the medium. *A. hesperidum* was also isolated using BAM in combination with membrane filtration (8). After the reclassification of the alicylic fatty acid possessing bacilli to the new genus * Alicyclobacillus*, the medium was renamed *Alicyclobacillus acidocaldarius* medium (AAM). However, the two names are often referred to interchangeably in the literature.

   Several enriched versions of BAM have been documented (53). Farrand et al. (121) proposed the addition of 1 ml of a trace element solution to BAM. Deinhard et al. (4) and Silva et al. (120) used BAM with the trace element with additional glucose (5 g/l). The modification used by Silva (122) and Silva et al. (123, 120) is composed of three solutions mixed after sterilization at 121°C for 10 min: (a) CaCl₂·2H₂O, 0.25 g; MgSO₄·7H₂O, 0.5 g; (NH₄)₂SO₄, 0.2 g; yeast extract, 2 g; glucose, 5 g; KH₂PO₄, 3 g; and distilled water, 500 ml adjusted to pH 4.0 with H₂SO₄; (b) 1 ml of SL-6 trace elements solution (ZnSO₄·7H₂O, 0.1 g; MnCl₂·4H₂O, 0.03 g; H₃BO₃, 0.3 g; CuCl₂·2H₂O, 0.2 g; CuCl₂·H₂O, 0.01 g; NiCl₂·6H₂O, 0.02 g; Na₂MoO₄·2H₂O, 0.03 g; distilled water, 1 l); (c) agar, 15 g; distilled water, 500 ml.

   Pinhatti et al. (55) assessed the ability of BAM and OSA to recover *A. acidoterrestris* from frozen orange concentrate. The concentrate was diluted to single strength and submitted to a heat shock at 80°C for 10 min. The frozen orange concentrates were incubated for enrichment at 50°C for 24 and 48 h. *A. acidoterrestris* were observed after pour plating and incubating at 50°C for 24 h. The plates were sealed in plastic bags to avoid drying of the medium. BAM gave slightly higher bacterial counts than OSA.

2. Orange Serum Agar (OSA)

   Orange serum agar has long been used for cultivation and enumeration of microorganisms associated with the spoilage of citrus products. Hays (124) described using OSA for the enumeration and isolation of organisms causing spoilage in frozen concentrated orange juice. Murdock et al. (125) used a similar orange serum agar containing 0.5% dextrose and 2% agar in their evaluation of plating media for citrus concentrates. Hays and Riester (126) recommended OSA (pH 5.5) for the study of off-flavor spoilage in frozen concentrated orange juice. OSA with an acidity of pH 5.5 supported most growth of spoilage microorganisms, and is widely accepted as the standard control medium to cultivate and enumerate microorganisms associated with citrus product spoilage.

   Orange serum agar was originally designed to isolate bacteria from citrus products, so this medium was also adapted for the isolation of *Alicyclobacillus* spp. Pettipher et al. (11) compared nutrient agar, tryptone soy agar, *Bacillus acidocaldarius* medium (BAM), potato dextrose agar (PDA), and OSA for recovery of *A. acidoterrestris*. BAM, PDA, and OSA supported growth while nutrient agar and trypticase soy agar did not. Compared with BAM and PDA, spread plating on OSA gave the highest yields. Jensen (61) reported improved growth of *Alicyclobacillus* spp. when OSA was supplemented with 0.5% sucrose.

3. Potato Dextrose Agar (PDA)

   Potato dextrose agar is a Standard Methods plating medium used for culturing yeasts and molds from dairy and other food products (127, 128). In a study of comparative methods and media used in the microbiological examination of creamery butter, Shadwick (129) investigated a number of media and reported that PDA gave the most consistent and highest count of yeast and mold in salted and unsalted butter. Potato infusion encourages development of fungi.

   Medium acidification is a frequently desirable procedure to inhibit bacterial growth and to facilitate yeast and mold counts. Standard Methods recommends the acidiification of PDA to pH 3.5 ± 0.1 after sterilization. The medium should not be heated after the acid is added because acid hydrolyzes agar and destroys the solidifying properties of agar.

   PDA is often acidified to pH 3.5 when used to isolate *Alicyclobacillus* spp. Many studies on *Alicyclobacillus* spp. were conducted with the use of PDA as the isolation medium and good recovery is reported. Splittstoesser et al. (37) observed that *Alicyclobacillus* spp. isolated from off-odor producing pasteurized fruit juice preferred PDA over many of the rich media usually used for cultivating sporeforming bacteria. Detection was reported after 24 h of incubation on PDA, pH 3.5 or 5.6, at 43°C. However, recovery was much lower at the near neutral pH. Splittstoesser et al. (50) also agreed that PDA adjusted to pH 3.5 was good selective medium for the detection of *Alicyclobacillus* spp. spores. Heat resistant molds such as *Byssoschlamys* were completely suppressed without decreasing recovery of *Alicyclobacillus* when incubated at 53°C. PDA (pH 3.5) was also used by the National Food Processors Association (NFPA) (106) and McIntyre et al. (42). The latter reported that sporulation was detectable within 24 h at 36°C and growth was dense if the incubation temperature was elevated to 50°C and 55°C.

4. Yeast-Starch-Glucose Agar (YSG agar)

   In Japan, a selective medium named yeast-starch-glucose agar (YSG agar) is used for detecting thermoacidophiles in acidic drinks (130). The medium composition is as follows: 2 g yeast extract, 2 g soluble starch, 1 g glucose, 15 g agar (pH adjusted to 3.7), and 11 distilled water.

   Goto et al. (9) isolated *A. herbarius* using membrane filtration and YSG agar. Dried hibiscus flowers were suspended in distilled water, left to settle, and the supernatant filtered through a membrane filter (ADVANTEC TOYO; mixed cellulose ester;
pore size 0.45 µm, diameter 47 mm). The filter was placed on YSG agar and incubated at 50°C for 5 d. Matsubara et al. (10) also used YSG agar, directly plating the acidic beverage sample onto the plate without filtration. Plates were incubated at 45°C for 3 d.

5. HGYE

Hiraishi et al. (35) used HGYE (131) for cultivation of newly isolated and authentic strains of Alicyclobacillus spp. The medium consisted of 0.4% glucose, 0.3% (NH₄)₂SO₄, 0.1% trypticase soy broth, 0.05% yeast extract, 0.05% MgSO₄·7H₂O, 0.01% K₂HPO₄, and 0.01% K₂SO₄; pH was adjusted to 3.0 with diluted H₂SO₄. The medium contained 3% agar when used as solidified medium.

6. K Medium or K Agar

K agar was first described by Walls and Chuyate (12). The composition is: 2.5 g yeast extract; 5.0 g peptone; 1.0 g glucose; 1.0 g Tween 80; 15 g agar; 990 ml deionized water. 25% (w/v) malic acid was filter-sterilized and used to adjust pH after autoclaving at 121°C for 15 min. According to their isolation media study, all tested Alicyclobacillus spp. isolates were able to grow on K agar (pH 3.7). Growth occurred at 1 to 2 d at 43°C as opposed to 5 days at 35°C. Walls and Chuyate compared K agar with other media typically used to isolate bacteria from acidic products, such as OSA, tomato juice special agar and PDA (12, 45). K agar exhibited significantly (p < 0.05) higher recovery of A. acidoterrestris than OSA at all temperatures tested. In all, K agar was superior to the other tested media, showing rapid growth and higher recovery. Among all temperatures tested, highest counts on K agar were observed at 43°C. When low numbers of spores were present, use of filtration to concentrate spores gave more consistent results than the use of a pre-incubation method to determine presence or absence of spores. Heat shocking juice samples also resulted in significantly (p < 0.05) higher counts. Isolation on K agar proved highly repeatable and reproducible. The exceptional recovery of A. acidoterrestris by K agar is also supported by Orr and Beuchat (52). In an attempt to observe the effects of disinfectants in killing A. acidoterrestris spores, K agar (pH 3.7) performed best compared to OSA and PDA in supporting the formation of colonies from chemically treated A. acidoterrestris.

Currently, many fruit juice manufacturers in the United States follow the procedures recommended by the NFPA as described by Walls and Chuyate (52) for assessing A. acidoterrestris. The procedure includes heat shocking at 80°C for 10 min, with/ without filtration, plating onto K agar (pH 3.7) and incubating at 43°C for 48 hr. However, many products containing Alicyclus spp. remain undetected and result in spoilage reported by consumers. This calls for a modification or development of an isolation procedure with higher sensitivity that can reduce the number of false negative fruit juice products.

MEMBRANE FILTRATION

Membrane filtration is commonly used to collect microorganisms from many different types of samples, including liquid and gases. Over the years, membrane filtration was applied in the detection of coliforms, pathogens, fungi, and viruses (132). The mechanism of membrane filtration is that particles larger than the membrane pore size can be retained (Figure 6).

One of the primary advantages of the membrane filtration method relates to the ability to test large sample volumes instead of being restricted by the volume that can be spread plated on an agar plate. Membrane filtration followed by growth on culture media to form visible colonies is a standard method for the detection of microorganisms.
the production result in membrane filters that vary greatly among manufacturers. It is crucial that the membrane filters currently used or available be evaluated for the ability to retain Alicyclobacillus spp. spores.

IDENTIFICATION METHODS

Presence/Absence Detection

A simple presence/absence detection method was proposed by Pettipher et al. (11). Juices were pre-incubated at 44°C for 48 h and streaked (10 µl) onto OSA plates. Growth detected after incubation at 44°C for 48 h was considered presumptive positive for A. acidoterrestris. This method is more sensitive than direct plating and can detect one cell in either 10 ml of concentrate or 100 ml of single strength fruit juice.

Prior to the proposal of this method, most researchers were focused on isolating and detecting the specific causative microorganism in spoiled fruit juices. Detection methods include DNA analysis or 16 rRNA sequence analysis. These methods can provide specific identification, but are too complicated and expensive for incorporation as routine quality control procedures for fruit juice manufacturers. The presence/absence detection reported by Pettipher et al. (11) provides an applicable alternative detection method for Alicyclobacillus spp. It also inspired subsequent developments of simple Alicyclobacillus spp. detection methods.

Microscopic Method

Pettipher and Osmundson (73) reported using a direct epifluorescent filter technique (DEFT) to isolate Alicyclobacillus spp. DEFT is a modification of membrane filtration, employing fluorescent dyes and microscopy (135). Typically, fruit juices are filtered through a 0.6 µm nucleopore polycarbonate membrane. The filter is stained with acridine orange and enumerated by epifluorescence microscopy. Pettipher and Osmundson (73) detected very high numbers (>5 × 10³ CFU/ml) of rod shaped bacteria in carbonated fruit drinks with the use of DEFT.

Off-Odor Production

Another pragmatic approach to detection of Alicyclobacillus spp. in fruit juices is by olfactory evaluation. The medicinal or disinfectant-like off-odor produced by Alicyclobacillus spp. is distinct and easy to detect. Colonies formed on isolation media that produce the distinctive off odor is presumptive positive for Alicyclobacillus spp. (73).

Rapid Detection Method

Conventional identification of Alicyclobacillus spp. uses standard biochemical and morphological tests, and usually requires several days to accomplish depending on the isolation procedure. Polymerase chain reaction (PCR) methods are widely used in the study of molecular evolution and are applied for the rapid identification of A. acidoterrestris (15, 24).
Reverse transcription polymerase chain reaction (RT-PCR) was used to detect *A. acidoterrestris*. Yamazaki et al. (136) utilized primers 5'-AC(G/A)GGTAGGCATCTCTGT-3' and 5'-AGGAGCTTTCCACTCTCTTGT-3' for specific identification of *A. acidoterrestris*. The primers were able to detect one bacterial cell per ml of fruit juice. The reported sensitivity of RT-PCR was no less than 10^2–10^6 CFU/ml if apple juice was directly extracted from Florurepore FHLP filters without any enrichment procedure. Increase of sensitivity to approximately 2 CFU/ml was observed with 15 h enrichment in modified *Bacillus acidoterrestris* medium. The researchers concluded that evaluation of *A. acidoterrestris* contamination in fruit juice or juice-containing beverages may be accomplished within 24 h using RT-PCR.

Randomly amplified polymorphic DNA (RAPD) assay is also a derivative application of polymerase chain reaction (PCR). RAPD obtains electrophoretic profiles of randomly amplified polymorphic DNA. A primer specific to the target microorganism and the lysed DNA is mixed with Taq polymerase. PCR is carried out and followed by electrophoresis. Bands that appear on the electrophoresis agarose gel can be analyzed and used to phage type microorganisms (135).

Yamazaki et al. (137) examined the possibility of utilizing the RAPD assay to identify *A. acidoterrestris*. Three primers, Ba10 (5'-AACCGCGAAC-3'), F-61 (5'-CCTGTGATGGGC-3'), and F-64 (5'-GCGGGCAAGTA-3') were identified to allow adequate discrimination from *A. acidoterrestris* related bacteria. To confirm the results, 41 thermoacidophilic isolates were identified using the RAPD assay and conventional biochemical/morphological methods. Results from both methods were identical, confirming the reliability and usefulness of RAPD in identifying *A. acidoterrestris*. RAPD also proved to be a rapid method, allowing exact identification within 6 h of the total viable count.

### INCUBATION TEMPERATURE

Various incubation temperatures were suggested for the recovery and isolation of *Alicyclobacillus*. The incubation temperature commonly used for bacterial cultures is in the range of 30–37°C and is used in some cases of *Alicyclobacillus* spp. isolation 7. However, incubation at this lower temperature often took 5–7 d, so elevated incubation temperatures were soon favored over this moderate temperature range.

Splittstoesser et al. (37) modified the incubation temperature to 43°C after observing better sporulation results at this temperature. Walls and Chuyate (12, 106) reported that K agar gave the highest spore counts at 43°C. Pettipher and Osmundson (73) also incubated OSA plates at 44°C. The temperature chosen prevented the growth of many non-thermophilic organisms, thus increasing the specificity of the recovered microorganism. Silva et al. (120, 123) and Matsubara et al. (10) used 45°C for incubation. Since *Alicyclobacillus* spp. is a thermophilic microorganism, higher temperatures were also used. Goto et al. (9) isolated *A. herbarius* by incubating at 50°C. Splittstoesser et al. (50) recorded that incubation at 53°C could inhibit heat resistant molds such as *Byssochlamys* without decreasing recovery of *Alicyclobacillus* spp. The highest temperature range reported in literature for *Alicyclobacillus* spp. growth is 55–60°C. This was used by Hiraishi et al. (35) for cultivation of newly isolated and authentic strains of *Alicyclobacillus* spp. However, though higher temperatures do increase the specificity of isolation procedure for *Alicyclobacillus* spp., 43 ± 1°C is by far the most widely used incubation temperature.

### MEDIA pH

Regardless of isolation medium, all related research have utilized acidification to isolate *Alicyclobacillus* spp. Acidification can inhibit the growth of other background microorganisms that may interfere with *Alicyclobacillus* spp. Splittstoesser et al. (50) adjusted PDA to pH 3.5 as was recommended by the American Public Health Association. In some cases, the medium was acidified to pH 4.0 (138). An intermediate pH 3.7 is suggested by Walls and Chuyate (12) for K agar.

### HEAT SHOCK

Heat shocking fruit juices prior to testing can result in increased viable count if the organism is present mainly as spores. Sublethal heat is a commonly used activation treatment for spores, and encourages germination and subsequent outgrowth. Different heat shock treatments are reported in various *Alicyclobacillus* research. In addition to the research results discussed previously, Pettipher and Osmundson (73) indicated that 80°C for 10 min resulted in increased counts if the microorganism is present mainly as spores. Pinhatti et al. (55) stressed the importance of heat shocking by concluding that the number of spores may consistently be underestimated even with enrichment procedures for long incubation times if heat shock were not applied prior to plating. Contrary to the majority of research, no significant \((p < 0.05)\) differences between heated and unheated spore suspensions were observed by Pontius et al. (26) and Silva et al. (120). Though it is possible that natural components of apple juice may promote germination of spores, the natural activation will take longer than sublethal heat activation. Regarding rapid detection of *Alicyclobacillus* spp., heat shocking is essential, though the difference among heat treatments is minimal.

### SOURCES OF *ALICYCLOBACILLUS*

*Alicyclobacillus* spp. origin can be divided into two general groups, one is acidic hot springs and the other is soil (4, 5, 8). Of the six identified species of *Alicyclobacillus*, *A. acidocaldarius* are detected only in thermal, acidic environments (1). Hiraishi et al. (35) attempted to isolate *Alicyclobacillus* spp. from hot springs in Japan and was successful. Other *Alicyclobacillus* spp. are soilborne and do not require the strict extreme requirements of acidity and high temperature for survival. It is the soil borne group of *Alicyclobacillus* spp. that often contaminate fruit juices.
FUTURE RESEARCH ON ALICYCLOBACILLUS SPP.

The diversity of the Alicyclobacillus species can be noted by the different characteristics presented in Table 1. Isolation attempts for this microorganism are equally diverse as can be seen by the numerous isolation media and procedures discussed previously. Variations in isolation procedures give different results, many of which underestimate the contamination of Alicyclobacillus spp., and result in consumer complaints of unsatisfactory products. The thickening concern of this microorganism in the fruit juice industry reflects the urgency for a more sensitive procedure to detect these spoilage bacteria.

The focus on Alicyclobacillus spoilage is currently centered on A. acidoterrestris, probably because it was first identified in spoiled apple juice. Much research was conducted on A. acidoterrestris, but the possibility of other Alicyclobacillus species to produce off-flavors and spoilage in fruit juices should not be overlooked. The recent discovery of the guaiacol producing A. acidiphilus (10) indicates the possibility of other Alicyclobacillus species to cause spoilage. Research on Alicyclobacillus should include other species of Alicyclobacillus rather than just A. acidoterrestris.

Future work on Alicyclobacillus spp. should involve developing a suitable isolation medium for all Alicyclobacillus spp. since the fundamental basis for Alicyclobacillus spp. related research is based on the isolation medium. A highly sensitive medium gives a better estimate of the actual contamination level. If used for quality control, high sensitivity recovery medium can better give an estimate of the actual contamination level. If used for quality control, high sensitivity recovery medium can better give a better estimate of the actual contamination level. If used

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