Algae *see* Single-Cell Protein: The Algae

Alicyclobacillus

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Introduction

Acidothermophilic microorganisms have been isolated since the late 1960s, mainly from acidic and hot sites. The microorganisms, originally classified as acidothermophilic *Bacillus*, were for the first time associated with spoilage of apple juices in the beginning of the 1980s in Germany. *Bacillus acidoterrestris* was reported as the causative agent of an off-flavor or taint in the spoiled apple juice.

Microorganisms with similar features of *B. acidoterrestris* were isolated continuously from a variety of sources. Then, 16S rRNA studies provided the basis for the distinction of the acidothermophilic strains from other members of genus *Bacillus*. In 1992, a new genus named *Alicyclobacillus* was created to accommodate acidothermophilic and spore-forming bacteria (Figure 1) characterized by the presence of unusual amounts of ω-alicyclic fatty acids and hopanoid in their membranes. The new genus was composed by three species: *A. acidocaldarius*, *Alicyclobacillus acidoterrestris*, and *Alicyclobacillus cycloheptanicus*.

Currently, more than 21 species, two subspecies, and two genomic species of *Alicyclobacillus* have been described (Figure 2). The species may vary in terms of sources of isolation, morphology, pH, and temperature ranges for growth, among other properties (Table 1).

Characteristics of *Alicyclobacillus* Genus

Alicyclobacillus spp. are mostly Gram-positive, rod-shaped, spore-forming, acidophilic, and moderately thermophilic bacteria belonging to Alicyclobacillaceae family. The species *Alicyclobacillus disulfidooxidans* is mesophilic, whereas *Alicyclobacillus sendaiensis* is the only Gram-negative species within the genus. Spores in *Alicyclobacillus* spp. can be terminal, subterminal, or central, with or without swollen sporangium. Spores

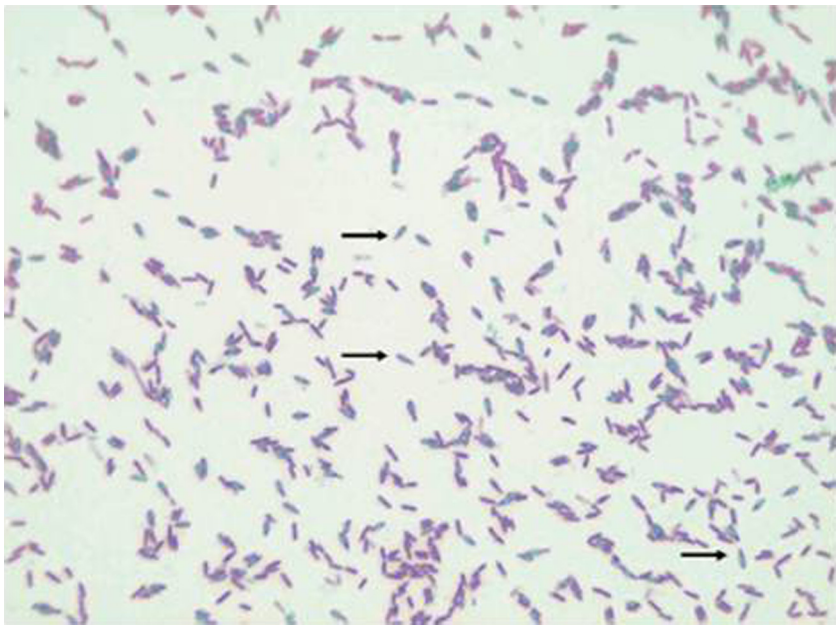

Figure 1 *Alicyclobacillus* spores and vegetative cells as observed in optical microscope (100×) after spore staining (5% green malachite and 0.5% safranin). Spores and vegetative cells are stained in green and purple, respectively. From Oteiza, J.M., 2011. *Alicyclobacillus acidoterrestris*. Revista Argentina de Microbiologia 43, 67–67 with permission.

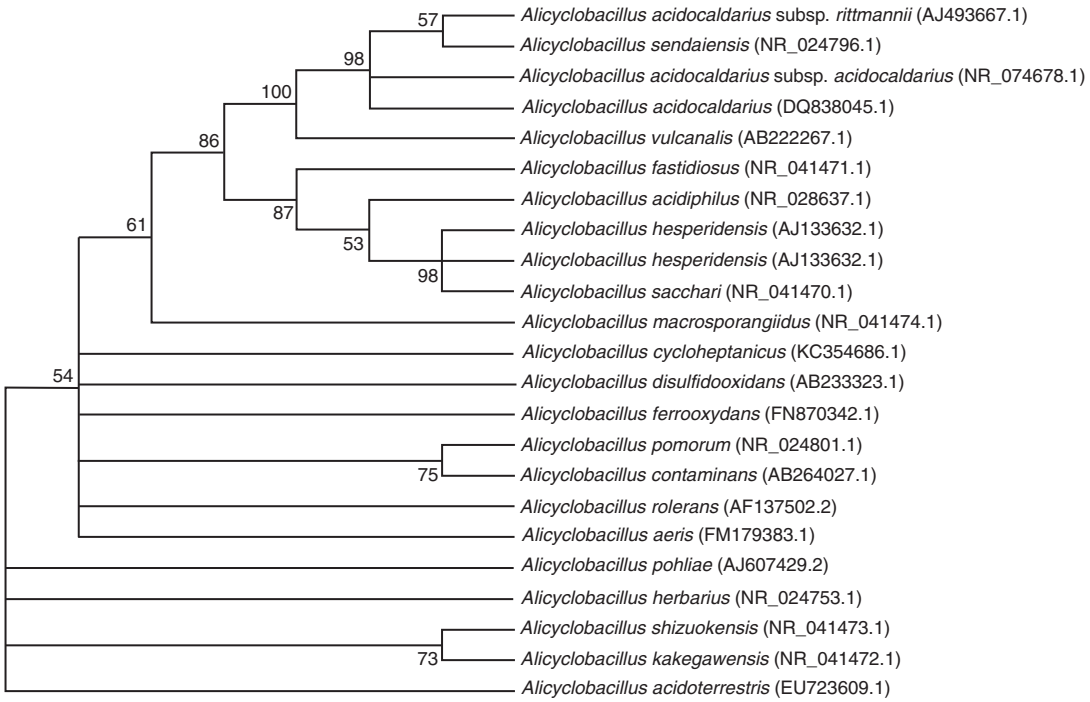

Figure 2 Phylogenetic dendrogram of *Alicyclobacillus* species based on the 16S rRNA genes, illustrating the genetic diversity within the genus. A neighbor-joining tree of *Alicyclobacillus* sequences reported in the GenBank is shown.

can be oval, ellipsoidal, or round and present high thermal and chemical resistances at acidic conditions.

A unique characteristic of *Alicyclobacillus* spp. (excepting *Alicyclobacillus pomorum*) is the presence of ω-alicyclic fatty acids, such as ω-cyclohexane and ω-cycloheptane, as the main lipids of membrane. The presence of ω-alicyclic fatty acids in their membrane is regarded to contribute for the heat resistance and thermoacidophilic behavior of *Alicyclobacillus* spp.

Although most species are aerobic, these microorganisms still can grow in aseptically packaged and canned acidic foods. *Alicyclobacillus pohliae* seems to present a facultative anaerobic metabolism. Most *Alicyclobacillus* are motile, with a wide growth temperature range (20–70 °C), with the optimum growth temperature being between 35 and 60 °C. The most concerning species for spoilage of acid foods, *A. acidoterrestris*, presents an optimum growth temperature between 35 and 53 °C, although it cannot grow in temperatures below 20 °C (Figure 3). Until now, the only three species able to grow in temperature below 20 °C are *A. disulfidooxidans*, *Alicyclobacillus tolerans*, and *Alicyclobacillus ferrooxydans*.

Species within *Alicyclobacillus* spp. can grow in a wide pH range (0.5–7.5), although the optimum range is mostly in the acidic region (<4.5). *Alicyclobacillus* spp. can grow mixotrophically or chemotrophically. Other factors affecting the behavior of *Alicyclobacillus* spp. include the soluble solid content, presence phenolic compounds, ethanol, salt content, and preservatives.

Soluble solid contents above about 20°Bx markedly inhibits the growth of *Alicyclobacillus* spp., whereas the presence of some phenolic compounds may explain the inability of *Alicyclobacillus* spp. to grow in certain substrates, such as red grape juice. In concentrations higher than approximately 5%, ethanol inhibits the growth of *Alicyclobacillus* spp. Therefore, *Alicyclobacillus* spp. are not a challenge for winery industry, but acidic beverages with lower ethanol content than 5% can be prone to spoilage. Preservatives, such as sodium benzoate, potassium sorbate, and nisin, can be effective in inhibiting the growth of *Alicyclobacillus* spp. vegetative cells, depending on the initial concentration. Among the organic acids, benzoic, butyric, and caprylic acids are the most effective, while tartaric, lactic, malic, citric, and acetic acids are the less effective in inhibiting the growth of *Alicyclobacillus* spp. Despite this, some compounds, such as sodium benzoate, can be only sporstatic against *Alicyclobacillus* spp. spores. Amounts of salt above 5–7% are detrimental for *Alicyclobacillus* spp. growth.

Different species of *Alicyclobacillus* have different nutritional and environmental growth requirements (Table 1). The growth parameters (growth rate, lag time, and maximum population) of *A. acidoterrestris* inoculated in orange juice processed and stored at different conditions and with two different initial levels can be seen in Table 2.

The growth probability of *A. acidoterrestris* can be affected by changes and interactions of temperature, pH, soluble solid contents, and the presence of preservatives (Figure 4).

Apple and orange juices correspond to the main fruit juices in which *A. acidoterrestris* can grow easily. This bacterium is also able to easily grow in tomato juice, grapefruit, pineapple, mango, and pear juices. On the other hand, the microorganism does not grow in prune, apple–grape, lemon, cranberry, red grape, and Concord grape juices.

The effectiveness of oxidizing agents in inhibiting *Alicyclobacillus* spp. varies with the chemical. For example, chlorine-

Table 1 Cultural, morphological, and colony characteristics of species belonging to the genus *Alicyclobacillus*

		Cultural characteristics			*Morphological characteristics*	
Alicyclobacillus *species*	*Source*	*pH range (optimum)*	T-*range (°C) (optimum)*	*Oxygen requirement*	*Gram stain*	*Shape*
A. acidiphilus	Acidic beverage	2.50–5.50 (3.00)	20–55 (50)	Aerobic	+	Rod
A. acidocaldarius	Thermal acid waters	2.00–6.00 (3.50–4.00)	45–71 (53–65)	Aerobic	+ To variable	Rod
A. acidocaldarius subsp. *acidocaldarius*	Subspecies automatically created according to Rule 40d (previously Rule 46) of the International Code of Nomenclature of bacteria (1990 revision). Characteristics, the same as for *A. acidocaldarius*.					
A. acidocaldarius subsp. *rittmannii*	Geothermal soil of Mount, Rittmann, Antarctica	250–5.00 (4.00)	45–70 (63)	Aerobic	+	Rod
A. acidoterrestris	Soil/apple juice	2.50–5.80 (4.50–5.00)	20–70 (36–53)	Aerobic	+ To variable	Rod
A. contaminans	Soil from crop fields in Fuji city	3.50–5.50 (4.00–4.50)	35–60 (50–55)	Aerobic	+ To variable	Rod
A. cycloheptanicus	Soil	3.00–5.50 (3.50–4.50)	40–53 (48)	Aerobic	+	Rod
A. disulfidooxidans	Waste, water, sludge	0.50–6.00 (1.50–2.50)	4–40 (35)	Aerobic	+ To variable	Rod
A. fastidiosus	Apple juice	2.50–5.00 (4.00–4.50)	20–55 (40–45)	Aerobic	+ To variable	Rod
A. ferrooxydans	Solfataric soil	2.00–6.00 (3.00)	17–40 (28)	Aerobic	+	Rod/coccus
Alicyclobacillus genomic species 1 (*A. mali*)	Solfataric soils of São Miguel, Azores	3.50–4.00	40–70 (60–63)	Aerobic	+	Rod
Alicyclobacillus genomic species 2	Soil near a geyser in Kirishima, Japan	2.00–6.50 (4.00–4.50)	35–70 (55–60)	Aerobic	+	Rod
A. herbarius	Herbal tea	3.50–6.00 (4.50–5.00)	35–65 (55–60)	Aerobic	+	Rod
A. hesperidum	Solfataric soils of São Miguel, Azores	3.50–4.00	35–60 (50–53)	Aerobic	+	Rod
A. kakegawensis	Soil from crop fields in Kakegawa city	3.50−6.00 (4.00−4.50)	40−60 (50−55)	Aerobic	+ To variable	Rod
A. macrosporangiidus	Soil from crop fields in Fujieda city	3.50–6.00 (4.00–4.50)	35–60 (50–55)	Aerobic	+ To variable	Rod
A. pohliae	Geothermal soil of Mount Melbourne, Antarctica	4.50–7.50 (5.50)	42–60 (55)	Aerobic, facultatively anaerobic	+	Rod
A. pomorum	Mixed fruit juice	3.00–6.00 (4.00–4.50)	30–60 (45–50)	Aerobic	+ To variable	Rod
A. sacchari	Liquid sugar	2.50–5.50 (4.00–4.50)	30–55 (45–50)	Aerobic	+ To variable	Rod
A. sendaiensis	Soil, Japan	2.50–6.50 (5.50)	40–65 (55)	Aerobic	–	Rod
A. shizuokensis	Soil from crop fields in Shizuoka city	3.50–6.00 (4.00–4.50)	35–60 (45–50)	Aerobic	+ To variable	Rod
A. tolerans	Oxidizable lead–zinc ores	1.50–5.00 (2.50–2.70)	<20–55 (37–42)	Aerobic	+	Rod
A. vulcanalis	Geothermal pool, Caso hot springs, California	2.00–6.00 (4.00)	35–65 (55)	Aerobic	+	Rod

NR, not reported.
Reproduced from Smit, Y., Cameron, M., Venter, P., Witthuhn, R.C., 2011. *Alicyclobacillus* spoilage and isolation – a review. Food Microbiology 28, 331–349 with permission.

Morphological characteristics		*Colony morphology*				
Size (length × width μm)	*Cell motility*	*Endospore characteristics*	*Sporangia swollen*	*Color*	*Shape*	*Size (diameter mm)*
0.9–1.1 × 4.8–6.3	Yes	Ellipsoidal to oval, terminal to subterminal	Yes	Creamy white, opaque	Round, smooth	1.1–3.8
1.5–3.0 × 0.5–0.8	Yes	Oval or ellipsoidal, 1.0–1.1 × 0.7–0.8 μm, terminal to subterminal	No to slightly	Unpigmented, cream yellow	Circular flat or convex, smooth, irregular margins	1.0–2.0
Subspecies automatically created according to Rule 40d (previously Rule 46) of the International Code of Nomenclature of bacteria (1990 revision). Characteristics, the same as for *A. acidocaldarius*.						
2.0–4.0 × 0.5–2.0	No	Central to terminal	No	Cream, opaque	Convex, circular, entire margins	0.8–1.0
2.9–4.3 × 0.6–0.8	Yes	Oval, 1.5–1.8 × 0.9–1.0 μm, terminal, subterminal and central	No to slightly	Creamy white to yellowish, translucent to opaque	Round	3.0–5.0
4.0–5.0 × 0.8–0.9	Yes	Ellipsoidal, subterminal	Yes	Nonpigmented (creamy white), opaque	Circular, entire, umbonate	3.0–5.0
2.5–4.5 × 0.35–0.55	Yes	Oval, 1.0–0.75 μm, subterminal	Slightly	Creamy white, opaque	Round, small, smooth	NR
0.9–3.6 × 0.3–0.5	No	Oval, 0.9–1.8 × 0.7–0.9, subterminal or terminal	Yes	NR	NR	NR
4.0–5.0 × 0.9–1.0	No	Ellipsoidal, subterminal	Yes	Nonpigmented (creamy white), opaque	Circular, entire, flat	3.0–4.0
1.0–1.5 × 0.4–0.6	No	NR	NR	Nonpigmented	Pinpoint, circular, entire	0.3–0.5
2.1–4.2 × 0.5–0.8	No	Terminal	No	Nonpigmented	NR	1.0–2.0
2.0–4.5 × 0.5–1.0	Yes	Ellipsoidal, terminal or subterminal	No	Creamy white, slightly mucous	Round	1.0–4.0
NR	Yes	Oval, subterminal	Yes	Not pigmented	Circular	2.0–3.0
2.1–3.9 × 0.5–0.7	No	Terminal	No	Not pigmented	NR	1.0–2.0
4.0–5.0 × 0.6–0.7	Yes	Oval, subterminal	Yes	Nonpigmented (creamy white), opaque	Circular, entire, flat	2.0–3.0
5.0–6.0 × 0.7–0.8	Yes	Oval, terminal	Yes	Nonpigmented (creamy white), opaque	Circular, entire, convex	2.0–4.0
1.5–2.5 × 0.4–0.6	NR	Round terminal	Yes	Cream colored	Entire, convex	1.5–2.0
2.0–4.0 × 0.8–1.0	Yes	Oval, subterminal	Yes	Not pigmented	Circular	3.0–4.0
4.0–5.0 × 0.6–0.7	Yes	Ellipsoidal, subterminal	Yes	Nonpigmented (creamy white), opaque	Circular, entire, umbonate	3.0–5.0
2.0–3.0 × 0.8	No	Round or ellipsoidal, terminal	Yes	White and semitransparent	Circular, convex	1.0
4.0–5.0 × 0.7–0.8	Yes	Oval, subterminal	Yes	Nonpigmented (creamy white), opaque	Circular, entire, convex	1.0–2.0
3.0–6.0 × 0.9–1.0	No	Oval, terminal or subterminal	Yes	NR	NR	0.3–0.5
1.5–2.5 × 0.4–0.7	NR	Terminal	NR	Semitransparent to white	Convex	1.0

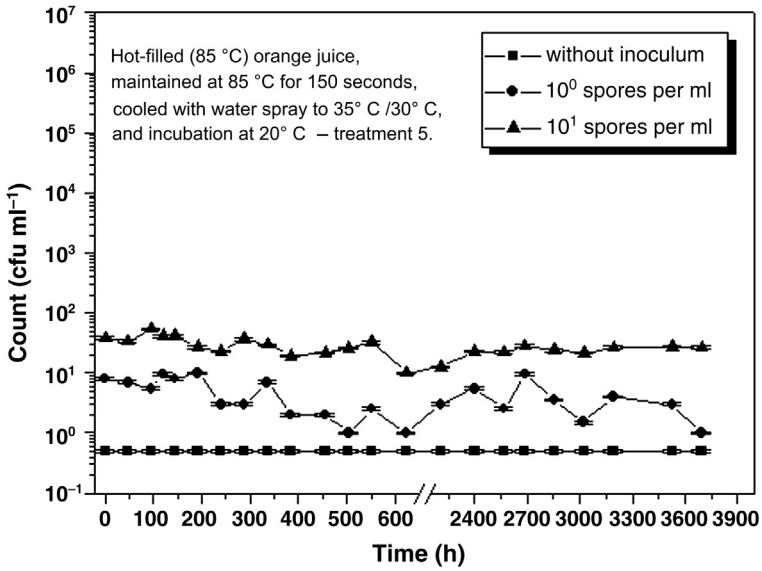

Figure 3 Inability of *A. acidoterrestris* CRA 7152 to grow in orange juice stored at 20 °C during 6 months. From Spinelli, A.C.N., Sant'Ana, A.S., Rodrigues-Junior, S., Massaguer, P.R., 2009. Influence of different storage temperatures on *Alicyclobacillus acidoterrestris* CRA7152 growth in hot-filled orange juice. Applied and Environmental Microbiology 137, 295–298 with permission.

Table 2 Predicted growth parameters for *A. acidoterrestris* in hot-filled orange juice stored under various conditions[a]

Treatment no.[b]	*Description*	*Treatment conditions*	*Inoculum levels (spores per ml)*	*λ (h)*	*μ log ((cfu ml^{-1})/h)*	*κ*	t *10^4 (h)*
1	Hot filling with quick cooling	Cooling to 30 °C at the bottle cold point and storage at 35 °C	$<10^1$	51.71 ± 3.73 de	0.093 ± 0.0085 ABC	4.20 ± 0.12 A	81 ± 1.4 EF
			10^1	62.73 ± 5.18 CD	0.104 ± 0.0332 AB	3.26 ± 0.01 BC	84 ± 5.7 DEF
2	Hot filling with slow cooling	Cooling to 30 °C for 48 h and storage at 35 °C	$<10^1$	75.19 ± 4.00 C	0.076 ± 0.01 BC	4.44 ± 0.05 A	116 ± 5.7 ABC
			10^1	74.25 ± 11.31 C	0.091 ± 0.0071 BC	3.52 ± 0.20 B	104 ± 5.7 BCD
3	Hot filling with quick cooling	Cooling to 25 °C at the bottle cold point and storage at 35 °C	$<10^1$	53.90 ± 3.51 de	0.079 ± 0.0078 BC	3.56 ± 0.17 B	95 ± 1.4 CDE
			10^1	41.14 ± 3.98 de	0.084 ± 0.0014 BC	2.69 ± 0.08 D	67 ± 1.4 F
4	Hot filling with slow cooling	Cooling to 25 °C for 48 h and storage at 35 °C	$<10^1$	100.4 ± 0.40 B	0.101 ± 0.0078 ABC	3.57 ± 0.13 B	132 ± 0.0 A
			10^1	105.54 ± 1.22 B	0.149 ± 0.0226 A	2.90 ± 0.08 CD	125 ± 12.7 AB
6	Cold filling	Filling and storage at 25 °C	10^1	270.95 ± 3.18 A	0.044 ± 0.0057 C	1.85 ± 0.01 E	_[c]

[a]Values are means ± standard deviations. Different capital letters in the same column indicate significant statistical differences according to a Tukey test ($p < .05$).
[b]Control samples for treatments 1–4 were stored for 288 h, and for treatments 5 and 6, they were stored for 6 months. Data on treatment 5 were not included since no growth was observed during the 6 months.
[c]Maximum population of *A. acidoterrestris* in orange juice did not reach 104 cfu ml^{-1} after 6 months of storage.

based disinfectants seem to be the most efficient, while peracetic acid–based disinfectants are the less efficient. Other compounds such as lysozyme can greatly affect *Alicyclobacillus* spores viability. Fatty acids and their esters (monolaurin, sucrose laurate, sucrose palmitate, and sucrose stearate) seem to be effective against vegetative cells and spores, while chitosan and essential oils have limited effects.

Alicyclobacillus spp. are inhabitants of hot springs and soil and the species have been isolated from several sources (Table 1). Nonetheless, soil seems to be the primary source of acidic food contamination by these microorganisms. Dusty, insects, birds, rain, flooding, and close contact with soil or soil particles seem to play an important role in the contamination of raw materials with spores of *Alicyclobacillus* spp. Water also has been described as an important source of raw material, equipments, and acidic foods contamination by *Alicyclobacillus* spp. Thus, good agricultural practices to reduce the contamination of raw materials entering food-processing plants by *Alicyclobacillus* spp. include the avoidance to pick up fruits from the ground and the use of good quality water in acidic food processing.

Alicyclobacillus Species

Species belonging to genus *Alicyclobacillus* spp. share several common characteristics as can be seen in Table 1. Although there are few markedly dissimilarities among the members of genus *Alicyclobacillus* spp. regarding cultural, morphological, and colony features, the main characteristic concerning the food industry is the ability to spoil acidic food products.

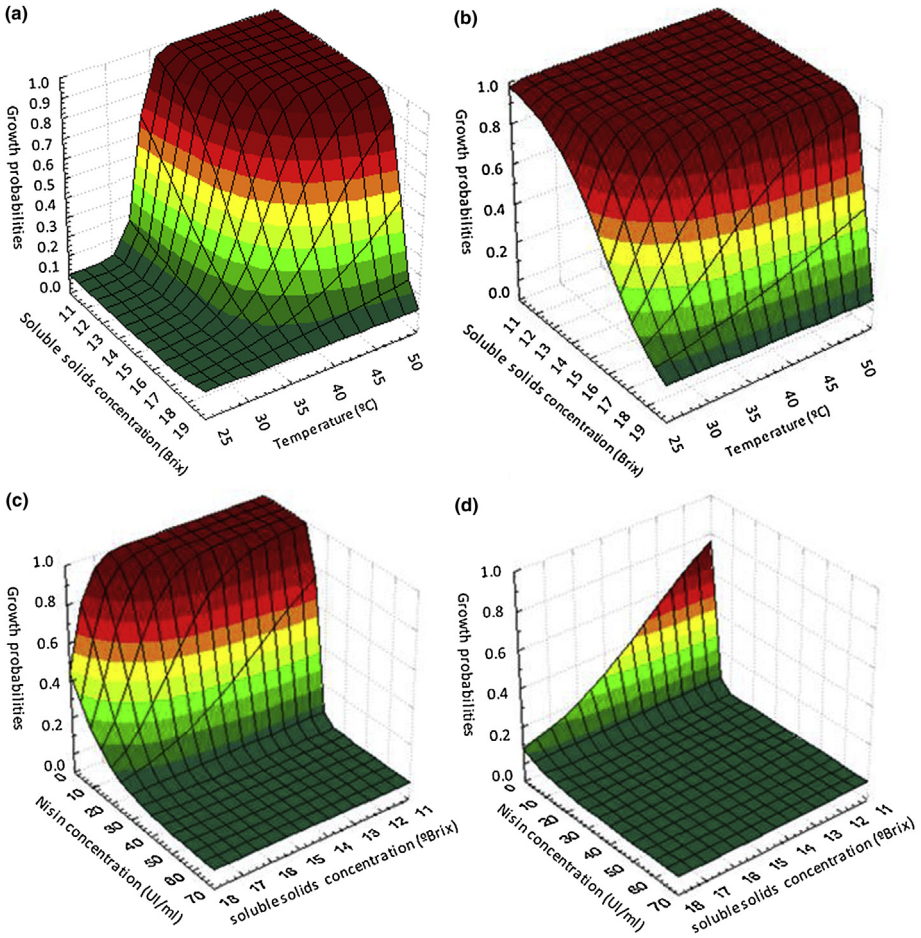

Figure 4 Growth probability of *A. acidoterrestris* CRA 7152 in apple juice as affected by soluble solid content and temperature at pH = 3.7 (a) and pH = 4.5 (b) and in apple juice as affected by nisin and soluble solid content at 45 °C (c) and 30 °C (d).

The spoilage of acidic foods by *Alicyclobacillus* spp. has been restricted mainly to few species in the genus. Although *A. acidoterrestris*, *A. pomorum*, and *Alicyclobacillus acidiphilus* have been isolated from spoiled acidic foods, *Alicyclobacillus herbarius*, *Alicyclobacillus hesperidum*, and *A. cycloheptanicus* can be a concern because of their ability to produce off-flavor compounds linked to spoilage. In spite of their ability to produce off-flavor compounds, the latter three species still were not isolated from spoiled acidic food products.

Although at least seven *Alicyclobacillus* species are of concern because of their spoilage potential, *A. acidoterrestris* is deemed to be the greatest challenge for acidic food industries. This is because *A. acidoterrestris* is the most frequent species isolated from acidic foods spoiled or not. Nonetheless, it should be regarded that not all *A. acidoterrestris* strains are deteriogenic. Therefore, the simple isolation of *A. acidoterrestris* from acidic foods should be evaluated with care. Despite this, a common practice at industrial level is to demand the absence of *Alicyclobacillus* spp. in batches of fruit concentrates to overcome the time required for isolation and identification of this microorganism to a species level as well as to determine its spoilage potential.

Spoilage of Foods by *Alicyclobacillus*

Alicyclobacillus spp. was first reported as the causative agent of acidic foods spoilage in the beginning of the 1980s. In an unusual hot summer, a huge spoilage outbreak of aseptically packaged apple juice was reported to be due to an acidothermophilic spore-forming *Bacillus*, further named *A. acidoterrestris*.

The spoilage by *Alicyclobacillus* is characterized by no changes in turbidity, lack of gas, or the presence of sediments, but with the presence of a strong off-flavor and -odor. The off-flavor and off-odor produced by *Alicyclobacillus* have been described with adjectives, such as 'smoky,' 'medicinal,' 'antiseptic,' 'disinfectant-like,' 'phenolic,' and 'hammy.' The presence of guaiacol (2-methoxyphenol) or halophenols, such as bromophenol (2,6-dibromophenol) and chlorophenol (2,6-dichlorophenol), is regarded as the cause of off-flavor and off-odor.

Although guaiacol is known as the main compound associated with *Alicyclobacillus* spoilage, a guaiacol-positive *Alicyclobacillus* strain can also produce halophenols. Halophenols can also be the only off-flavor compounds produced by deteriogenic *Alicyclobacillus*. Therefore, the spoilage potential of

this bacterium should not be based only on its ability to produce guaiacol but also halophenols. Another important characteristic to be taken into account is that the qualitative and quantitative production of off-flavor compounds may vary within strains and species of *Alicyclobacillus*.

As guaiacol is pointed as the main compound associated with *Alicyclobacillus* spoilage, the understanding of its metabolism is of major relevance. The knowledge of guaiacol production pathway can be useful either to develop strategies to control deterioration by *Alicyclobacillus* or for early detection of spoilage.

Guaiacol and halophenols can be formed either by chemical reactions taking place during food processing or by microbial synthesis. Although also possible, the synthetic pathway for halophenols formation by *Alicyclobacillus* has not been studied. Hypothesis are that halophenols are formed through reactions involving a phenolic precursor, halide ions, hydrogen peroxide, and halogenizing enzymes, such as haloperoxidases. On the other hand, microbial synthesis of guaiacol is associated with the metabolism of ferulic acid (Figure 5).

Ferulic acid is an abundant phenolic compound found in plant cell walls and a precursor for production of aromatic compounds. From ferulic acid, 4-vinylguaiacol, vanillin, or vanillic acid can be formed. Also, vanillic acid can be formed directly from vanillin. Thus, the catabolism of vanillic acid leads to the formation of guaiacol (Figure 5). Therefore, the natural existence of 4-vinylguaiacol, ferulic acid, or their precursors in foods seems to be a requirement for the formation of off-flavor in acidic foods by *Alicyclobacillus*.

The formation of off-flavor in acidic foods by deteriogenic *Alicyclobacillus* is affected by: (1) strain and species of *Alicyclobacillus*, (2) concentration of *Alicyclobacillus*, (3) presence of vegetative cells or spores, (4) storage temperature, (5) availability of oxygen/head space, and (6) substrata. Among these, concentration of *Alicyclobacillus* as vegetative cells, composition of substrata, and storage temperature seem to play the major role for the occurrence of spoilage. Normally, detectable amounts of guaiacol can be found when the concentration of vegetative cells is above 10^4 cfu ml^{-1}. The time taken to reach this population will vary with the processing, storage, and initial load (Table 2).

Once 4-vinylguaiacol, ferulic acid, or their precursors are present in an acidic food, the production of off-flavor compounds, such as guaiacol and halophenols, can take place. The sensory threshold of guaiacol and halophenols will depend on the substrate, but they are in the range of 2–2.5 ppb and 0.5–30 ng l^{-1}, respectively. Guaiacol seems to be the key compound associated with *Alicyclobacillus* spoilage possibly because of its high volatility and production in higher amounts in comparison to halophenols.

The presence of off-flavor and off-odor compounds associated with *Alicyclobacillus* spoilage can be verified through chemical, sensory, instrumental, and analytical methods. Analytical approaches normally are used when the purpose is to quantify the off-flavor compounds. Analytical methods include chromatography-based techniques, such as liquid, gas chromatography, and mass spectrometry. Chemical methods include colorimetric detection of guaiacol present in the substrate in a reaction based on peroxidise enzyme activity (Figure 6). Instrumental methods include the application of electronic noses for early, rapid, and automated detection of acidic foods contamination by deteriogenic *Alicyclobacillus*. Sensory methods have been used mainly with qualitative purposes and stand out as the most sensitive method for spoilage due to *Alicyclobacillus*. Through sensory methods, the threshold for food spoilage by this bacterium can be determined.

Despite the methods available for the detection of off-flavors produced by *Alicyclobacillus*, the recognition of the spoilage in the early stages is somehow hard to accomplish because there are no major changes easily perceivable such as drops in pH, alteration of color, presence of sediments, and packaging collapse. Most commonly, the spoilage caused by *Alicyclobacillus* is realized by the consumer when opening the product's packages. Therefore, this spoilage can be responsible for major economic losses and distrust of brands and companies.

Occurrence of *Alicyclobacillus* in Raw Materials, Ingredients, and Final Products

The populations of *Alicyclobacillus* in soil, their primary source, can be as high as 10^6 cfu g^{-1}. From soil, this bacterium can contaminate raw materials, the processing environment, and final products. Water has also been identified as an important source of contamination of acidic foods by *Alicyclobacillus*.

The incidence and populations of *Alicyclobacillus* in raw materials (e.g., fruits) will depend on the season, type of fruit, and harvest conditions, among other factors. Nonetheless, the concentration of spores in fruit surfaces can be between 1 and 10 spores per fruit. Therefore, considering the level of contamination in the fruits and the high chemical resistance of *Alicyclobacillus* spores, the role of fruits as route of food-processing contamination is highlighted.

Once inside the food-processing unity, *Alicyclobacillus* spores probably will be present in the final products because of its chemical and thermal resistances. During peeling, the microorganism certainly will be transferred to the pulps and then to other ingredients (e.g., essential oils). At the industrial level, condensate from evaporators seems to be an important source of *Alicyclobacillus* spores as they are added to final products. Levels as high as 10^3–10^6 MPN ml^{-1} of *Alicyclobacillus* spores can be found in industrial condensate water. The contamination of essential oils, which are further used for production of flavorings, is a great concern because these ingredients normally are added to a wide variety of foods at post-thermal-processing steps. Thus, this contamination can compromise the microbiological stability of acidic foods and beverages in which *Alicyclobacillus* spores find conditions to germinate, further outgrow, and produce off-flavor compounds.

Alicyclobacillus spores have been found in a wide variety of fruit juices, carbonated beverages, canned acid products, and fruit juice concentrates. Incidence may vary from very low levels to 100% of fruit juice concentrate samples. Populations of this bacterium in fruit juice concentrate are normally $<10^2$ spores per ml. Nonetheless, higher counts can be found depending on the preharvest contamination, washing and processing conditions, and juice composition (Table 3). Depending on the food composition, for example, soluble

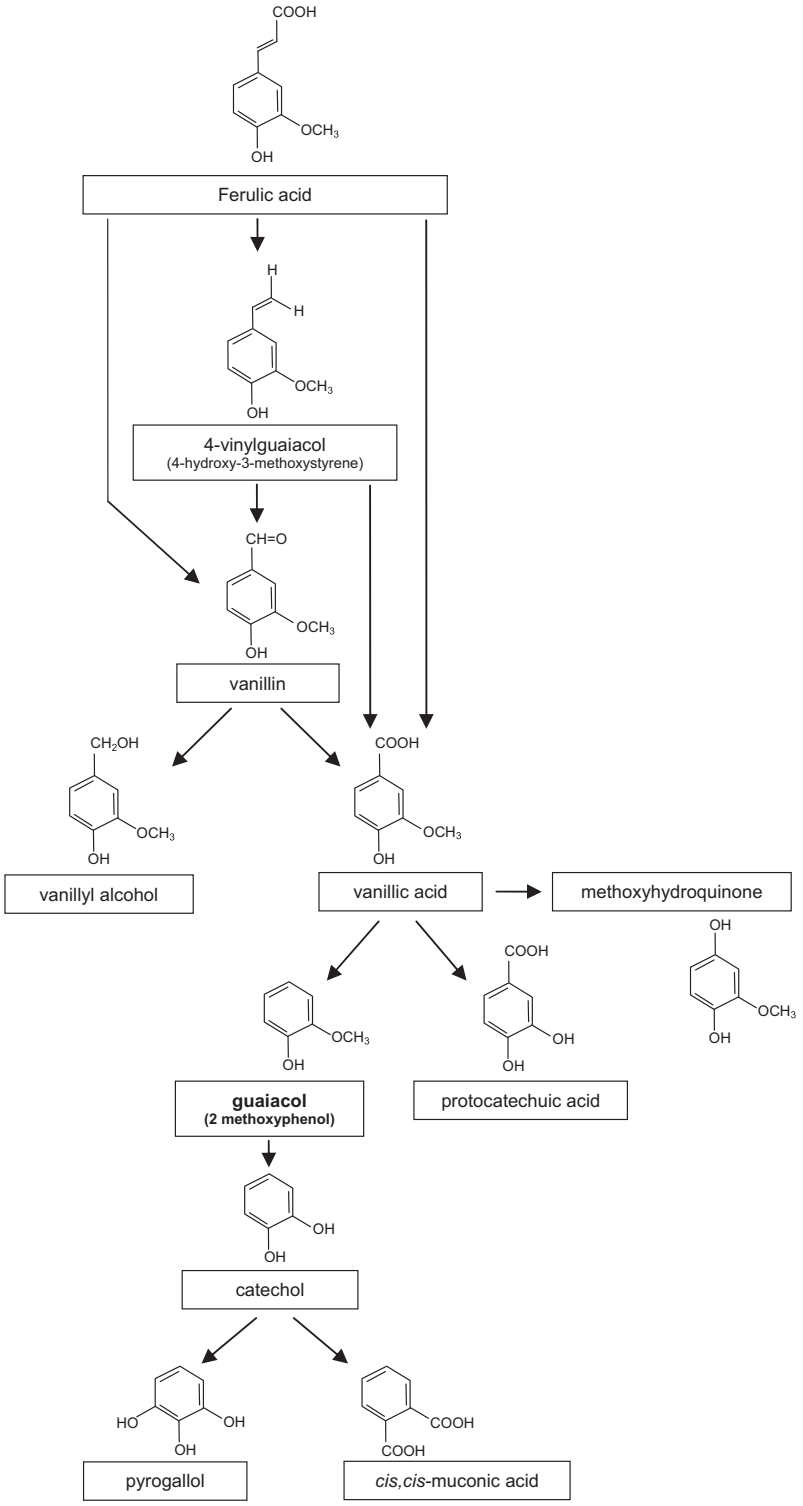

Figure 5 Microbial production pathways of guaiacol and other products through the metabolism of ferulic acid. From Smit, Y., Cameron, M., Venter, P., Witthuhn, R.C., 2011. *Alicyclobacillus* spoilage and isolation – a review. Food Microbiology 28, 331–349 with permission.

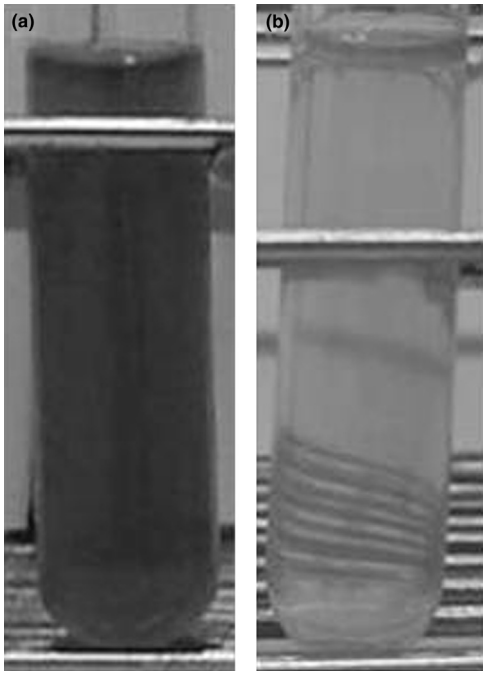

Figure 6 Visual judgment of the samples (a) positive and (b) negative for guaiacol. Considering a gray scale of colors, light gray refers to negative results and dark gray refers to positive results.

solid contents, *Alicyclobacillus* will be able to grow and spoil the product.

Inactivation of *Alicyclobacillus* in Foods

The use of high-quality raw materials, efficient fruit washing, and well-designed thermal processing can be considered three key points to ensure production of shelf-stable fruit juices. Fruit washing can be particularly efficient to reduce population of most yeasts, molds, and vegetative bacterial cells found at surface fruits. With the emergence of *Alicyclobacillus*, however, the efficiency of fruit washing has been challenged. The spores of *A. acidoterrestris* have been shown to be highly resistant to chemical compounds, such as chlorine dioxide and hypochlorite in different concentrations (Figure 7). Disinfectants commonly applied in fruit washing, such as hydrogen peroxide, chlorine, and acidified sodium chlorite lead to no more than one log reduction in the populations of *A. acidoterrestris* spores present on fruit surfaces. Therefore, measures should be taken to avoid or reduce fruit contamination by *A. acidoterrestris* to optimize the efficiency of washing process.

Alicyclobacillus spp. can withstand thermal process of acidic foods because of their highly thermal-resistant spores. The thermal resistance of *A. acidoterrestris* spores has been determined for a series of single-strength and concentrate fruit juices (Table 4). The thermal inactivation curve of *A. acidoterrestris* spores in cupuacu nectar at 90, 95, 100, 105, and 110 is shown in Figure 8.

As expected, the heat resistance of *A. acidoterrestris* spores is influenced by several factors, including strain, species, pH of the heating medium, temperature, soluble solid content, presence of cations such as calcium and manganese, sporulation conditions (pH of the medium, composition, and temperature), presence of antimicrobial compounds, and spore age.

The focus of heat-resistance studies on *A. acidoterrestris* is explained by its high association with fruit juice spoilage outbreaks. Also, because of this, *A. acidoterrestris* spores are considered to be the main target of thermal processing for acidic products. Despite this, it has been shown that thermal processing of single-strength orange juice (holding at 92 °C for 10 s, followed by hot fill at 85 °C with holding time of 20 s and them cooling to 35 °C in 30 min) leads to <0.3 log reduction (γ) (Table 5).

The emergence of *Alicyclobacillus* spp. led to drastic changes in the design of thermal processing applied to acidic foods and fruit juices. The evolution in the requirements of fruit juice pasteurization intensity to reach a hypothetical 5-log reduction for lactic acid bacteria (D_{60} °C = 1.7 min, z = 9 °C), heat-resistant fungi (D_{90} °C = 3.1 min, z = 7.4 °C), and *A. acidoterrestris* ($D_{94.6}$ °C = 6.3 min, z = 7.7 °C) are illustrated in thermal history shown in Figure 9.

Detection and Quantification of *Alicyclobacillus*

Several methods have been developed and applied with the aim of isolating and quantifying *Alicyclobacillus* spp. Detection or quantification will be used depending on the concentration of *Alicyclobacillus* expected in the sample. Samples expected to contain a low concentration of these microorganisms are preferable subjected to filtration or enrichment procedures. On the other hand, samples contaminated with populations of *Alicyclobacillus* spp. above 10^1 cfu ml^{-1} or g can be subjected to direct plating (spread or pour plating) after proper dilution. Quantification can also be done either through membrane filtration and most probable number (MPN). In the case of the former, membranes are placed in appropriate culture medium that is further incubated at appropriate conditions. In the latter case, MPN is particularly useful when recovery of injured cells and spores or further quantification is required.

The type of culture media, culture media pH, incubation conditions, and sample preparation are also relevant factors to be considered for *Alicyclobacillus* detection and enumeration. Despite this, some compounds, such as sodium benzoate, can present an inhibitorious effect against *Alicyclobacillis* spp. spores.

Another key step for proper isolation and quantification of *Alicyclobacillus* is the application of heat shocks. Heat shocks are applied to trigger the germination and outgrowth of dormant spores. The concentration of *Alicyclobacillus* in culture media is always higher when heat shocks are used. Therefore, the application of these treatments before plating is of paramount importance both for isolation and enumeration purposes. The efficiency of heat shock in activating spores may be dependent on few particularities, such as substrate, the purpose of the study, and conditions to which the spores previously have been exposed. Nonetheless, heat shock conditions tested include 60 °C/10 min, 60 °C/30 min, 60 °C/60 min, 70 °C/10 min, 70 °C/20 min, 80 °C/5 min, 80 °C/10 min, 80 °C/30 min, and

Table 3 Heat resistance of *Alicyclobacillus* endospores in high-acid concentrated fruit products

Concentrated juice	*Soluble solid (°Bx)*	*pH*	*Temperature (°C)*	D-*value (±SD) (min)*
Black currant (light)	26.10	2.50	91	3.84 (±0.49)
Black currant	58.50	2.50	91	24.10 (±2.70)
Grape (concord)	30.00	3.50	85	76.00
			90	18.00
			95	2.30
Grape (concord)	65.00	3.50	85	276.00
			90	127.00
			95	12.00
Mango	NR	4.00	80	4.00 (±1.50)
			85	25.00 (±0.10)
			90	11.66 (±1.80)
			95	8.33 (±2.00)
Lemon (clarified)	50.00	2.28	82	17.36
			86	18.06
			92	7.60
			95	6.20
	50.00	2.80	82	25.81
			86	22.01
			92	15.35
			95	11.32
	50.00	3.50	82	33.66
			86	68.95
			92	16.87
			95	12.63
	50.00	4.00	82	21.95
			86	35.16
			92	23.19
			95	9.72
Lemon (nonclarified)	50.00	2.45	82	15.50
			86	14.54
			92	8.81
			95	8.56
	68.00	2.28	82	15.50
			86	14.54
			92	8.81
			95	8.55
	68.00	2.80	82	50.50
			86	31.67
			92	39.30
			95	22.02
	68.00	3.50	82	38.00
			86	95.15
			92	59.50
			95	17.22
	68.00	4.00	82	27.48
			86	58.15
			92	85.29
			95	23.33

D-value is the time at a determined temperature to cause 1 log cycle reduction in the target microorganism. The *Z*-value is the change in temperature needed to cause 1 cycle reduction in the *D*-value.
SD, standard deviation; NR, not reported.
Reproduced from Steyn, C.E., Cameron, M., Witthuhn, R.C., 2011. Occurrence of *Alicyclobacillus* in the fruit processing environment – a review. International Journal of Food Microbiology 147, 1–11 with permission.

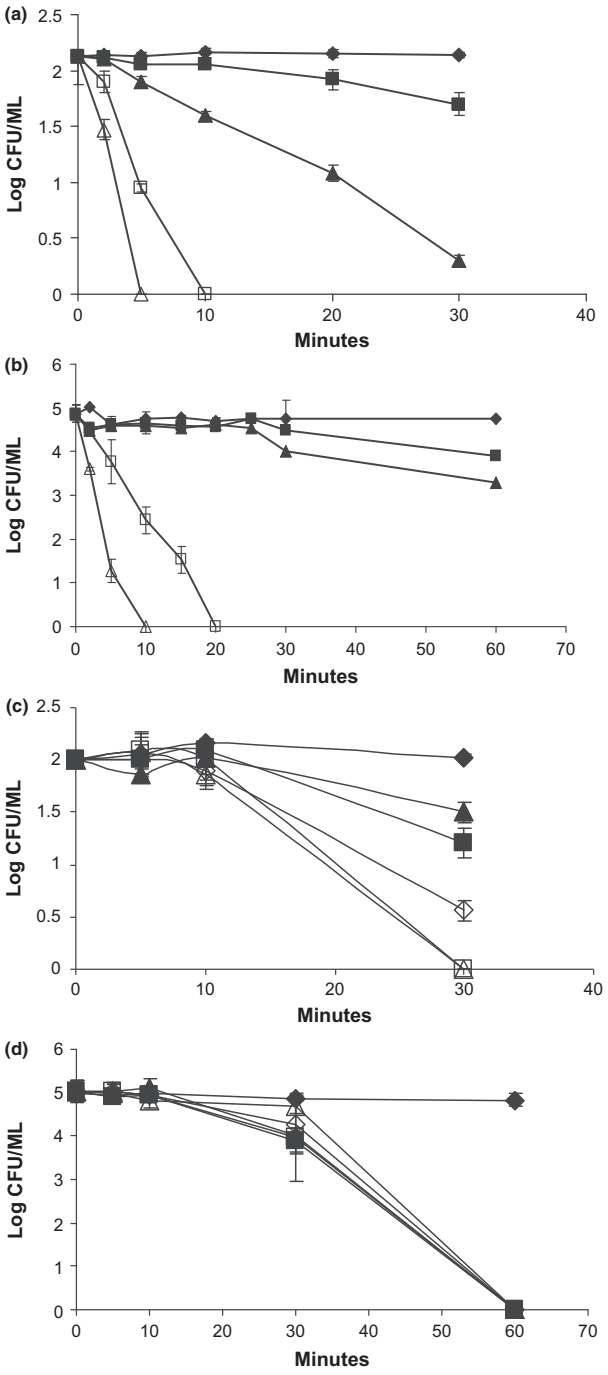

Figure 7 Survival of *Alicyclobacillus* spp. spores at 2 (a) and 5 (b) log cfu ml^{-1} inoculum levels following exposure to 0(◆), 5(■), 10(▲), 50(□), and 100(Δ) ppm chlorine dioxide in suspension and following exposure to 0(◆), 100(■), 200(▲), 500(◇), 1000(□), or 2000(Δ) ppm hypochlorite in suspension ($n = 9$; error bars represent standard deviations).

Table 4 Mean number of decimal reductions for *A. acidoterrestris* CRA 7152 in hot-fill orange juice with and without holding at 85 °C for 150 s

Inoculum level (A. acidoterrestris *spores per ml*)	*Holding at 85 °C for 150 s*	N_0 *(spores per l)*	N_f *(spores per ml)*	*Decimal reductions* (γ)
10^2	Yes	2.8 ± 0.1	2.8 ± 0.1	0.03 ± 0.1[b]
10^3	No	3.6 ± 0.5	3.3 ± 0.6	0.25 ± 0.1[a]
10^3	Yes	3.8 ± 0.1	3.7 ± 0.1	0.03 ± 0.1[b]

Different letters in the same column indicate significant statistical difference according to the Tukey test ($p < .05$).

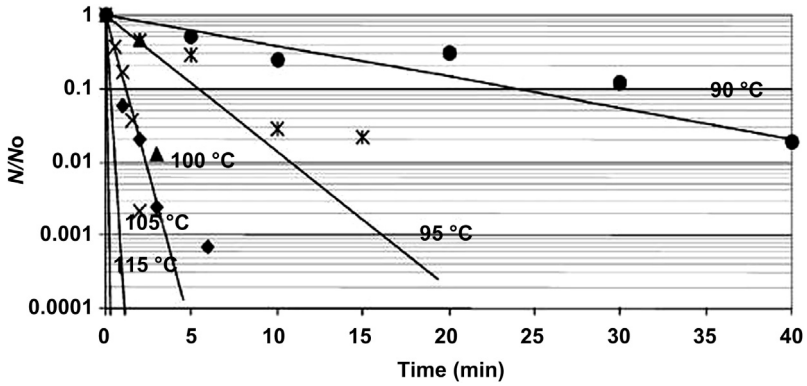

Figure 8 Thermal inactivation kinetics of *A. acidoterrestris* spores in Cupuacu nectar (pH 3.2 and 18 °Bx). Reproduced from Vieira, M.C., Teixeira, A.A., Silva, F.M., Gaspar, N., Silva, C.L.M. (2002). *Alicyclobacillus acidoterrestris* spores as a target for cupuaçu (*Theobroma grandiflorum*) nectar thermal processing: Kinetic parameters and experimental methods. International Journal of Food Microbiology 77(1–2), 71–81 with permission.

Table 5 Media for isolation and cultivation of *Alicyclobacillus* spp.

Substrate		*Composition*	*Use*
K agar[a]		Yeast extract, 2.5 g l^{-1}; bacteriological peptone, 5.0 g l^{-1}; glucose, 1.0 g l^{-1}; Tween 80, 1 g l^{-1}; malic acid; agar. The malic acid was added as a 2.5% (w/v) solution (pH 3.7)	Apples, juices
APDA	(acidified potato dextrose agar)	Potato extract, 4.0 g l^{-1}; dextrose, 20 g l^{-1}; agar. Acidified to pH 3.5 through a sterile solution of tartaric acid (10%)	Juices
OSA	(orange serum agar)	Tryptone, 10.0 g l^{-1}; yeast extract 3.0 g l^{-1}; dextrose, 4.0 g l; K_2HPO_4, 2.5 g l^{-1}; orange juice 200 ml; agar. Acidified to pH 3.5 through a sterile solution of malic acid (25%)	Juices, cupuacu nectar
YSG	(yeast extract starch glucose agar)	Yeast extract, 2.0 g l^{-1}; soluble starch, 2.0 g l; glucose, 1.0 g l^{-1}; agar. Acidified to pH 3.7 through sulfuric acid 1 N	Dried hibiscus flower
HGYE	(Hiraishi glucose yeast extract agar)	Glucose, 4 g l^{-1}; trypticase soy broth, 1.0 g l^{-1}; yeast extract, 0.5 g l^{-1}; $(NH_4)_2SO_4$, 3.0 g l^{-1}; $MgSO_4*7H_20$, 0.5 g l^{-1}; K_2HPO_4, 0.1 g l^{-1}. Acidified to pH 3.0 through sulfuric acid 1 N	Cell and spore counting
BAT-BAM	(*B. acidoterrestris* thermophilic medium *B. acidocaldarius* medium)	Yeast extract, 2 g l^{-1}; glucose, 5.0 g l^{-1}; $CaCl_2$, 0.25 g l^{-1}; $MgSO_4$, 0.5 g l^{-1}; $(NH_4)_2SO_4$, 0.2 g l^{-1}; K_2HPO_4, 3.0 g l^{-1} 1 ml of trace elements solution ($ZnSO_4*7H_2O$, 0.10 g l^{-1}; $MnCl_2*4H_2O$, 0.03 g l^{-1}; H_3BO_3, 0.30 g l^{-1}; $CoCl_2*6H_2O$, 0.20 g l^{-1}; $CuCl_2*2H_2O$, 0.01 g l^{-1}; $NiCl_2*6H_2O$, 0.02 g l^{-1}; $Na_2MoO_4*2H_2O$, 0.03 g l^{-1}); agar. Acidified to pH 4.0 through sulfuric acid 1 N	Suggested by International Fruit Union (IFU) as the standard medium to detect alicyclobacilli in juices
ALI	(*Alicyclobacillus* medium)	The composition is the same of BAM medium. Acidified to pH 4.0 through sulfuric acid 1 N	Orange and pear juices, nectar juice
AAM	(*A. acidoterrestris* medium)	Yeast extract, 2.0 g l^{-1}; glucose, 2.0 g l^{-1}; $(NH_4)_2SO_4$, 0.2 g l^{-1}; $MgSO_4*7H_20$, 1.0 g l^{-1}; $CaCl_2*2H_20$, 0.50 g l^{-1}; KH_2PO_4, 1.2 g l^{-1}; $MnSO_4*4H_20$, 0.5 g l^{-1}; agar. Acidified to pH 4.0 through a sterile solution of malic acid (25%)	Cell and spore recovery
SK agar		K agar supplemented with Tween 80 (1 ml); Ca^{2+} (0.5 g l^{-1}) and acidified to pH 4.0 through a sterile solution of tartaric acid (10%)	Recovery of low number of spores in juices
MEA	(acidified malt extract agar)	Malt extract, 17.0 g l^{-1}; peptone, 3.0 g l^{-1}; agar. Acidified to pH 4.5 through a sterile solution of citric acid 1:1 (w/w)	Evaluation of cells and spore number from juices or laboratory media

[a]The acidification of the media has to be performed after autoclaving, to avoid agar hydrolysis.
Reproduced from Bevilacqua, A., Sinigaglia, M., Corbo, M.R., 2008. *Alicyclobacillus acidoterrestris*: new methods for inhibiting spore germination. International Journal of Food Microbiology 125, 103–110 with permission.

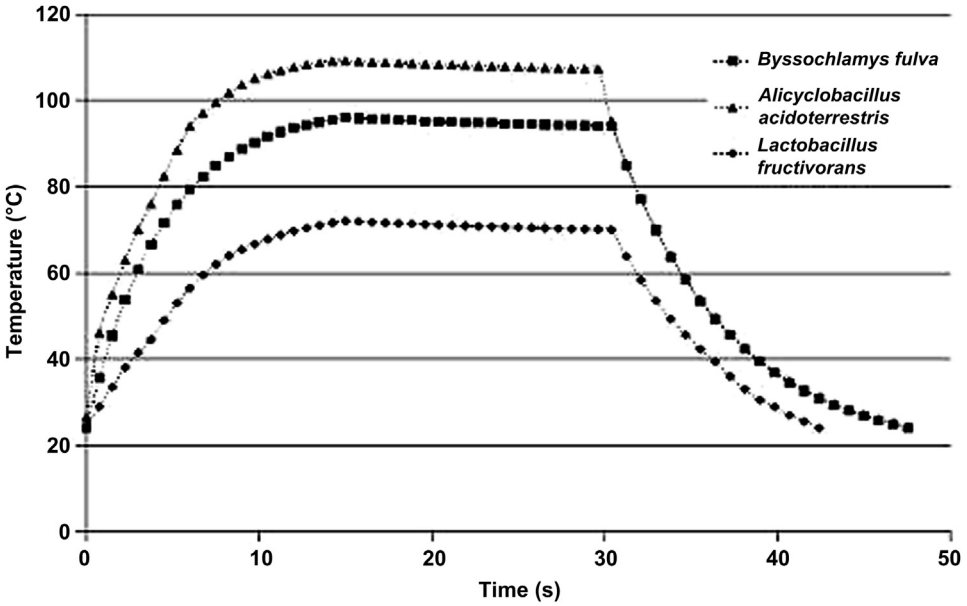

Figure 9 Comparison of pasteurization histories to obtain a hypothetical 5-log reduction of different microbial targets in fruit juice. Reproduced from Lima Tribst, A.A., De Souza Sant'ana, A., De Massaguer, P.R., 2009. Review: microbiological quality and safety of fruit juice past, present and future perspectives microbiology of fruit juices tribst et al. Critical Reviews in Microbiology 35(4), 310–339 with permission.

100 °C/5 min. Although all these conditions have been tested and may serve specific purposes, 80 °C/10 min corresponds to the most acceptable and applied heat shock treatment.

Although culture medium–based methods are greatly used with the purpose to isolate and enumerate *Alicyclobacillus*, more sensible, selective, reproducible, and rapid methods are required by industries to assess the quality of raw materials, processing environment, and final products. Thus, there has been observed an increased interest in the development of molecular biology–based and instrumental-based methods, such as real-time PCR, 16S rRNA gene sequence, and Fourier transform infrared spectroscopy (FT-IR) spectroscopy. PCR-based methods have been developed to detect genes required for production of off-flavor compounds, such as guaiacol. Other methods also used for detection, enumeration, and identification of *Alicyclobacillus* are based on immunological reactions, such as ELISA.

Because of the great challenge posed by *Alicyclobacillus*, the development of rapid, simple, and cost-effective methods continues to be major need for quality-control programs of acidic food industries.

Final Remarks

Because of its characteristics, strategies to control and avoid the spoilage by *Alicyclobacillus* must include the application of good manufacturing practices in the field, efficient fruit washing and selection, and strict control of time and temperature of pasteurization, and be combined with adequate storage conditions. Therefore, control strategies of *Alicyclobacillus* by acidic food industries should be based on an integrated approach from farm to market.

See also: **Fruit and Vegetables:** Introduction; Fruit and Vegetable Juices; **Heat Treatment of Foods:** Principles of Canning; **Heat Treatment of Foods:** Spoilage Problems Associated with Canning; **Heat Treatment of Foods:** Ultra-High-Temperature Treatments; Heat Treatment of Foods – Principles of Pasteurization; **Metabolic Pathways:** Production of Secondary Metabolites of Bacteria; **Spoilage Problems:** Problems Caused by Bacteria.

Further Reading

Bevilacqua, A., Sinigaglia, M., Corbo, M.R., 2008. *Alicyclobacillus acidoterrestris*: new methods for inhibiting spore germination. International Journal of Food Microbiology 125, 103–110.

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

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

Steyn, C.E., Cameron, M., Witthuhn, R.C., 2011. Occurrence of *Alicyclobacillus* in the fruit processing environment – a review. International Journal of Food Microbiology 147, 1–11.