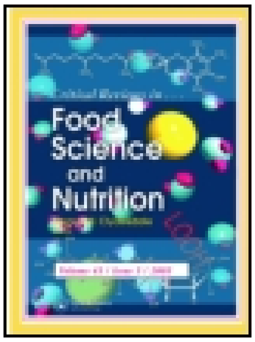

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CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
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REVIEW

Alicyclobacillus spoilage and control - a review

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ABSTRACT
In the last few decades Gram positive non pathogenic, rod shaped, thermo-acidophilic and acid-tolerant spore-forming bacteria such as *Alicyclobacillus* spp. have been identified as the causative agent in spoilage of commercially pasteurized fruit juice. In particular, *A. acidoterrestris* is considered a major producer of off-flavors. The spores of *A. acidoterrestris* possess the ability to survive commercial pasteurization processes, to germinate and grow in low pH environments and to produce volatile, unpleasant odorous compound (guaiacol) in fruit juices. The flat sour type of spoilage (without gas production or package swelling) is characterized as having a "medicinal," "smoky," and "antiseptic" off-flavor and makes the final juice product unacceptable. Spoilage by *Alicyclobacillus* is a major concern for producers since many of the new methods, which can destroy spores in the absence of chemical additives, may not destroy *Alicyclobacillus*. Although *A. acidoterrestris* is not pathogenic to humans, it can result in significant economic losses to juice processors because of its odor. The present review includes the taxonomy of *Alicyclobacillus* spp., their general characteristics, their resistance to heat and possible off-flavor production pathways. Particular emphasis is given to commonly used control measures, including physical, chemical and biological treatments currently available for removal of *Alicyclobacillus* spp.

KEYWORDS
Alicyclobacillus spp.; Thermo-acidophilic spore-forming bacteria; fruit juices; spoilage; control

Introduction

In 1982, huge spoilage of a pasteurized apple juice occurred in Germany during distribution and storage in what was an unusually long warm season (Cerny et al., 1984). The deterioration was characterized as a slight cloudiness in the juice and a distinct off-flavor similar to that of disinfectant, due to the production of chemical compounds guaiacol,2,6-dibromophenol and 2,6-biclorophenol.

The spoilage was due to a bacterium that was able to produce highly heat-resistant spores capable of surviving the usual pasteurization treatments, and of multiplying at temperatures between 45 and 50 °C with an optimum of 40–42 °C. The spoilage was attributed to an acid-tolerant organism "related to" *Bacillus acidocaldarius* but, later studies showed that the organism was *Alicyclobacillus acidoterrestris* (Goto et al., 2007). This was the first documented commercial fruit juice spoilage outbreak caused by *A. acidoterrestris*.

Alicyclobacillus in aseptically packaged juice fruit is now recognized as a significant spoilage organism in the fruit juice industry and the seriousness of this situation is increasingly being appreciated. In the present review, data will be presented on the taxonomy of *Alicyclobacillus* spp., their characteristics, factors that affect their growth and death, suggested off-flavor production pathways and ability to spoil various fruit-juice beverages. Particular emphasis will be given to commonly used control methods.

Taxonomy

Bacteria that show facultative thermophilic, obligatory acidophilic behavior and are spore formers were first isolated in 1967 from water sources (hot springs) in Japan (Uchino and Doi, 1967) and later from soil (Hippchen et al., 1981). Initially, they were placed in the genus *Bacillus*, because they form endospores in a manner similar to other bacilli. Strains isolated from hot springs were termed *Bacillus acidocaldarius* (Darland and Brock, 1971), while strains isolated from soil were termed *B. acidoterrestris* (Deinhard et al., 1987). A third thermo-acidophilic bacillus, distinct from *B. acidocaldarius* and *B. acidoterrestris,* was described by Poralla and König (1983). It differed from *B. acidocaldarius* and *B. acidoterrestris* in that it contained primarily ω(omega)-cycloheptane fatty acids in its membrane (instead of ω-cyclohexane fatty acid contained in *B. acidocaldarius* and *B. acidoterrestris*) and it was subsequently classified as a new species, *Bacillus cycloheptanicus* (Poralla and König, 1983; Deinhard et al., 1987). Subsequently, Wisotzkey et al. (1992), proposed a new genus to comprise the species *B. acidocaldarius*, *B. acidoterrestris* and *B. cycloheptanicus*. The genus was called *Alicyclobacillus* due to large amounts of ω-alicyclic fatty acid as the major natural membranous lipid component. In fact, phylogenetic analysis based on the comparisons of the 16S rRNA sequences showed that the three strains were sufficiently different from other *Bacillus* spp. to warrant reclassification in a new genus

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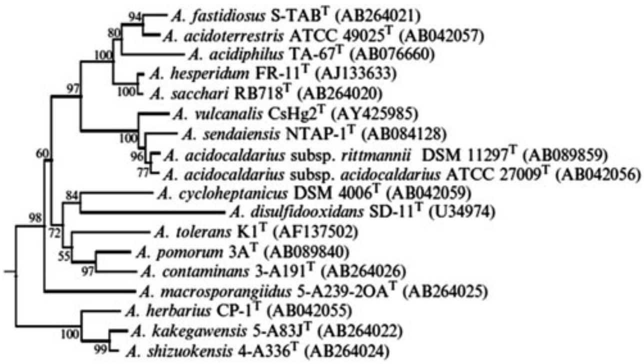

Figure 1. Phylogenetic tree indicating the relationships of the *Alicyclobacillus* species based on 16S rRNA gene sequence comparisons. *Bacillus subtilis* was used as an outgroup organism. (da Costa et al. 2009).

within the Gram-positive lineage of bacteria with low guanine + cytosine (G + C) content.

In recent years, the *Alicyclobacillus* genus has been updated constantly. From the three species first described (*A. acidoterrestris*, *A. acidocaldarius* and *A. cycloheptanicus*), when the genus was created in 1992 (Wisotzkey et al., 1992), there are 17 species (Figure 1) reported in the Bergey's Manual of Systematic Bacteriology (da Costa et al., 2009).

Since 2009, four new *Alicyclobacillus* species, *A.aeris*, *A. consociatus*, *A. ferrooxydans*, and *A. pohliae,* have been validly named (Stackebrandt, 2014). The latest described species are: *A. cellulosilyticus* (Kusube et al., 2014), *A. tengchongensis* (Kim et al., 2014), *A. dauci* (Nakano et al., 2015), *A. fodiniaquatilis* (Zhang et al., 2015), and *A. montanus* (López et al., 2018).

According to several researchers *A. acidoterrestris* is the predominant spoilage *Alicyclobacillus* species due to its high occurrence in spoiled products and fruit juice processing environments, and its ability to produce taints in fruit juices (Matsubara et al., 2002; Chen et al., 2006; Walker and Phillips, 2008; Danyluk et al., 2011). Consequently, *A. acidoterrestris* is recognized as the target species for quality control in the fruit juice industry (Goto et al., 2008; Osopale et al., 2016). Other *Alicyclobacillus* species, like *A. acidocaldarius* (Wisotzkey et al., 1992) *A. acidiphilus* (Matsubara et al., 2002; Goto et al., 2008), *A. herbarius* and *A. hesperidum* (Goto et al., 2008), *A. cycloheptanicus* (Gocmen et al., 2005), *A. pomorum* (Goto et al., 2003), and *A.dauci* (Nakano et al., 2015) are rarely encountered, although they can be relevant to the fruit juice industry as a cause of off-flavors. Furthermore, Zhang et al. (2013) were the first to show that *A. contaminans* could produce guaiacol (11.65 ppb) in pH adjusted kiwi fruit juice.

Characteristics of *Alicyclobacillus* spp.

Alicyclobacillus species are Gram positive, nonpathogenic, rod-shaped, thermophilic, and acidophilic spore-forming bacteria. All strains that have been examined produce endospores which tend to be terminal or subterminal and have an ovoid morphology; some species have swollen sporangia, but others do not (da Costa et al., 2009). All species are obligate aerobic, but *A. pohliae* is sometimes facultatively anaerobic (Imperio et al., 2008). Cerny et al. (2000) found that the presence or absence of a headspace in the packaging system did not significantly influence the growth of *A. acidoterrestris*. In contrast, the results obtained by Walker and Phillips (2005) demonstrate that headspace, storage temperature and adequate agitation of fruit juice containers play an important part in the growth and detection of *A. acidoterrestris* in apple juice. Growth rates in partly filled containers are higher than in full containers at temperatures at, or in excess of, 35 °C emphasizing the aerobic nature of *A. acidoterrestris*. If the sample is shaken before sampling, detection of growth of *A. acidoterrestris* is generally higher than if the container is not shaken and sampled from either top or bottom. The amount of moving and shaking would allow headspace oxygen to be incorporated into the juice, again emphasizing the aerobic nature of *A. acidoterrestris*. According Walker and Phillips (2005) filling the empty headspace of containers with an inert gas, such as nitrogen, may slow the growth of *A. acidoterrestris*. The results of Siegmund and Pöllinger-Zierler (2007) confirmed that a limited oxygen supply slowed the growth rate of *A. acidoterrestris.*

Soil is considered to be the main *habitat* of *A. acidoterrestris* and also the most important source of contamination of acidic products. Studies have suggested that contamination of fruit juices is most likely caused by the fruit being contaminated by soil during harvest or by unwashed or poorly washed raw fruit used in processing facilities (Chang and Kang, 2004). Another possibility is that soil can be carried into the manufacturing facilities by employees. In addition to soil, water can also be a major source of contamination (Huang et al., 2015). Chen et al. (2006) isolated several strains of *Alicyclobacillus* from cleaning water, distilled water,apple juice, and apple juice concentrate of a local juice-processing factory in Shaanxi, China. Groenewald et al. (2009) isolated several *A. acidoterrestris* strains from wash water, water at the evaporator inlet, flume water, and soil outside the juice-processing plant.

Alicyclobacillus spp. can be grouped into three categories in terms of their growth temperature ranges (da Costa et al., 2009): (i) the strains of *A. acidocaldarius* that have a growth temperature range of about 45–70 °C, with an optimum growth temperature of about 65 °C; (ii) species that have a temperature range, 20–65 °C, with optimum for growth of 40–55 °C, including *A. acidoterrestris, A.acidophilus*, *A. herbarius, A. hesperidum*, *A. pomorum*, *A. contaminans*, *A. cycloheptanicus, A. dauci*, *A. sendaienensis*, *A. vulcanalis*, *A. tolerans*, *A. fastidiosus*, *A. kakegawensis*, *A. macrosporangiidus*, and *A. shizuokensis*; (iii) species that have growth temperature range of about 4–55 °C and optimum growth temperatures of about 35–42 °C including *A. disulfidooxidans* and *A. tolerans*, formerly classified in the genus *Sulfobacillus*.

As mentioned above, the name of *Alicyclobacillus* derives from the presence of ω-alicyclic fatty acids as the major component in their membranes (Hippchen et al., 1981; Poralla and König, 1983; Albuquerque et al., 2000). Researchers have speculated on the function of ω-alicyclic fatty acids, suggesting that they contribute to the heat resistance and thermo-acidophilic nature of these bacteria. Closely packed rings of ω-alicyclic fatty acids form a protective coating of the cellular membrane, explaining the resistance of these bacteria to acid environments and high temperatures (Pontius et al., 1998; Walls and Chuyate, 1998; Merle and Montville, 2014).

Although some species of *Alicyclobacillus,* particularly *A. acidoterrestris*, can cause serious spoilage problems, there are not a safety concerns. Walls and Chuyate (2000) tested if *A. acidoterrestris* was pathogenic by injecting spores directly into mice or by feeding spore-inoculated juice to guinea pigs. No death or illness symptoms were reported in the mice, indicating that *A. acidoterrestris* is not pathogenic at the levels tested. Also, no reported illnesses have been attributed to the consumption of juice that had been spoiled by *A. acidoterrestris*. Similarly, there is no evidence proving that *A. consociatus*—identified and reported in May 2013—is pathogenic, although it was isolated from human blood of a 51 year old old woman (Glaeser et al., 2013).

Endospore heat resistance

Since *A. acidoterrestris* is the *Alicyclobacillus* species mostly associated with spoilage, heat resistance of its spores has been intensively studied (Jovetta et al., 2011; Groenewald et al., 2013). The *D* value (the time necessary—at a specific temperature—to reduce the overall microbial population by 90%) of *A. acidoterrestris* in fruit juices (apple juice, grape-juice, concord grape juice, orange juice, grapefruit juice, a clear apple drink, an orange drink, apple nectar without ascorbic acid, apple nectar with ascorbic acid and mango pulp) at 90 °C range from 5.95 to 23.10 min (Smit et al., 2011). For different strains of *A. acidoterrestris,* the D_{95} values (in apple juice, grape juice, berry juice, orange juice, a fruit drink, a fruit nectar, concord grape juice, Cupuaçu extract, grapefruit juice, mango pulp, clarified lemon juice, and non-clarified lemon juice) are usually between 1 and 10 min (Tianli et al., 2014). The *z*-values (the temperature increase necessary to reduce by 1 log cycle the time needed to achieve a 1 log reduction) ranged from 6.90 to 21.27 °C in different fruit products (De Carvalho et al., 2008) and from 5.90 to 10.00 °C in buffers (Bahçeci and Acar, 2007; Smit et al. 2011) (Table 1).

Several factors determine the heat resistance of *Alicyclobacillus* endospores including temperature, pH, TSS (total soluble solids) content (°Brix), water activity, species/strain, dipicolinic acid content and divalent cations and some antimicrobial compounds in products such as ascorbic acid which is used as an antioxidant to prevent browning in fruit nectars.

Table 1. Some *D* (min) and *z* (°C) values for spore of *A. acidoterrestris* reported in literature.

				D value (min)			
Heating medium	pH	SSC[a]	*T* (°C)	*D*	SD[b]	*z* value (°C)	References
Apple juice	3.5	11.4	85	56	14	7.7	Splittstoesser et al. (1994)
			90	23	7.5		
			95	2.8	0.7		
Grape juice	3.3	15.8	85	57	13	7.2	Splittstoesser et al. (1994)
			90	16	4.1		
			95	2.4	0.9		
Berry juice	3.5	NR[c]	88	11	NR	7.2	Walls (1997)
			91	3.8	NR		
			95	1	NR		
Wine	NR	NR	75	33	NR	10.5	Splittstoesser et al. (1997)
			85	0.57			
Orange juice drink	4.1	5.3	95	5.3	NR	9.5	Baumgart et al. (1997)
Fruit drink	3.5	4.8	95	5.2	NR	10.8	Baumgart et al. (1997)
Fruit nectar	3.5	6.1	95	5.1	NR	9.6	Baumgart et al. (1997)
Citrate buffer	2.5-6.9	NR	95	1	NR	NR	Yamazaki et al. (1997)
Model fruit juice	3.1	NR	91	31	NR	10.0	Pontius et al. (1998)
			97	7.9	NR		
	3.7	NR	91	54	NR	7.7	
			97	85	NR		
Cupuaçu extract	3.6	11.3	85	17.5	1.10	9.0	Silva et al. (1999)
			91	5.35	0.57		
			95	2.82	0.27		
			97	0.56	0.03		
Orange juice	3.5	11.7	85	65.6	5.5	7.8	Silva et al. (1999)
			91	11.9	0.6		
Blackcurrant concentrate	2.5	58.5	91	24.1	2.7		Silva et al. (1999)
Apple juice	3.5	10	85	117.6	9.4	10.9	Ceviz et al. (2009)
			90	32.8	1.84		
			95	20.8	1.98		
	4.0	10	85	105.3	5.52	16.4	
			90	36.1	4.53		
			95	27.8	1.70		
Orange juice	3.5	20	85	88.5	8.06	11.4	Ceviz et al. (2009)
			90	35.5	3.11		
			95	16.7	2.97		

[a]SSC, Soluble solids content.
[b]SD, Standard deviation.
[c]NR, Not reported.

Temperature

D-value decreased with an increase in temperature, which indicates decreased heat resistance. *D*-values decreased substantially when temperature was increased from 85 °C to 90 °C, and the highest *D*-values were recorded in blackcurrant concentrate (24.1 min at 91 °C) (Table 1) and lemon juice concentrate (12.63 min at 95 °C) (Silva et al., 1999; Maldonado et al., 2008).

pH

Conflicting results have been reported on the effects of pH. Silva et al. (1999) observed that pH had an effect on heat resistance, with a linear decrease in D-values being observed with a progressive decrease in pH. Vice versa, Murakami et al. (1998) found that pH did not have a significant influence on heat resistance, as there were no significant differences ($p > 0.05$) between the *D*-values of *A. acidoterrestris* AB-1 endospores in McIlvaine buffer at pH values ranging from 3.00 to 8.00 at a given temperature.

Total soluble solids (TSS) content

An increase in the TSS content led to an increase in *D*-values and higher heat resistance. In blackcurrant concentrate, when increasing the TSS from 26.1 to 58.5°Brix, the D_{91} values increased from 3.8 to 24.1 min (Silva et al., 1999; Silva and Gibbs, 2001). This explains the greater difficulty of destroying *Alicyclobacillus* endospores in fruit juice concentrates than in a single-strength juice.

Water activity

Bacterial spores are more heat resistant as water activity decreases and Silva et al. (1999) suggested that water activity may be used instead of TSS in future studies because different sugars generate different water activities at the same concentrations and could have different effects on *D*-values.

Species/strain

Heat resistance may differ among different species or even different strains of the same species (Tianli et al., 2014). Four different strains of *Alicyclobacillus acidoterrestris* (strain DSM 2498, strain 46, strain 70, and strain 145) showed four different D_{95} values (2.7 , 2.5 , 8.7 , and 3.8 min, respectively) under the same conditions of TSS content and pH (Eiroa et al., 1999). The heat resistance of *Alicyclobacillus* can be explained by the fact that, depending on the species, the ω-cyclohexane fatty acids of the cell membranes can vary from 15 to 91% of the total fatty acid content (Hippchen et al., 1981).

Dipicolinic acid content and divalent cations

The mineralization of divalent cations (especially Ca^{2+}) with dipicolinic acid (DPA) to form the Ca-DPA complex also affect the heat resistance of spores. 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 (Chang and Kang, 2004).

Antimicrobial compounds

According to Bahçeci and Acar (2007), at a fixed pH of 3.5, an increasing of the ascorbic acid concentration resulted in a slight decrease in *D*-values especially at the low temperatures tested, although it was not found to be significant within the concentration studied. On the other hand, Cerny et al. (2000) reported that the addition of 100 mg/L of ascorbic acid into apple juice stimulated the growth of *A. acidoterrestris*, whereas 150 mg/L or higher concentration of ascorbic acid inhibited their growth.

According to some researchers, the high heat resistance of *A.acidoterrestris* spores make it suitable to be used as the reference microorganism to design pasteurization processes for acidic fruit products (pH <4.6), just as the design of food sterilization processes is based on *Clostridium botulinum* spores for low acid canned foods, that is, food with pH >4.6 (Silva and Gibbs, 2004). For low-acid canned foods, the food industry uses, as the criterion, a 12 logarithmic reduction in the number of *C. botulinum* spores, but for high-acid fruit products there are no specific parameters for juice pasteurization (FDA, 2001). This because each type of juice and juice products have unique characteristics.

Spoilage

Alicyclobacillus spp. can inflict significant economic losses to juice processors because visual detection of spoilage is very difficult and—unlike the typical juice spoilage—since gas is not produced during growth, and therefore swelling of containers does not occur and no substantial change in fruit juice pH occurs (Merle and Montville, 2014). *Alicyclobacillus* spp. operate in a manner similar to another group of spore-forming bacteria, the flat sour organisms, for example, *Bacillus coagulans* and *Geobacillus stearothermophilus*, which spoil low acid canned foods but without visible evidence of the deterioration of the product. In both cases, the impact on the unsuspecting consumer can be quite unpleasant and result in brand damage for the product involved (Gordon, 2017). Spoilage of juice products can occur long before the expiration date and companies often do not realize a spoilage incident has occurred until they receive consumer complaints (Chang and Kang, 2004). The spoiled products may be either pasteurized, hot-filled, ultra-heat-treated, canned, or carbonated, which are all characterized by a specific off-flavor and off-odors described as a "medicinal, smoky, phenolic, or antiseptic" (Walls and Chuyate, 1998; Orr et al., 2000; Chang and Kang, 2004; Huang et al., 2015), with normal or light sediment. The compounds associated with off-flavors caused by *Alicyclobacillus* species include three main odiferous chemicals: guaiacol, 2,6-dibromophenol (2, 6-DBP) and 2,6-dichlorophenol (2,6-DCP). Guaiacol is the predominant metabolite associated with the spoilage in fruit juice, although 2,6-DBP and 2,6-DCP have been also isolated from products with large populations of *Alicyclobacillus* spp.

Guaiacol

Guaiacol (2-methoxyphenol) is a phenolic compound with the formula $C_6H_4(OH)(OCH_3)$. Guaiacol is accepted to be the major metabolite associated with off-flavors and off-odors in fruit juices spoiled by *A. acidoterrestris* (Springett, 1996). The postulated pathway of guaiacol formation is during ferulic acid metabolism (a common compound in fruit juices) where (i) ferulic acid is decarboxylated to 4-vinylguaiacol or transformed to vanillin; (ii) 4-vinylguaiacol is oxidized to vanillin; (iii) vanillin is oxidized to vanillic acid and (iv) vanillic acid is decarboxylated to guaiacol (Peleg et al., 1992; Huang et al., 1993; Mathew et al., 2007). Another possible precursor of guaiacol is tyrosine. Apple juice contains approximately 4.1 μg tyrosine/ml juice and orange juice contains 3.4–13.5 μg tyrosine/ml juice. However, this reaction has not been extensively studied (Jensen, 1999; Smit et al., 2011). The human sensory threshold of guaiacol is low, so it is easily detected by olfactory evaluation. It has been reported that the threshold concentration of guaiacol in water is 0.021 ppm for odor and 0.013 ppm for taste detection, while in 12% aqueous ethanol olfactory perception was reported to be 0.03 ppm (Chang and Kang, 2004). In the case of *A. acidoterrestris* spoilage, there is a critical level in cells that must be present before taint compounds are detectable organoleptically. Bahçeci et al. (2005) reported that in apple juice inoculated with 10^3 CFU/ml, guaiacol production only started after approximately 30 h, once a cell concentration of 10^4 CFU/ml had been reached, while guaiacol production started immediately when apple juice was inoculated with 10^5 CFU/ml. Chang et al. (2015) studied extrinsic factors (temperature, pH and oxygen concentration) that can affect the production of guaiacol by two strains of *Alicyclobacillus* (isolates 1016 and 1101). Maximum production of guaiacol by isolate 1016 was detected within 9 h when incubated at 43 °C and pH 4.0, under microaerophilic conditions. Isolate 1101 produced detectable amounts of guaiacol within 8 h at pH 5.0. Their results indicate that the production of guaiacol, contrary to common belief, is a rapid reaction under desirable conditions specific to each isolate (Chang et al., 2015).

Halophenols

Although guaiacol is considered the predominant off-odor compound, researchers also detect halophenols, 2,6-DBP and 2,6-DCP produced by *A. acidoterrestris* in spoiled juices (Chang and Kang, 2004). Halogenated phenolic compounds are well known as one of the most common causes of off-flavors in foods. Their occurrence in food can be either from chemical contamination or microbial synthesis. The taste threshold in water of 2,6-DCP is 6.2 ppt and 0.5 ppt for 2,6-DBP. In juices, the taste threshold is reported to be 0.5 ppt for 2,6-DBP and 30 ppt for 2,6-DCP (Chang and Kang, 2004; Smit et al., 2011).The metabolic pathways of the halophenols are still not clear.

Control

Physical treatments

Thermal methods

The conventional heat treatment normally applied to pasteurize fruit juices accomplished by a hot-fill and hold process (88–96 °C, 2 minutes), generally destroys the heat-liable spoilage organisms such as aciduric bacteria, yeasts, and some types of molds. Bacterial spores are unlikely to be destroyed by pasteurization treatments, but their germination would be inhibited by the low pH, lower than 4 in most cases (Silva et al., 1999; Chang and Kang, 2004). Though spores and 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 (Palop et al., 2000). Since *A. acidoterrestris* is acidophilic, the spore can germinate in acid media (such as apple juice or fruit-based drinks and beverages at pH values as low as 2.5) and grow when temperatures are higher than 20 °C. The pasteurization will not inactivate the *Alicyclobacillus* spores, but serves as a heat treatment that will stimulate their germination, which follows outgrowth of the organism (Gouws et al., 2005; Groenewald et al., 2009; Osopale et al., 2016). If incubation conditions are favorable after the heat treatment and spore outgrowth, flavor taints will develop (Jensen, 1999; 2000). *A. acidoterrestris* is a thermophilic microorganism; hence it does not grow below 20 °C (Jensen and Whitfield, 2003; Spinelli et al., 2009). Storing commercial pasteurized fruit juices at temperatures below 20 °C could be enough to prevent germination and outgrowth of spores and provide a potential control measure for the industry, avoiding spoilage by *Alicyclobacillus* spp. (Chang and Kang, 2004). Unfortunately, the conditions prevailing in the supply chain of pasteurized fruit drinks are out of the manufacturers' direct control and often deviate from specifications (Heyndrickx, 2011), especially during the warmer months or in tropical and semitropical regions (Roig-Sagues et al., 2015; Kakagianni et al., 2018). In addition, pasteurized fruit juices are not commonly distributed under refrigeration conditions of 4–8 °C, because chilling these products would be a major new cost factor (Chang and Kang, 2004).

As *A. acidoterrestris* is an aerobic microorganism, the absence of air is essential to rule out the development of *Alicyclobacillus* spp. (and consequently guaiacol production). Whatever the heat treatment used, according to Gordon (2017), a good vacuum and hermetic seal in a container must be ensured, as well as rapid cooling down to room temperature or below. This leads to a sufficient post-process vacuum that that excludes air in the headspace of the bottled product. This means that if sufficient focus is placed on ensuring the development of a good vacuum and a hermetic seal, even though *Alicyclobacillus* spp. would survive the process, their growth would be impaired.

If thermal processes, other than pasteurization, are stringent enough to destroy heat resistant *Alicyclobacillus* spores in fruit juices they may not be feasible as they are potentially damaging to the product quality by degrading taste, color and flavor, as well as loss of nutrients (Danyluk et al., 2011). Therefore the efforts of processors, together with the scientific community, in attending consumer demands for high quality food and dealing with raising economic standards has triggered the development of novel non-thermal technologies to inhibit spore germination and growth of *A. acidoterrestris* in fruit juices (Bevilacqua et al., 2008a; Steyn et al., 2011).

Non-thermal methods

*High hydrostatic pressure (*HHP*).* High-pressure processing is a nonthermal method that does not cause heat-related deterioration phenomena like loss of vitamins and nutrients, discoloration and textural and organoleptic changes. HHP treated foods are therefore often of superior quality than thermally processed foods. Many researchers have studied the application of HHP for inactivating *A. acidoterrestris* vegetative cells and spores (Lee et al., 2002; Alpas et al., 2003; Lee et al., 2006a; Silva et al., 2012; Vercammen et al., 2012; Sokołowska et al., 2012). The application of HHP, combined with an elevated temperature, can efficiently inactivate bacterial spores, for example, approximately by 2 log reduction of *A. acidoterrestris* spores after processing with 200 MPa at 65 °C for 10 minutes (Silva et al., 2012), and by more than 5.5 log reduction at 70 and 90 °C using pressures ranging from 207 to 621 MPa (Lee et al., 2002). The mechanism of inactivation of bacterial spores through high pressure occurs in two steps: (i) high pressure induces the spores to germinate, and (ii) the elevated temperature causes the inactivation of germinated spores (Setlow, 2003; Luu et al., 2015). More recently, Porębska et al. (2016) investigated the effect of HHP and SCCD (supercritical carbon dioxide) on inactivation and germination of spores of *A. acidoterrestris* strains. After 40 minutes of SCCD treatment at 60 MPa and 75 °C, germination of *A. acidoterrestris* spores in apple juice (11.2°Brix) was 3.9 log of which 3.4 log were inactivated. On the contrary, under the same conditions, in apple juice concentrate (70.7°Brix), germination and inactivation of the spores was 1.5 log and 0.9 log, respectively. According to Porębska et al. (2016) these results demonstrate that SCCD and HHP combined with moderately elevated temperatures may be a useful technique for inactivation of *A. acidoterrestris* spores in single strength juices. Unfortunately, these treatment temperatures are not lethal to spores which have not germinated and, furthermore, the inactivation of *A. acidoterrestris* spores under high pressure and supercritical carbon dioxide was shown to be suppressed by a high TSS content in apple juice concentrates.

Ultra-high-pressure homogenization (UHPH)**.** Ultra-high-pressure treatments are considered to be the most promising of these new food processing technologies because of improvements in high-pressure machines and the acceptance of pressure-processed foods (Dumay et al., 2013). The treatment is based on the same design principles as the conventional homogenization used in the dairy industry but involves much higher pressures (>100 MPa). Depending on the nominal pressure level, the technology will be called high-pressure homogenization (HPH, up to 150–200 MPa) or ultra-high-pressure homogenization (UHPH, up to 350–400 MPa). The antimicrobial effectiveness of HPH and UHPH against foodborne pathogens has been widely studied (Brinez et al., 2006; Diels and Michiels, 2006) but very few studies deal with *A. acidoterrestris* inactivation using HPH or UHPH. The antimicrobial effectiveness of HPH on controlling *A. acidoterrestris* was investgated by Bevilacqua et al. (2007), using three different strains (DSMZ 2498, γ4 and c8). HPH caused a significant reduction ($p < 0.05$) of the initial cell number (1–2 log CFU ml^{-1} at the highest pressures) in γ4 and DSMZ 2498 strains, 0.25 log reduction of initial cell number was observed for c8 strain. In addition, the cells were more sensitive than the spores. Bevilacqua et al. (2007) concluded that the susceptibility of *A. acidoterrestris* to HPH was strain-dependent with DSMZ 2498 appearently the most susceptible strain, and c8 strain was the most resistant strain.

*UV-C light inactivation***.** The ultraviolet (UV)-C light is the region of the electromagnetic spectrum that ranges from 200 to 280 nm (Bintsis et al., 2000; Lado and Yousef, 2002). It has germicidal effects on microorganisms such as bacteria, yeasts, molds and viruses as a consequence of DNA damage (Caminiti et al., 2012), and has also been approved to treat food surfaces and to clear fruit juices (FDA, 2000). Advantages associated with UV-C radiation used as a nonthermal method for microorganism control are that no known toxic or significant nontoxic by-products are formed during the treatment and the treatment requires very little energy when compared to thermal pasteurization. Furthermore, UV treatment of apple, pineapple, and orange juices does not change their taste or color profiles (Keyser et al., 2008). Tremarin et al. (2017) studied the influence of UV-C radiation with seven different intensities (0.34, 0.86, 2.59, 5.59, 8.45, 11.50 and 13.44 W/m^2) on *A. acidoterrestris* inactivation in apple juice. Commercial juices were artificially inoculated with bacterium, with initial loads of about 10^7 CFU/mL; then exposed to UV-C radiation and the impact of the treatment on microbial loads was assessed throughout the exposure times. Results showed that the log-survival of *A. acidoterrestris* was decreased linearly with treatment time, for all seven intensities tested. When the most severe intensity was used, the number of spores decreased most with some 5-log reduction after 8 min of treatment. The authors concluded that UV-C radiation is a promising treatment with a substantial impact on the loads of *A. acidoterrestris* in apple juice, especially when high intensities were used. However, the limiting factor on the application of UV remains its low penetration into food matrices resulting in low absorption coefficients.

Irradiation. Irradiation technology involves the application of gamma rays (from a radioisotope source Cobalt-60), X-rays or electrons (Mahapatra et al., 2005). Although gamma irradiation has been applied to various fruit juices to control food borne pathogenic bacteria as well as enzymes (Foley et al., 2002; Song et al., 2006; Alighourchi et al., 2008), little information is available on the effects of gamma irradiation on the reduction of *A. acidoterrestris* spores in fruit juices. Lee et al. (2014) evaluated the effects of gamma irradiation on reduction of *A. acidoterrestris* spores in different concentrations of reconstituted apple and orange juices. Spores of *A. acidoterrestris* were inoculated into three concentrations of apple (18, 36, and 72°Brix) and orange (11, 33, and 66°Brix) juices and subjected them to five radiation doses (1, 3, 5, 7, and 10 kGy). No significant reductions ($p > 0.05$) in spores were observed after the 1-kGy treatment any of the apple and orange concentrations. Spores in 18, 36, and 72°Brix apple juice exposed to 10 kGy were reduced to 4.34, 3.9, and 3.84 log CFU/ml, respectively. Similar results were observed for orange juice. The researchers concluded that gamma irradiation is effective in inactivating A. *acidoterrestris* spores in apple and orange juices and this technology could be applied by the fruit juice industry to replace conventional methods used to control spores of *A. acidoterrestris*. Unfortunately the major limitations of irradiation processing of food is its slow acceptance by the consumers, due, *inter alia*, to a perceived association with radioactivity and the possibility of generating off-odors.

Microwaves and ultrasonic waves. Ultrasound or ultrasonic waves (UWs), in its most basic definitions, refers to electromagnetic waves with a frequency of 20 kHz or more, that can cause microbial cell death by a phenomenon called cavitation (Chen, 2012). Yuan et al. (2009) investigated the effect of ultrasonic treatments on *A. acidoterrestris* in apple juice. They found that inactivation of the cells was more pronounced at higher power levels and as the exposure time increased. Approximately 60% of the cells were inactivated after treating the apple juice with 300-W ultrasound for 30 min. The reduction reached more than 80% when the juice was processed for 60 min. However, reductions in the sugar content, hazing and browning of the juice, and increased acidity were noted after ultrasonic treatments. Evelyn and Silva (2016) found that HPP (high pressure processing) enhanced the thermosonication (TS, use of ultrasound and heat) for the inactivation of *A. acidoterrestris* spores in orange juice. In particular, the TS of orange juice pretreated with 600 MPa HPP for 15 min was the best technique, of those tested, in inactivation *A. acidoterrestris spores*, allowing a 3 log reduction after 42 min. Although the use of ultrasound might not be able to serve as a standalone unit operation to inactivate *A. acidoterrestris*, it could be employed as a secondary operation following thermal inactivation in order to ensure effective destruction of residual cells. However, furthers studies are needed for development of microwaves (MW) and UW technology for commercial applications.

*Ohmic heating technique (*OHT*).*The ohmic heating is treatment based on the passage of an electrical current through a food that contains sufficient water and electrolytes to generate heat within the product. The effectiveness of ohmic and conventional heating for reducing spores of *A acidoterrestris* was investigated in commercial pasteurized orange juice by Baysal and Icier (2010). The kinetic parameters (*D*-value and *z*-value) were determined during ohmic and conventional heating. At 30 V/cm, *D*-values at 70, 80 and 90 °C were 58.48, 12.24, and 5.97 min, respectively. *D*-values at corresponding temperatures for conventionally heated spores were 83. 33, 15.11, and 7.84 min, respectively. These results of Baysal and Icier (2010) showed significantly ($p < 0.05$) higher lethality for spores treated with ohmic heating than for spores treated with conventional heating. More recently, Kim et al. (2017) examined the sporicidal effect of an ohmic heating (OH) system with five sequential electric fields and compared it with that of conventional heating. Apple juice (50 kg) inoculated with *A. acidoterrestris* spores was subjected to OH (electric field strength =26.7 V/cm; frequency =25 kHz) at 85–100 °C for 30–90 s. The effect of conventional heating was also examined under these conditions. OH treatment at 100 °C for 30 s resulted in total inactivation of the inoculum, with no recovery of viable cells (initial population =4.8–4.9 log CFU/ml), whereas 3.6–4.9 log CFU/ml of the spores survived conventional heating. These results suggest that the OH system is superior to conventional heating for rapid sterilization (30 s) of apple juice to assure microbiological safety in the absence of chemical additives. OH did not significantly alter the quality (°Brix, color, and pH) of commercial apple juice ($p < 0.05$). Kim et al. (2017) concluded that OH treatment would be effective from a practical perspective because (i) it can be used to treat bulk samples (in this case 50 kg), (ii) it can sterilize apple juice in a short time (30 s), (iii) chemical additives are unnecessary and (iv) it does not cause a significant deterioration of both °Brix and color of commercially processed apple juice. In future studies these encouraging results should be accompanied by sensory analyses to determine if ohmic pasteurization has any negative organoleptic effects on fruit juices.

Chemical treatments

Ozone (O_3). Gaseous ozone is a powerful antimicrobial substance due to its potential oxidizing capacity and has been declared as Generally Recognised as Safe (GRAS) by the Food and Drug Administration of the USA for direct application on food products (FDA (Food and Drug Administration), 2001). Torlak (2014) found that the level of *A. acidoterrestris* spores in apple juice decreased by 2.2 and 2.8 log after 40 min when the juice was bubbled with continuous stream at two constant concentrations (2.8 and 5.3 mg/l, respectively) of ozone at 4 °C. According to FDA, a minimum 5 log reduction of spoilage and potentially pathogenic bacteria is most commonly associated with fruit juices and is considered as a primary performance standard for non-thermal processing methods (Tiwari and Muthukumarappan, 2012). Although reduction values obtained in apple juice after 40 min of ozonation did not meet the FDA's requirement of a 5 log reduction, Torlak (2014) concluded that the efficacy of ozone against *A.*

acidoterrestris spores can be increased by increasing the concentration and exposure time. Treatment of apple juice with the gaseous ozone can also be a complementary microbial reduction method for the control of *A. acidoterrestris* spores.

Chlorine dioxide (ClO_2). Application of this sanitizing agent has recently received attention due to its potential advantages over chlorine-based sanitizers, because the FDA has allowed the use of aqueous ClO_2 in washing fruits and vegetables (FDA (Food and Drug Administration), 1998). Danyluk et al. (2011) studied the survival of a cocktail of spores of five *Alicyclobacillus* spp. onto the fruit surface of grapefruit, guava, limes, mangoes, oranges and pineapple, which were then washed with 0, 50, or 100 ppm aqueous ClO_2. Significant reductions ($p < 0.05$) due to chlorine dioxide were only seen on the citrus fruits. While Lee et al. (2006b) reported that the visible effects on the fruit treated with high levels of ClO_2 gas were unacceptable, no significant ($p > 0.05$) changes in visual quality were seen between control (untreated) and treated fruit by Danyluk et al. (2011). However, as *Alicyclobacillus* spp. spoilage is associated with juices rather than fresh fruit, visible damage to the fruit skin by ClO_2 may be acceptable if *Alicyclobacillus* spp. contamination can be controlled in juices and purees. Recently Cai et al. (2015) determined the potential of combining chlorine dioxide (ClO_2) with ultrasound or shaker processes to reduce *A. acidoterrestris* spores on the apple. The results showed that ClO_2 in combination with shaker was the most effective in reducing *A. acidoterrestris* spores on apples (Cai et al., 2015). After treatment with 200 mg/l ClO_2 plus shaker (200 rpm) for 20 min, the viable spores remaining on the surface of the apple were reduced to undetectable levels (<1.7 log10 CFU/apple). According to Cai et al. (2015) this study demonstrates that the combination of ClO_2 and shaker is an effective approach for controlling *A. acidoterrestris* spores on apples and minimizing the risk of contamination in apple juice.

Organic acids, potassium sorbate and sodium benzoate Hsiao and Siebert (1999) studied the effectiveness of some organic acids against *A. acidoterrestris.* Although experiments were performed on cells, and no data were available on spores, using the minimum inhibitory concentrations, it was possible to derive a hierarchy of the acids effectiveness: benzoic > butyric-caprylic > acetic ≫ citric-malic-lactic-tartaric acids. The use of chemical preservatives, like potassium sorbate and sodium benzoate, may be added to control growth of *A. acidoterrestris.* The level of these additives that are allowed in beverages marketed under European Union (2004) legislation is 1500 mg/L. Walker and Phillips (2008) found that sodium benzoate and potassium sorbate were effective against *A. acidoterrestris* but the levels for inhibition of each preservative were higher for vegetative cells than spores. In particular sporulation was inhibited even when vegetative cells survived. Sodium benzoate, with its broad antibacterial range, non-volatility and water solubility, is widely used as a fruit beverage preservative. Its antimicrobial activity is due to the undissociated acid, the effective concentration of which declines as the pH level increases. The antimicrobial activity of potassium sorbate also comes from the undissociated acid but, unlike benzoic acid, this is less affected by pH and consequently it can be used at pH levels higher than 3.0 (Walker and Phillips, 2008).

*Poly dimethyl ammonium chloride (*PDAC*).* Recently Osopale et al. (2017) investigated the antimicrobial activity of PDAC, an emerging disinfectant, that is recommended for the disinfection of drinking water for poultry, hatching eggs and the air in poultry houses, indicating the chemical's low level of toxicity against living tissues. PDAC solution is becoming widely used for general disinfection beyond the poultry applications and therefore may be useful in the fruit juice industry for the control of *A. acidoterrestris* contamination. According to Osopale et al. (2017), whose study is on the activity of PDAC against *A. acidoterrestris* under laboratory conditions, there is need for further *in situ* studies for ascertaining the effectiveness of this promising disinfectant in cleaning fruit and production surfaces during industrial processing of fruit juices.

Alternative natural compounds

Natural antimicrobials of microbial origin (bacteriocins)

Nisin. Nisin is one of the bacteriocins with well-documented inhibitory effect against Gram-positive bacteria and spore formers such as *Bacillus* and *Clostridium* spp. It is essentially nontoxic to humans and therefore currently recognized as a safe food preservative in many countries (Gharsallaoui et al., 2016). Its use against *A. acidoterrestris* cells and spores has been extensively studied (Yamazaki et al., 2000; Peña et al., 2011; Huertas et al., 2014). At present, nisin is the only compound currently used in fruit juices for the control of *A. acidoterrestris*, given its identification as an effective agent in preventing spore outgrowth (Cleveland et al., 2001). Nisin can be added directly to fruit juices, as suggested by Komitopoulou et al. (1999) and Yamazaki et al. (2000), or incorporated into biodegradable polylactic acid polymer film, used in packaging, where it is released in a controlled manner during storage (Jin and Zhang, 2008; Barbosa et al., 2013). One study found that nisin can inhibit many human intestinal bacteria (Le Blay et al., 2007). To solve this challange, some reports have produced nano material conjugates of nisin that can inhibit resistant bacteria and then be easily separated from the food product (Adhikari et al., 2012). Song et al. (2017) have recently described a novel approach in which iron oxide nanoparticles (IONPs) were conjugated with nisin (IONPs–nisin). IONPs–nisin inhibited target strains via pore formation in the membrane, which is the same as with nisin. The advantage of this approach is that the IONPs are ferromagnetic in nature and can be rapidly separated by an external magnetic after being deployed as antimicrobials. However, more systematic research should be done before its practical application for the food sector.

Enterocin AS-48. Enterocin AS-48, produced by *Enterococcus faecalis* A-48-32, was active against one *A. acidocaldarius* and three strains of *A. acidoterrestris* (Grande et al., 2005). Examination under an electron microscopy of vegetative cells and endospores treated with enterocin AS-48 revealed substantial cell damage and bacterial lysis as well as disorganization of endospore structure supporting the hypothesis that the bacteriocin is adsorbed into the spores has negatively charged surface groups. This interaction with *A. acidoterrestris* would suggest a sporicidal rather than the sporostatic mechanism of action that was suggested for *Bacillus cereus* (Grande et al., 2005).

Bificin C6165. Bificin C6165, produced by *Bifidobacterium animalis* subsp. *animalis*, is another new bacteriocin that has been shown to control *A. acidoterrestris* in fruit juices. Vegetative cells of *A. acidoterrestris* were inactivated by bificin C6165 at 40 µg/ml, the inhibitory effect being better at a lower pH (pH 3.5) and a higher temperature (45 °C). Although the addition of bacteriocin contributed to the reduction of the thermal resistance, no significant ($p > 0.05$) activity of bificin C6165 was observed against the endospores of *A. acidoterrestris* in commercial apple juice (Pei et al., 2014).

Other bacteriocins. Several other bacteriocin, in addition to those described above, have been proposed against *Alicyclobacillus* spp., including warnericin produced by *Staphylococcus warneri* RB4 (Minamikawa et al., 2005), bovicin HC5 purified from *Streptococcus bovis* HC5 (De Carvalho et al., 2008), paracin C extracted from *Lactobacillus paracasei* (Pei et al., 2013; 2017) and plantaricyclin A produced by *Lb. plantarum* (Borrero et al., 2018). Despite the suitable physicochemical and antimicrobial properties of the bacteriocins, their utilization is limited by the high cost of extraction and purification (Huang et al., 2015).

Natural antimicrobials of animal origin. *Lysozyme.* Lysozyme is as a common natural and "green" antibacterial agent present in various biological fluids and tissue such as saliva, tears, eggs and milk. It is often used to kill Gram-positive microorganisms, particularly thermophilic spore formers. The required concentrations are generally 20 μg/ml (Voundi et al., 2017). Similar to nisin, lysozyme has Generally Recognized as Safe (GRAS) status. It has been used as a food preservative in cheeses, cow's milk, beer, fresh fruits and vegetables, fish, meat and wine (Antolinos et al., 2011). The bioactivity of lysozyme against *A. acidoterrestris* has been studied in laboratory conditions. Conte et al. (2006) evaluated the effectiveness of a polymeric matrix film with immobilized lysozyme on the surface against both a single strain and a culture cocktail of *A. acidoterrestris* at 44 °C. By monitoring the viable cell concentration under three different packaging conditions, it was possible to demonstrate that the active film was equally effective against both the single strain and the culture cocktail and that it maintained this efficacy at various medium volumes. The same microbial tests were also conducted on viable spores of *A. acidoterrestris* inoculated both into a laboratory medium and apple juice. The results indicate that these viable spores were better inhibited than cells by lysozyme immobilized on the active film in both investigated media. Although similarly to nisin, the activity of lysozyme is dose-dependent and varies within the external conditions applied (Sokołowska et al., 2012; Molva and Baysal, 2017).

Chitosan. Chitosan is the N-deacetylated derivative of chitin, which can be extracted from the shells of crabs, shrimps and crawfishes. Chitosan can be applied as an antimicrobial agent against fungi, bacteria, and viruses (Rabea et al., 2003; Sivakumar et al., 2016). Falcone et al. (2003) studied the effectiveness of chitosan against *A. acidoterrestris* spores as a combined hurdle with thermal processing. They found that the optimal amount to add to the medium was 1.4 g/l, because lower amounts were not effective in controlling spore germination and higher amount could result in the formation of flakes. More recently the antimicrobial effect of chitosan on *A. acidoterrestris* spores during the clarification process of apple juice was determined by Taştan and Baysal (2017), but the microbial reduction of *A. acidoterrestris* was not found to be significant.

Natural antimicrobials of plant origin

*Essential oils (*EOs*).* EOs are aromatic liquids obtained from plant materials, mainly herbs and spices (Burt, 2004). Carvacrol, cinnamaldehyde, eugenol, citralgeraniol and D-limonene are the active components of EOs, which have been shown to possess antimicrobial activity against *A. acidoterrestris*. The antimicrobial activities of leaf extracts from 26 species of *Eucalyptus* toward *A. acidoterrestris* were measured by Takahashi et al. (2004). Extracts from *E. maculata* exhibited potent antimicrobial activities against *A. acidoterrestris* with low inhibitory concentrations ranging from 7.0 to 31 g/l. Bevilacqua et al (2008b) used cinnamaldehyde, eugenol and limonene (0.05–0.5 g/l) against the spores of two different strains of *A. acidoterrestris* that had been isolated respectively from soil and spoiled pear juice. Cinnamaldehyde was the most effective of the three compounds and a concentration of 500 ppm inhibited completely spore germination for 13 days. Eugenol appeared less effective than cinnamaldehyde, as it inhibited significantly spore germination only at the highest concentration (500 ppm), while limonene was not effective in inhibiting spore germination. Similar results on the ineffectiveness of limonene against *Alicyclobacillus* were reported by Huertas et al. (2014). Maldonado et al. (2013) studied lemon essential oil as a natural compound against a strain of *A.acidoterrestris* in malt extract broth (MEB medium) and in lemon juice concentrate (LJC). The results showed that lemon essential oil (0.08–0.12–0.16%) completely inhibited the germination of *A. acidoterrestris* spores in both the MEB medium and LJC for 11 days. Although the use of EOs against *A. acidoterrestris* appears to be promising, further investigations are required, in order to assess the effect of high doses of some EOs on intestinal cells. Additionally the cost and odors created by high concentrations of EOs, should also be considered (Tajkarimi et al., 2010).

Rosemary extracts. Piskernik et al. (2016) investigated the antimicrobial activities of two commercial rosemary (*Rosmarinus officinalis*) extract formulations, V20 and V40, against *Alicyclobacillus* strains inoculated into apple juice. The addition of the rosemary extracts at their minimum inhibitory concentrations (MICs) did not change the color, odor, taste or opacity of the apple juice. Growth kinetics studies with these rosemary extracts indicated a reduction in the vegetative cells for *A. acidoterrestris*, *A. hesperidum*, and *A. cycloheptanicus* in both BAT (*Bacillus acidoterrestris*) broth and apple juice. Moreover, *A. acidoterrestris* spores showed that the MICs of these rosemary extracts had relatively low effects on spore numbers in BAT broth, but had a spore number inhibition index >15% in apple juice. A fourfold increase in the rosemary extract concentrations showed the opposite effects with a greater reduction in spores in *A. acidoterrestris* broth (inhibition index >60%) than in apple juice (inhibition index <10%). According to Piskernik et al. (2016) rosemary extracts applied at their MICs might be a promising alternative for the control of *Alicyclobacilli* in apple juice.

Papain and bromelain. Papain and bromelain are proteolytic enzyme derived from papaya (*Carica papaya*) and pineapple (*Ananas comosus*), respectively. Some studies have demonstrated the antibacterial effects of papain and bromelain against several species of bacteria, as well as their absence of toxicity and mutagenicity (da Silva et al., 2010; Pavan et al., 2012) . dos Anjos et al. (2016) studied the antibacterial effect of papain and bromelain on *A. acidoterrestris* and found that for this microorganism the MIC of papain was 0.98 μg/ml and the MBC (minimum bactericidal concentration) was 3.91 μg/ml, while the MIC of bromelain was 62.5 μg/ml and the MBC was 250 μg/ml. The four times concentration of MIC for both the enzymes was sufficient to eliminate 4 logs of the microorganism after 24 hours of incubation. The synergistic activity of the enzymes revealed a fractional inhibitory concentration (FIC) level of 0.16. The combination of these enzymes with nisin revealed an FIC of 0.25 for papain and 0.19 for bromelain, indicating synergism between both compounds. The application of enzymes in reconstituted orange juice contaminated with *A. acidoterrestris* was found to be effective, after 48 hours of incubation, at three different temperatures, where the initial microbial population was eliminated. The conclusion of dos Anjos et al. (2016) was that both papain and bromelain have an antibacterial effect on *A. acidoterrestris.* Furthermore, dos Anjos et al. (2018) studied the microencapsulation of papain and bromelain with alginate and chitosan. Microencapsulation was performed by spray drying and the compounds were then exposed to high temperatures and their inhibitory and bactericidal activity against five different species of *Alicyclobacillus* was then evaluated. The results showed that papain microencapsulated with chitosan or alginate maintained low minimum inhibitory concentration values after exposure to heat treatment, demonstrating its effectiveness and potential application as a biopreservative.

Control of biofilm formation

Microbial biofilm formation is a multistage process in which cells adhere to a surface (initial reversible adhesion) and then produce an extracellular matrix (containing polysaccharides, proteins and DNA) that leads to an irreversible adhesion (Donlan, 2002; do Prado et al., 2018). dos Anjos et al. (2013) showed that *A. acidoterrestris* vegetative cells have the capacity to form biofilms on stainless steel (6 log CFU/cm^2) and nylon (6.43 CFU/cm^2) surfaces after 5 days contact at 45 °C in BAT broth. Soil is considered to be the main source of contamination of fresh fruit during harvesting, as *Alicyclobacilli* are soil-borne microbes (Steyn et al., 2011) and thus, fruit will almost inevitably carry the bacteria or spores to processing plants. Microbial colonization of food processing equipment surface may occur with the formation of biofilms of vegetative bacteria (Podolak et al., 2009; Shemesh et al., 2014; Tyfa et al., 2015). Biofilm formation on surfaces is undesirable because they increase the risk of cross-contamination and spores that contaminate the final product may originate just from biofilms that have been formed on the surfaces during industrial processing. Only disinfection processes can control microbial surface contamination. dos Anjos et al. (2013) evaluated the adhesion and biofilm formation of *A.acidoterrestris* on industrial orange juice processing equipment and the bactericidal efficacy of peracetic acid, sodium hypochlorite and quaternary ammonia on stainless steel, nylon and polyvinyl chloride surfaces. They found that peracetic acid was significantly ($p < 0.05$) the most effective in removing biofilms from all surfaces and also reduced bacterial counts by 3 log CFU/cm^2 on the surface of polyvinyl chloride. The other sanitizers also reduced the bacterial counts by 2 log CFU/cm^2. Quaternary ammonia exhibited the optimal minimum sporicidal concentration, preventing spore germination after only 15 s of contact at a concentration of 82 ppm. dos Anjos et al. (2013) suggested that the daily use of quaternary ammonia as the basis of sanitizing is the most strongly indicated treatment. To eliminate and prevent the formation of biofilms, the ideal is to establish a high frequency of application of peracetic acid to all surfaces. Working in this way with these two sanitizers, the industry can achieve suitable sanitation.

Conclusion

Due to their heat and acid tolerance, *Alicylobacillus* spp. endospores are capable of tolerating fruit juice pasteurization and can subsequently germinate in the acidic fruit juices. *A. acidoterrestris*, in particular, is the species primarily responsible for the reported spoilage incidents, being able of producing compounds such as 2-methoxyphenol (guaiacol), 2,6-dibromophenol and 2,6-dichlorophenol, which are responsible for unpleasant changes in the odor and taste of fruit juices. These changes have been described as a "medicinal" or "antiseptic" odor and taste. Juice processors only recognize spoilage by this bacterium when they were informed after consumer complaints. This is because there were no apparent signs (swelling of the container due to gas production or increased turbidity and sediment), which could led to rejection of the spoiled product before shipping. A variety of techniques and methods have been designed to prevent or control *Alicyclobacillus* contamination during juice manufacture. Chemical antimicrobials such as oxidizing agents, organic acids and salts are used to inactivate *A. acidoterrestris* spores on the surface of fruits or on the equipment used in processing or to inhibit the growth of the bacterium in the fruit juice. The efficacy of alternative natural compounds, like bacteriocins, essential oils and extract, has been intensively studied. According to current knowledge, the addition of any of these compounds might at least reduce the viability of the *Alicyclobacilli* and sustain the quality and product shelf stability. However, because consumers prefer fruit juice products that are "natural" or free from chemicals additives, there is a need for new methods that can destroy spores in the absence of chemical additives. The use of nonconventional approaches to reduce the contamination by *Alicyclobacilli* could be considered as a promising way for the juice industry. Nevertheless, as these approaches are currently successful at the laboratory scale, these techniques require to be scaled up to a production level before they will be commercially accepted. Still the inactivation of this spore-forming microorganism remains an open topic for further research. At present, adequate alternatives for the fruit industry, in order to avoid problems derived from *Alicyclobacillus,* are: (i) follow the "*Alicyclobacillus* Best Practice Guideline" issued by the European Fruit Juice Association (AIJN, 2008) for its reduction and control in the production, packaging and distribution of fruit juices, juice concentrates, purees and nectars (ii) ensure a good vacuum and hermetic seal in the container using rapid cooling to room temperature or below and therefore an anaerobic environment in the finished product and (iii) the application of correct cleaning and sanitation methods in production plants, using quaternary ammonium sanitizers daily and establishing a high frequency of application of peracetic acid to all surfaces (dos Anjos et al., 2013; Gordon, 2017).

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