Revew article

Activity of compounds of natural origin against *Alicyclobacillus acidoterrestris*, a common fruit juices contaminant

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Abstract: *Fruit product industry struggles with emerging problem of microbial contamination with Alicyclobacillus acidoterrestris. This acidothermophilic, soil-borne and sporeforming bacterium posses ability to survive commercial pasteurization and thus may cause fruit juices spoilage. Even modern technologies are not effective enough to eliminate A. acidoterrestris from the industrial environments. The green consumer attitude and safety standards suggest adaptation of natural and safe solutions. This paper summarizes the bioactivity of compounds of natural origin which could serve as anti-alicyclobacilli preservation agents maintaining stability of fruit juices.*

Keywords: *Alicyclobacillus acidoterrestris, essential oils, bacteriocins, fruit products spoilage, antimicrobial activity.*

Introduction

The history of *Alicyclobacillus* sp. has started in the seventies of the XX century. In 1967 an aerobic and acidophilic endospore forming microorganism had been isolated from Tokohu hot springs (Japan) and basing on morphological features both of the cells and colonies, characterized as a member of *Bacilli* class [1, 2]. Afterwards, since 1971, similar microorganisms have been isolated from differentiated habitats simultaneously overthrowing the assumption that these specific bacteria are able to survive in acidothermophilic environments only [3]. The analysis of isolates cell wall structure showed the presence of specific chemical compounds – ω-cyclohexane fatty acids and hopanoids which are not typical components of *Bacillus* sp. cells. Moreover, the G+C content of isolates varied substantially from other bacilli, therefore, the new species, *Bacillus acidocaldarius*, has been established [3]. Further researches conducted between 1980-1984, resulted in isolation of similar bacteria from non thermal and non acidic source (soil), as well as, from aseptically packed apple juice [4, 5].

Deinhard *et al.* (1987a) investigated their mutual relationship proceeding physiological and biochemical tests for acidophilic and thermophilic *Bacillus* isolates [6]. The presence of ω-alicyclic fatty acids in cell membranes appeared to be non specific and not limited only to *B. acidocaldarius*, therefore, a new bacterial strain, *Bacillus acidoterrestris*, was introduced [6]. The species expressed different physical and metabolic properties than *B. acidocaldarius* like ability to grow in lower temperature, lower G+C content or utilization of eritritol, sorbitol and xylitol. In addition, Deinhard *et al.* (1987b) conducted subsequent study on a new soilborne isolate containing ω-cycloheptane fatty acids in cell membranes creating next species – *Bacillus cycloheptanicus* [7]. Finally, Wisotzkey *et al.* (1992) performed the comparative analysis of 16S rRNA for three species and revealed their high identity (around 95.0%) and affinity to the same genus with indicating low identity to other *Bacillus* sp. [8]. Therefore, they proposed the establishment of a new genus in *Bacilli* class, *Alicyclobacillus*, which gathered all known acidothermophilic bacilli with high G+C content and ω-alicyclic fatty acids as the major membrane component of the cells. Moreover, the nomenclature of the three species has been changed as *Alicyclobacillus acidoterrestris, A. acidocaldarius* and *A. cycloheptanicus*.

Table 1. List of species and subspecies of *Alicyclobacillus* genus

Species	References
A. acidiphilus	**[17]**
A. acidocaldarius	**[3, 8, 18, 19]**
A. acidocaldarius subsp. *acidocaldarius*	**[3, 8]**
A. acidocaldarius subsp. *rittmanni*	**[20]**
A. acidoterrestris	**[6, 8]**
A. aeris	**[21]**
A. cellulosilyticus	**[9]**
A. contaminans	**[22]**
A. cycloheptanicus	**[1, 8, 7]**
A. dauci	**[10]**
A. disulfidooxidans	**[23, 24]**
A. fastidiosus	**[22]**
A. ferrooxydans	**[25]**
A. herbarius	**[26]**
A. hesperidum	**[27]**
A. kakegawensis	**[22]**
A. macrosporangiidus	**[22]**
A. pomorum	**[28]**
A. sacchari	**[22]**
A. sendaiensis	**[29]**
A. shizuokensis	**[22]**
A. tengchongensis	**[30]**
A. tolerans	**[24]**
A. vulcanalis	**[31]**

Until now the research on taxonomic diversity of *Alicyclobacillus* sp. has been intensively studied. Current status of *Alicyclobacillus* genus after reclassification of several species counts for 22 species and 2 subspecies (Table 1.) with the newest ones isolated from cedarwood chips and spoiled mixed juice product [9, 10]. The reclassification included an geothermal isolate form Antarctica soil (originally *Alicyclobacilus pohliae*) and clinical isolate from women blood sample (originally *Alicyclobacillus consociatus*) into *Effusibacillus pohliae* and *Effusibacillus consociatus* respectively [11-14]. However, *A. acidoterrestris* recognized as a common threat for fruit and vegetable products industry was deeply characterized [13, 15, 16].

Alicyclobacillus acidoterrestris – species characterization

A. acidoterrestris is considered as a representative of *Alicyclobacillus* genus belonging to *Alicyclobacilaceae* family in *Bacillus* order. First reports on this bacterium appear in 1987, when Deinhard *et al.* isolated an unknown acidothermophilic microorganism from soil [6]. Later, a similar species was isolated from a spoiled pasteurized apple juice [5]. Current studies on morphological and physiological analyses present that *A. acidoterrestris* is an aerobic, motile, rod-shaped bacterium able to form oval endospores located centrally or terminally and may cause cell deformation [2]. The size of the endospores varies between 1.5-1.8 μm in length and 0.9-1.0 μm in width whereas cell size reaches 2.9-4.3 μm × 0.9-1.0 μm [2, 4, 6, 32]. These bacteria remain Gram-positive, however, may stain as Gram-variable as well [7, 8].

A. acidoterrestris is an acidothermophilic bacterium growing in low pH and elevated temperature. The growth is observed in pH between 2.0 to 6.0 with the optimum rate at pH 4.0-5.0, however, soilborne isolates are able to survive at pH 7.0 [33, 34]. On the other hand, the optimum temperature for *A. acidoterrestris* is 40-53°C but the cells remain active in temperature range 20-70°C [4, 6, 33-35]. Major components of cellular membranes of cells and spores are ω-alicyclic fatty acids, mainly ω-cyclohexane $C_{17:0}$, $C_{19:0}$, ω-cycloheptane, iso $C_{15:0}$, $C_{17:0}$ and anteiso $C_{15:0}$, $C_{17:0}$ acids [16, 29]. The genetic sequence data for 16S rRNA analysis for the microorganisms show that the overall guanine and cytosine content reaches 52.2 mol% of the genome [8, 36].

A. acidoterrestris is considered as a major threat for fruit processing industry. The bacterium is natural inhabitant of soil where it may survive inconvenient conditions. However, *A. acidoterrestis* might contaminate surface of vegetables and fruits especially when windfalls are collected for fruit products processing [15]. Several reports from all over the world have confirmed the presence of alicyclobacilli in acidic beverages (iced tea, juice drinks, etc.) and fruit juices made from pears, grapefruits, mango, white grapes, lemons, tomatoes and others with apple juice most often contaminated [16, 34, 37].

Juices contaminated with alicyclobacilli very rarely manifest with taint and/or precipitate, thus the spoilage cannot be detected easily [16]. The most characteristic feature of a spoiled product are specific off-flavors identified as disinfectant-like or medicinal smell [35]. This is associated with chemical metabolites produced by *Alicyclobacillus* sp., predominately guaiacol and halophenols: 2,6-DCP (2,6-dichlorophenol) and 2,6-DBC (dibromophenol) [38, 39]. The precursors of guaiacol are ferulic acid and its metabolic pathway products (vanillic acid and vanillin) as well as tyrosine [40]. Still the concentration of volatile metabolites is strictly related to a kind of juice and a number of bacterial cells [39].

The attempts to prevent *Alicyclobacillus* sp. contamination have been undertaken worldwide, however, specific acidothermophilic spores are extremely hard to eliminate. Conventional and unconventional methods involve application of techniques like high temperature (pasteurization), elevated pressure (HPH – high pressure homogenization), addition of preservation agents, ultrasounds, infrared radiation, clarification, high hydrostatic pressure (HHP) or ultrafiltration [39, 41-45]. Moreover, a promising technique authorized by U.S. Food and Drug Administration (USFDA) seems to be cleansing surface of fruits with aqueous chlorine dioxide (ClO_2) and its addition to condensation water used for sanitizing technological installations [46]. On the contrary, chemicals which are approved by FDA as food additives are several enzymes, plant and bacterial metabolites which are regarded as natural compounds of confirmed antimicrobial potential. This include bacteriocins, lysozyme and plant derivatives – extracts and essential oils.

Bacteriocins active against *Alicyclobacillus* sp.

Bacteriocins are considered as proteinaceous metabolites produced by Gram-positive and Gram-negative bacteria, expressing activity against microorganisms [47, 48]. Due to their high bactericidal and bacteriostatic potential, food products with bacteriocins are less susceptible to spoilage. Moreover, majority of these chemical compounds express thermostability and remain active in relatively wide pH range, therefore, are recognized as natural preservatives and could be regarded as food products additives. However, current status of bacteriocins Generally Recognized as Safe (GRAS) accounts for nisin only [47-50].

Nisin

One of bacteriocins with well-documented inhibitory effect against Gram-positive bacteria and pathogens is nisin, a peptide produced by *Lactococcus lactis* subsp. *lactis*. Its antibacterial activity towards *A. acidoterrestris* has also been extensively studied [43, 51, 52]. Nisin is regarded as a potential preservative and suitable natural additive to fruit drinks and beverages of high acidity [53, 54]. Due to stability in both low pH and elevated temperature, nisin can be added directly into juices prior to pasteurization in order to control both *A. acidoterrestris* spores germination and growth of vegetative cells [53]. Moreover, Buonocore *et al.* (2004) reported that release and maintenance of nisin biological properties could be achieved during product storage [55]. The attempts of nisin incorporation as a component of active packaging have been considered as well [56]. The amount of nisin and its effectiveness varies substantially among the studies [43, 53, 54]. These differences are caused by variety of media applied, chemical composition of juices and particular *Alicyclobacillus* sp. strain.

Nisin in concentration 100 IU/ml was proved to be effective enough to prevent growth of *A. acidoterrestris* spores and vegetative cells in apple, orange and grapefruit juices even at 45°C. Furthermore, at ambient temperature lower concentration of nisin (50 IU/ml) enhanced thermal sensitivity of *A. acidoterrestris* spores, thus prevented their outgrowth in a higher extent [51]. The 100 IU/ml dosis of nisin was also confirmed as a suitable apple and orange juice preservative in other studies [57-59].

Analogous dependence, therefore, weaker activity towards vegetative cells was observed by Yamazaki *et al.* (2000) [53]. In addition, greater acidity facilitated improvement of nisin bacteriostatic activity. Both for vegetative cells and spores cultivated on YPGA medium, the Minimum Inhibitory Concentration (MIC) values were 1.56-25 IU/ml and 25-100 IU/ml at pH 3.4 and 4.2, respectively. On the contrary, the inhibitory effect for suspension of spores only was <0.78-12.5 IU/ml at pH 3.4 and 25-100 IU/ml at pH 4.2. No outgrowth of spores was observed after 7 days of incubation for orange and mixed fruit juices with 25 IU/ml addition of nisin. However, similar effect was not achieved for the clear apple juice even with nisin dose equal to 600 IU/ml [53].

As suggested by Splittstoesser *et al.* (1994), the chemical composition of a juice itself may have an influence on microorganisms cultivation [60]. The higher the content of soluble solids and thus lower water activity (a_w), the lower concentration of nisin is required for *A. acidoterrestris* growth inhibition [58]. Researches considering operation on several parameters (soluble solids concentration, pH, temperature) indicate that independently on the parameter, nisin at concentration 70 IU/ml is an effective inhibiting agent in both apple and orange juices of pH 3.5-5.5 and 11-19°Brix incubated at the temperature range 25-50°C [61].

On the other hand, Sokolowska *et al.* (2012) presented results of nisin effect on *A. acidoterrestris* strains isolated from polish fruit products [54]. Bacterial inhibition was observed both in 25 and 45°C with MIC value for spores germination 100-1500 IU/ml whereas for vegetative cells 50-1250 UI/ml. The results are not in agreement with study presented by Komitopoulou *et al.* (1999) [51] and Yamazaki *et al.* (2000) [53] and simultaneously indicate that polish strains of *A. acidoterrestris* are rather sensitive to higher concentration of nisin [54].

The potential bactericidal and bacteriostatic activity of nisin was corraborated in Ruiz *et al.* (2013) study considering Brazilian *A. acidoterrestris* [62]. The MIC and Minimum Bactericidal Concentration (MBC) values were assigned for 15.6 μg/ml and 31.25 μg/ml bacteriocin concentration respectively.

Moreover, the mixture of nisin and chloroform extract of *Piper aduncum* tested resulted in a synergistic effect [62].

Nisin is regarded as the bacteriocin with biological activity against *A. acidoterrestris* most intensively tested since 1999. However, apart from nisin other bacteriocins have been suggested for commercial application as biopreservative substances preventing spoilage caused by this acido-termophilic bacteria.

Enterocin AS-48

Enterocin AS-48 is a cyclic peptide extracted from *Enterococcus faecalis* A-48-32. The research by Grande *et al.* (2005) [63] confirmed antibacterial activity of enterocin against *Alicyclobacillus* sp. inoculated in fresh–made and commercial acidic juices. The concentration of bacteriocin of 2.5 µg/ml reduced the risk of *A. acidoterrestris* overgrowth in natural apple and orange juices. Spores germination in commercial juices (apple, grapefruit, orange, peach and pineapple) incubated in temperature 37°C for 90 days was inhibited and the viable cell count was reduced below the detection limit. Similarly, for enterocin-treated juices inoculated with vegetative cells at least bacteriostatic effect for 60 days of incubation was maintained. Furthermore, as suggested by Grande *et al.* (2005), addition of enterocin AS-48 to commercial acidic juices could be combined with heat treatment of lower intensity [63].

Warnericin RB4

In another study Minamikawa *et al.* (2005) has presented antagonistic effect of newly characterized bacteriocin extracted from *Staphylococcus warneri* RB4 – warnericin RB4 [64]. Bacteriocin was tested for thermal and acidic stability as well as against broad microorganisms spectrum. Warnericin expressed antibacterial activity towards *Micrococcus luteus*, *A. acidoterrestris* and *A. acidocaldarius* strains. Despite strong anti-alicyclobacilli potential there are limited data on additional studies.

Bificin C6165

Bifidobacterium animalis subsp. *animalis* CICC 6165 produces a specific IIa class bateriocin – bificin C6165. Its antimicrobial activity against twenty strains of *Alicyclobacillus* sp. was tested [65]. The bacteriostatic effect was observed for bificin concentration at 32-64 AU/ml whereas at the concentration 512 AU/ml no viable cells were detected. The activity of bificin against alicyclobacilli was maintained only in acidic pH. Bificin thermostability up to 80°C seems to be promising in its industrial application as a biopreservative of fruit juices [65].
In the subsequent research by Pei *et al.* (2014) [66], the activity of bificin C6165 was tested against *Alicyclobacillus* sp. in diluted apple juice. Vegetative cells of *A. acidoterrestris* required at least 40 µg/ml of bificin to be reduced below the detection limit. On the contrary, for commercial fruit juices inoculated with endospores suspension, their inactivation was observed at the concentration of

80 µg/ml. The same amount of bificin added to commercial apple juice was not effective. Similar tendency, therefore lack of activity for commercial apple juice, was observed in Yamazaki *et al.* (2000) study but with nisin addition [53].

Paracin C

Lactobacillus paracasei CICC 20241 is a particular strain in group of LAB (lactic acid bacteria) classified as a probiotic producer of bacteriocin, paracin C. In the research conducted by Pei *et al.* (2013), the biological properties as well as antimicrobial activity against *A. acidoterrestris* have been investigated [67]. Paracin C appeared to express effectiveness comparable to this of nisin, therefore, both bacteriocins are regarded as thermostable agents maintaining stability in acidic and neutral pH [51, 53, 67]. Bacteriostatic effect was observed for vegetative cell culture at the concentration 80 AU/ml whereas concentration of 160 AU/ml seemed to be the MBC value. Moreover, the amount of paracin equal to 400 AU/ml resulted in reduction of *A. acidoterrestris* endospores number by 6 log cfu/ml [67].

Biovicin HC5

Biovicin HC5 is produced by *Streptococcus bovis* HC5 which expresses antimicrobial activity against many spoilage and pathogenic bacteria contaminating food products [68]. Its stability in high temperature and acidic environment has been proven, thus biovicin seems to have potential against thermoacidophilic bacteria as well [68, 69]. De Carvalho *et al.* (2008) observed that biovicin in concentration 80 AU/ml expressed bactericidal and sporicidal effect in mango pulp [68]. Moreover, biovicin increased the heat susceptibility of spores and thus the lethality of *A. acidoterrestris* cells [68].

Controlling enzymes – lysozymes

Lysozymes are considered as a particular group of hydrolytic enzymes which posses an ability to 'lyse' (digest) bacterial cell wall [70]. Therefore, they might play particular role in prevention of microbial growth in food products and simultaneously enhance prolongation of food shelf stability [71, 72]. The bioactivity of lysozyme against *A. acidoterrestris* has been studied in laboratory conditions as well [55, 73-75].

Although the reports confirm its inhibition against alicyclobacilli growth, the exact mechanism seems not to be precisely specified. Bevilacqua *et al.* (2014) indicates that lysozyme could initially promote spore germination and later act on the outgrown spores causing the reduction of their amount [73]. Still the researches proceeded for both vegetative cells and spores sustain that endospores remain more sensitive to the enzyme [75]. Moreover, similarly to nisin, the exact activity of lysozyme is dose-dependent and varies within external conditions applied [73-75].

Plant derivatives acting against alicyclobacilli

Plants are the most abundant source of natural antimicrobials, essential oils, plant extracts and hydrolates [76-78]. Essential oils are regarded as oily mixture of various chemical compounds which gained GRAS status (Generally Recognized As Safe) and approval of Food and Drug Administration (FDA) as food additives [78, 79]. They compose of several active components, however, the most potent are terpenes, alcohols, phenols, aldehydes and ketones [79]. Apart from essential oils, their by-products are collected during the production as well. These includes hydrolates and plant extracts, however, the oils themselves are applied mostly.

Essential oils are reported to exhibit biological activity against microorganisms. This might be expressed either by microbial growth inhibition or as elimination and cidal effect [77, 79]. Fruit juices are commonly spoiled by *Alicyclobacillus* bacteria, however, several researches have considered application of essential oils or their active constituents as natural antimicrobial agents [52, 80-82]. According to Maldonado *et al.* (2013), lemon essential oil seems to be potential inhibitor of alicyclobacilli even up to 11 days of incubation in culture media [81]. However, the efficiency of the oil could depend on its exact chemical composition and production method [81]. On the contrary, Huertas *et al.* (2014) [52] and Bevilacqua *et al.* (2010) [82] conducted the research involving active components of essential oils only. According to study by Huertas *et al.* (2014) on citrus essential oils components, only 0.69 mM of citral could decrease the amount of *A. acidoterrestris* spores by 1 log cfu/ml, whereas limonene even at concentration 3.7 mM appeared to be ineffective. However, combining two natural antimicrobials citral and nisin (1.5 mg/L and 0.69 mM respectively) with heating in 95°C for 2 minutes led to complete elimination of the bacteria [52]. Research by Bevilacqua *et al.* (2010) considered the most active components of cinnamon and clove essential oils – eugenol and cinnamaldehylde, added into the apple juice [82]. The preservative effect was achieved for this mixture at concentration 40 ppm of eugenol and 20 ppm of cinnamaldehyde, however, the better stability of juice was observed for their doubled concentrations [82].

Apart from essential oils, plant extracts were tested for their antimicrobial potential as well [83,84]. Bevilacqua *et al.* (2013) compared the bioactivity of citrus extracts on *A. acidoterrestris* spores number during thermal processing [83]. The reduction of spores by 5 log cfu/ml was observed for all three extracts tested (lemon, neroli, bicitro) at concentration 500 ppm. Bioactivity of these extracts seemed to be strain dependent, however, higher concentrations applied should also suppress the growth of any additional microbiota. The MIC values achieved for bicitro have fallen in the range 250-500 ppm, whereas for lemon extracts lower concentration was required to maintain bacteriostatic effect (160-250 ppm). Neroli seemed, however, inadequate as an apple juice additive due to substantial alternation of juice organoleptic features [83].

Molva and Baysal (2015) investigated the effect of grape seed extract on *A. acidoterrestris* vegetative cells and spore viability [84]. The addition of 0.23-3.6% extract to apple juice stored in 37°C for 14 days resulted in the decrease of microbial population by 3-4 log cfu/ml, which seems to be promising [84].

An interesting research was proceeded by Alberice *et al.* (2012) and involved usage of chemically active glycosides saponins [85]. The inactivation of alicyclobacilli was tested for both commercial saponin and purified methanolic extracts from *S. saponaria* and obtained results seemed comparable. Treatment with 100 mg/L commercial saponin reduced the amount of spores by 2.34 log, whereas 500 mg/L purified plant extract maintained juice stability longer than for 5 days. According to these studies, saponins may be also used in preservation of fruit juices.

Summary

Presence of *A. acidoterrestris* is an emerging problem for the fruit products industry. This acidothermophilic and sporeforming microorganisms is able to spoil pasteurized juices causing them inadequate for the consumption. Therefore, prevention and elimination methods are of a very high concern. Current technologies involving thermal treatment, adaptation of high pressure, sanitizing of industrial installations are not effective enough. The best solution seems to be direct action oriented towards *A. acidoterrestris* cells and spores by chemical agents. However, incorporation of chemical substances into food products should be strictly controlled and stay in agreement with FDA standards. Current trends for green consumption requires rejection of synthetic preservative substances and thus natural plant substances are appreciated. Nevertheless, fruit juices industry should consider only these substances which will not only maintain microbiological shelf stability of products but also will not affect product organoleptic features. Essential oils and extracts as well as bacteriocins are compounds of natural origin with proved antimicrobial activity. Moreover, majority of them had gained GRAS status and may be added directly into the food. Bacteriocins and plant metabolites effectiveness against *A. acidoterrestris* has been intensively studied since decades. According to current knowledge, the addition of any of these compounds might at least reduce the viability of the bacteria and sustain the quality and product shelf stability. Still the activity of natural plant and microbial substances remain an open topic for further researches.

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