Biotechnology and Food Science

Review article

The possibility of thermal inactivation of *Alicyclobacillus acidoterrestris* spores in fruit and vegetable juices

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Abstract: *For a long period of time the thermal processing has been considered as the only way to reduce the initial spore number of Alicyclobacillus acidoterrestris and prevent the spoilage of beverage especially the acidic one. The effect of temperature on the inactivation of bacterial spores is well documented. However is still not yet fully explored the interactions between the components of the tested environment. Alicyclobacillus acidoterrestris spores being resistant to the pasteurization treatment conditions normally applied to acidic fruit products can germinate and grow causing spoilage. Visual detection of spoilage is very difficult because Alicyclobacillus acidoterrestris does not produce gas during growth and incipient swelling of containers does not occur. Knowledge of the survival curves of Alicyclobacillus acidoterrestris spores suspended in natural and model environments is necessary to design pasteurization processes for high acidic fruit products. Therefore, this microorganism was suggested as the target to be used in the design of adequate pasteurization processes.*

Keywords: *Alicyclobacillus acidoterrestris, thermal inactivation.*

Introduction

In recent years, the number of spoilage incidents caused by alicyclobacilli has increased as different types of food have been contaminated. At present, alicyclobacilli are more widespread than originally observed and are becoming an industry-wide problem. Since 1982, when aseptically packaged apple juice was spoiled in Germany, interest in the *Alicyclobacillus spp.* bacteria has greatly increased.

Alicyclobacillus acidoterrestris is a thermoacidophilic, nonpathogenic, spore-forming and aerobic microorganism which has been isolated and identified in several spoiled commercial pasteurized fruit juices, such as orange and apple juices. The characteristic that distinguishes it from other bacterial spores is the presence ω-alicyclic fatty acids (ω-cyclohexane or ω-cycloheptane fatty acids), which are the major components of its cellular membrane [1-3]. Some scientists believe that the presence of these specific compounds may be responsible for the exceptional resistance of *Alicyclobacillus* bacteria to low pH and high temperatures. The results of their research show that *Alicyclobacillus* tolerates the standard conditions of the pasteurization process in the fruit and vegetable industry very well. Therefore, the usefulness of typical pasteurization to ensure the quality of commercial products seems to be questionable.

Table 1. Characteristics of *Alicyclobacillus acidoterrestris* [6,7]

Parameter		Value
Growth temperature range (ºC)		30-60
Growth pH range		2.0-6.0
Water activity minimum for growth		0.97
$D_{95ºC}$ (min)	Range	1.0-5.3
	Mean	2.8
z-Value (ºC)	Range	7.2-12.9
	Mean	9.7

The growth of heat resistant acidophilic bacteria of the genus *Alicyclobacillus* spp. leads to substantial losses for the producers of fruit juices and beverages [4,5]. The basic characteristics of *Alicyclobacillus acidoterrestris* spores were given by Bevilacqua *et al*. [6], and Silva and Gibbs [7].

It should be noted that the spoilage of beverage or food is not only caused by the presence of microorganisms capable of creating spores. Both thermal resistance as well as the ability to sporulate is determined by a highly acidic environment [8]. The spores of *Clostridium botulinum* are not able to germinate and grow, and more importantly, cannot produce dangerous toxins in foods with a pH < 4.6. *Geobacillus stearothermophilus* bacteria are highly heat resistant, spore-forming, and also acidophilic, but do not multiply in environments with a pH of less than 4 [9]. Therefore, the manufacturers of fruit juices are guided chiefly by the pH of the end product in setting the parameters of the technological process (mainly pasteurization), as the end product has a fairly low pH of < 4.0. This is due to the fact that low pH limits any increase in the concentration of most microorganisms in the medium.

Consequently, both the choice of temperature and the time of exposure also need to take into account the presence of acidophilic microorganisms, that is, those capable of growing in low pH environments. These include some yeasts, molds, non-spore-forming bacteria, *Geobacillus stearothermophilus* and *Alicyclobacillus acidoterrestris* spores.

Discussion of literature data on thermal inactivation of *Alicyclobacillus acidoterrestris* spores

The largest problem in the case of fruit juices is posed by difficult-to-notice negative changes in their quality resulting from the presence of *Alicyclobacillus spp.* bacteria, including *A. acidoterrestris*. Despite pasteurizing juices, heat resistant spores can still grow in concentrates obtained from them or in the end products. A low initial concentration of spores does not cause any visible change in the appearance the fruit product. However, after some time, by the decomposition of juice ingredients (mostly carbohydrates, proteins and vitamins), these bacteria produce an unpleasant smell evocative of disinfectants (2.6-dibromofenol and 2-methoxyphenol that is guaiacol), induce changes in pH, color and texture, and can also contribute to the emergence of a white precipitate, and that practically without gassing. Eventually, the concentrate or juice becomes unsuitable for consumption and must be disposed of [10,11].

A. acidoterrestris spoilage has been observed mainly in apple juice, but also in pear, orange and tomato juices [8,12,13]. Cases of microbial contamination of processed fruit have been recorded around the world (*e.g.* in Germany, the United States, Japan, Australia and the UK), especially during warm springs and summers [12,14,15].

Splittstoesser *et al.* [8] studied the impact of many commonly available fruit juices on the growth of bacteria of the genus *Alicyclobacillus*. The results showed that microflora grew better in tomato juice as compared to apple juice. However, nowadays there are many more cases of spoilage of apple juice, which is the most popular fruit juice.

The germination and growth of *A. acidoterrestris* under an acidic environment was also observed by Pettipher *et al.*[16] in orange juice stored at 44°C for 24 h, and by Komitopoulou *et al.*[10] in apple, orange and grapefruit juices stored at 30ºC. Similar results were obtained by Walls and Chuyate [17] after 1-2 weeks of storage at 35ºC for apple, orange, white grape, tomato, and pear juices. No growth was observed in red grape juice due to the high content of polyphenols.

Impact of dry matter content

Table 1 indicates that for *A. acidoterrestris* bacteria to multiply in real or model environments water activity must be high, at least 0.97 [6,7].

Therefore, it can be inferred that a fruit concentrate with a high content of solids (SS), and thus lower water activity, should be fully safe and free from contamination by *A. acidoterrestris* bacteria, but there is no information about it in the literature. Only Splittstoesser *et al.* [8] reported that concentrating juices for storage had an inhibitory effect on the growth of *A. acidoterrestris*, for example in apple juice concentrated from 12.5 °Bx (a_w = 0.992) to 38.7 ºBx (a_w = 0.96).

Ceviz *et al.* [18] carried out inactivation of *A. acidoterrestris* spores suspended in apple and orange juices changing the content of solids from 10 ºBx to 20 ºBx at pH 3.5 and 4.0. At such low solid content levels, neither the conversion of solids nor different juice pH levels led to shortening of the D-values.

Maldonado *et al.* [4] examined samples of lemon juice with a pH of 2.28-3.5, dry matter content from 50 to 68°Bx, heated to 82-95°C. They showed that with increased dry matter content and pH, the decimal reduction time in the lemon juice also increased (50°Bx, D_{82} = 22minutes; 68°Bx, D_{82} = 28minutes).

Conclusions similar to those offered by Murakami *et al.* [19] were presented by Silva *et al.* [20], who examined a number of juices and juice drinks with different carbohydrate contents (5-60°Bx) and various pH levels (2.5-6.0). Juice products with *A. acidoterrestris* bacteria were heated to 85-97°C. A linear relationship between the D-value and sugar content and pH levels was discovered. The lower the sugar content and environmental pH were, the lower the decimal reduction time was. However, no linear relationship was observed during the heating of the medium to different temperatures. The D-value was much higher at lower temperatures. The authors also highlight the fact that pH change does not play a significant role in the inactivation of these organisms, which is mostly dependent on the content of solids and the use of different temperatures. The decimal reduction time in a sample with a high dry matter content was higher than that in samples with a low dry matter content, which confirms the protective properties of sugars. In addition, Parish and Goodrich [21] report that significant concentrations of *Alicyclobacillus* spores are present in citrus fruit concentrates.

The effect of temperature and pH values

It was shown that heat damages spores, which causes them to be more sensitive to various environmental factors.

Spinelli *et al.* [1] conducted inactivation of *A. acidoterrestris* spores in orange juice in two stages. Preliminary activation was carried out at 80°C for 150s and proper inactivation at 92ºC. The decimal reduction time was substantially shortened, from 0.25min to 0.03min. However, the key issue in the study was cooling of the samples immediately after heating. The slow cooling of the product did not bring the expected results. It was proved that slow cooling and storage at a relatively high temperature (30-35°C) favors the multiplication of *A. acidoterrestris* bacteria.

Eiroa *et al.* [22] conducted preliminary activation of *A. acidoterrestris* spores in orange juice at different temperatures and for various periods of time. The best results were obtained with a 20 minute activation at 70°C. The decimal reduction time ranged from 60.8-94.5minutes at 85ºC, to 10-20.6min at 90°C, to 2.5-8.7 min at 95°C.

Splittstoesser *et al.* [8] did not conduct a preliminary stage of activation in apple juice. The D-value ranged from 16 to 23 minutes at 90°C, and 2.4-2.8 min at 95°C, which suggested that activation does not play a significant role in accelerating the process of spore inactivation. Thus, the thermal activation of *A. acidoterrestris* spores seems to be an unnecessary and uneconomic process.

The thermal resistance of bacterial spores is largely influenced by the composition of the environment. One of the important factors is the pH of the heated medium. Thus, applying a combination of temperature, heating time and low pH is likely to enable an adequate pasteurization process and thereby obtain a juice or fruit nectar of the appropriate level of microbiological purity.

The extent to which pH reduces the thermal resistance of bacterial spores is dependent on various factors such as temperature, the tested strain, the composition of the environment and water activity.

As reported in literature data, spores are more resistant in solutions with a pH higher than their optimum growth pH [2]. However, Murakami *et al.* [19] found no significant effect of pH on the decimal reduction time of *A. acidoterrestris* spores. The resistance of *Alicyclobacillus spp.* to high temperatures and low pH enables its presence in a wide spectrum of foods. The mechanism of action of pH on the thermal resistance of spores is not exactly understood. It is suggested that reduced thermal resistance of spores can cause a change in pH inside the cell, which modifies the stability of proteins and enzymes [23].

Murakami *et al.* [19] conducted a study on the effects of pH on the growth of spore-forming acidophilic *A. acidoterrestris* bacteria, but they did not notice a significant reduction in thermal resistance at different pH. The authors do not agree, then, that low pH greatly reduces the value of the decimal reduction time in the case of *A. acidoterrestris*. A comparison of the impact of citric and phosphoric buffers at a low concentration (20 mM) clearly showed that they had no effect on the D-values, which were 12.9 and 13.6 min., respectively, but the buffers at a higher concentration (100mM) led to differences of about 2 minutes in the D-values in favor of the citric buffer, and amounted to 12.3 and 14.4 min., respectively. However, applying the same environment with a different pH significantly affected the D and z-values. This may be due to the differences in the process of sporulation and activation of vegetative cells in comparison to other species.

Furthermore, the results they obtained in a model environment and the use of solutions with different contents of fructose and HCl were not confirmed in the real environments of fruit juices. Perhaps this is due to the influence of other components, such as polyphenols, on the germination and growth of bacterial cells [20].

The heat resistance of *A. acidoterrestris* was not affected by the addition of different divalent cations (Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Sr^{2+}) to the sporulation medium [24]. The survival curves of spores do not show the existence of different physiological states of spores as is the case with *Geobacillus stearothermophilus*. There is no activation shoulder and tailing and the curves are linear, which is also confirmed by literature data. The addition of organic acids (malic, citric, and tartaric acid) to the environment at the decimal reduction time in the literature also found no evidence [13,20].

The addition of ascorbic acid in doses of 50-100mg/l promotes the growth of *A. acidoterrestris*, while higher doses in the range of 150-200 mg/l inhibit its growth. Depending on the type of juice, it was sufficient to reduce oxygen content to 0.1% (apple and white grape juice) or create anaerobic conditions (orange juice) to inhibit the growth of *A. acidoterrestris* [25].

Many studies have shown that lowering the pH of the pasteurized environment decreases the decimal reduction time for *A. acidoterrestris* spores suspended in model solutions (buffers) and in real environments (*e.g.* fruit and vegetable juices), while others emphasize that pH does not affect the process of spore inactivation. There is no information in the literature about the unique effect of pH on the survival of *A. acidoterrestris* spores. In addition, *A. acidoterrestris* spores are able to survive typical pasteurization conditions widely used in the industry. In view of the discrepancies concerning the effect of pH on the process of spore inactivation, it is necessary to carry out tests to determine the optimum parameters of the pasteurization process.

Unconventional methods of *Alicyclobacillus acidoterrestris* spore inactivation

Researchers are still looking for solutions to obtain products of the required microbiological purity while maintaining their labile components.

The literature offers also other ways of preventing the spoilage of fruit juices by *A. acidoterrestris* spores. Some authors recommend using mainly the principles of GMP, while others suggest the addition of various disinfectants such as ClO_2 during the washing of raw materials [11,26]. Walker and Phillips [27] demonstrated that the addition of sodium benzoate and/or potassium to *A. acidoterrestris*-contaminated orange juice in an amount of 0.1 mg/ml and 0.5 mg/ml caused a decrease in the number of cells by 10% and by up to 40%, respectively. Nisin had no effect on the reduction of *A. acidoterrestris* spores at all [5].

However, much research has been devoted not only to the introduction of components designed to reduce the thermal resistance of spores (*e.g.* organic acids, sugars, salts, other bacteriocins, etc.) to the heated medium, but also to the modification of the process of pasteurization (unconventional methods).

In recent years, the use of high hydrostatic pressure to inactivate *A. acidoterrestris* spores has been increasingly recommended by many researchers [3,28,29].

Elevated pressure limits the germination of spores and inactivates those which have germinated. Wuytach *et al.* [30] notes that high pressure limits the germination of spores in a temperature-dependent manner. Lee *et al.* [29] conclude that sugars and salt present in fruit juices have a major impact on the inactivation process. The higher the concentration of these compounds in the juice, the smaller the effect of high pressure. Therefore, researchers believe that the fruit and vegetable processing industry, which mainly manufactures concentrates, should not introduce high pressure pasteurization to the traditional thermal methods.

Bae *et al.* [31] conducted a study on apple juice contaminated by *A. acidoterrestris*, holding it at 65-70°C under the pressure of 8-12atm for 10 to 40min. in the presence of supercritical carbon dioxide. This procedure resulted in a sterile product which showed no loss of labile components, while the pH and dry matter content remained unchanged. However, the disadvantage of this method is a substantial investment cost for the industry.

Nakauma *et al.* [14] studied citrus juices inoculated with *A. acidoterrestris*. In order to inactivate the spores they used radiation along with high temperatures (85-95ºC). The authors demonstrated that this process of thermal modification prevents the multiplication of the strain in acidic juices and beverages, and has a destructive influence on the DNA of bacterial cells.

On the other hand, Giuliani *et al.* [32] proposed to carry out the inactivation of *A acidoterrestris* spores in asparagus cream with olive oil by microwaves. Microwaves have been used in the sterilization process in a wide variety of foods such as pasta, bread, delicatessen products and vegetables [15,33]. In this case, the initial concentration of *A. acidoterrestris* spores decreased by about 20% within 5-7 minutes of being microwaved. The effect of microwaves depends on both the time of their operation and intensity, and therefore it is important to select the right parameters so as not to cause irreversible adverse changes to the product.

The most effective way to eliminate bacteria *Alicyclobacillus spp.* is ultrafiltration. However, due to the high cost of this operation, it is easier for manufacturers to adjust the parameters of thermal pasteurization [25].

Conclusions

Substances such as fats, proteins and sugars present in a pasteurized environment have mostly protective properties. The addition of other substances such as organic acids, sodium benzoate or potassium in an environment subjected to an elevated temperature causes a reduction in the number of heat resistant spores suspended in it, and prevents the growth of surviving cells during storage. The survival of spores held at a temperature of pasteurization depends both on the physical and chemical parameters of the environment in which they are suspended. These parameters include: temperature and time of exposure to that temperature, water activity, pH, and the presence of mineral salts and other substances.

Acidification of food is often used as a method for inhibiting the multiplication of microflora, and especially heat resistant microorganisms, because low pH significantly reduces thermal resistance, and many species of spores are unable to develop in such conditions. In research into acidic beverages (pH = 3), the use of high temperatures in the pasteurization process (90 to 95ºC) did not inhibit the growth of acidophilic strains such as *Alicyclobacillus acidoterrestris* sufficiently to prevent juice spoilage. Therefore, it is very important to determine the parameters of heat treatment, in particular of such products as concentrates, juices and juice drinks, considering not only the initial pH of the environment, but also the possibility of inactivation of *Alicyclobacillus spp*. The limited effect of pasteurization is mainly the result of the applied temperature and heating time, but raising the level of these parameters affects the quality of juices, changing their chemical composition and organoleptic qualities. During thermal treatment, heat resistant spores are subject to activation, which in turn leads to their rapid germination in the product after pasteurization.

The effect of temperature on the inactivation of bacterial spores is well documented. However, there are many discrepancies regarding the effect of pH, because other factors influencing the process of inactivation of *Alicyclobacillus acidoterrestris* spores include dry matter content and the composition of the analyzed environment. There is no literature data regarding the additional impact of such a single component of the environment and its characteristics.

Due to the fact that that excessive heat treatment leads to adverse changes in the processed food, analysis of various possibilities to destroy spores is a very important issue. For the industry, of particular importance is knowledge about the conditions of the pasteurization process, to make the end product completely safe. While *Geobacillus stearothermophilus* or *Clostridium botulinum* spores are often used to determine the right parameters of sterilization, in the case of pasteurization *Alicyclobacillus acidoterrestris* spores have not been conclusively recognized as a biological indicator for this process.

The use of unconventional methods has turned out to be most successful in reducing the degree of contamination with *Alicyclobacillus acidoterrestris* bacteria, but studies have been conducted on a laboratory scale. It would be important to implement new technologies on an industrial scale in order to verify whether the proposed solutions can be successfully applied in real conditions and systems.

Another important issue, still not yet fully explored, is the interactions between the components of the tested environment. The results of the described analyses suggest the need for research into the inactivation of spores of a particular microorganism suspended both in real environments (*e.g.* fruit products) and in model conditions such as buffers at different pH levels or in single component solutions (*e.g.* carbohydrates). The latter are most often used in scientific research due to the constancy of the composition of the environment.

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