Resistance of *Alicyclobacillus acidoterrestris* Spores and Biofilm to Industrial Sanitizers

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**ABSTRACT**

This study evaluated the adhesion and biofilm formation of *Alicyclobacillus acidoterrestris* on industrial orange juice processing equipment and the bactericidal efficacy of peracetic acid, sodium hypochlorite, and quaternary ammonia after biofilm formation. The efficacy of these sanitizers against the spores of this microorganism was also evaluated. Stainless steel and nylon surfaces exhibited higher cell adhesion levels than did polyvinyl chloride surfaces. Peracetic acid was the most effective in removing biofilms from all surfaces (*P* < 0.05) and also reduced bacterial counts by 3 log CFU/cm² on the surface of polyvinyl chloride, but the other sanitizers also reduced the bacterial counts by 2 log CFU/cm². Quaternary ammonia exhibited the optimal minimum sporicidal concentration, preventing spore germination after only 15 s of contact at a concentration of 82 ppm. The flow cytometry results indicated that the spores and cells had low incidences of plasma membrane lysis after treatment with sanitizer, suggesting that lysis is not the principal mode of action for these sanitizers on *A. acidoterrestris*.

The genus *Alicyclobacillus* is composed of gram-positive bacilli containing cyclic fatty acids as a major component of the cell membrane that forms spores, making them resistant to the thermal processes used during food processing. The pH required for growth ranges from 2.2 to 6.0, while the temperature can range from 35 to 55°C. These bacteria produce some components, such as 2,6-dibromo-phenol, 2,6-dichlorophenol, and 2-methoxyphenol, the latter known as guaiacol, which causes an unpleasant smell and taste with the medicinal or antiseptic characteristics of such foods as citrus juices (15, 20, 22, 23).

Through the presence of spores in juice concentrate, these microorganisms are frequently linked to the spoilage of reconstituted orange juice that has been stored at high temperatures after pasteurization (3, 20). This genus is widely distributed in the soil; thus, fruit will inevitably carry the bacteria or spores to the industrial processing plant. The first case of juice spoilage caused by a spore-forming acidophile was reported for apple juice (pH 3.15) bottled aseptically in Germany in 1982 (3).

Orange juice is the only product for which Brazilian production accounts for more than 50% of the worldwide supply, and it accounts for 85% of the country’s exports. Orange juice even surpasses the production of coffee, beef, chicken, and sugar (4). In addition to being a source of pride for the country, citrus production brings in billions of dollars to Brazil’s economy via exports. Thus, orange juice concentrate exports contribute significantly to the country’s trade balance, and product spoilage can cause severe economic damage.

To control bacteria during juice processing, manufacturers wash the fruit, spray them with sanitizers, and extract and pasteurize the juice. However, both spores and biofilms can be resistant to these methods and remain in the final product. Spores in orange juice concentrate encounter conditions suitable for vegetative growth when the product is reconstituted and pasteurized (3).

Several factors, such as concentration and time of contact between the microorganisms and the sanitizer, can affect the sanitizer’s bactericidal ability; however, conclusive data on the activities of the sanitizers used to wash the fruit against spores and biofilm of *Alicyclobacillus* spp. are lacking.

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 (19). The cells in the biofilm exhibit coordinated group behavior that increases their resistance to sanitizers, making them a problem for the food industry (9, 16). However, studies on *Alicyclobacillus* adhesion and biofilm production remain limited.

This study sought to evaluate *Alicyclobacillus acidoterrestris* adhesion and biofilm formation on orange juice processing equipment (stainless steel, polyvinyl chloride [PVC], and nylon) and to measure the efficacy of peracetic acid, calcium hypochlorite, and quaternary ammonia in...
biofilm removal and inactivation of the microorganism’s spores.

MATERIALS AND METHODS

Microbial strain. *A. acidoterrestris* DSMZ 3922T CBMAI 0244T, provided by the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen, or DSMZ), was used in this study. The bacteria were obtained by transferring cells from a BAT medium plate (8) to a test tube containing 5 ml of liquid BAT medium. The suspension was incubated at 45°C for 72 h or until a sporulation level of approximately 80% was observed in the cells using a phase-contrast microscope. The culture was then transferred to a tube and centrifuged at 9,500 × g for 1 min, and the pellet was washed 3 times with sterile distilled water. The pellet was later resuspended in 1 ml of sterile distilled water, aliquoted into 2-ml Nunc cryotubes (Thermo Fisher Scientific, Waltham, MA), and stored at −20°C. The viability of the spore suspension was determined by dilution and plate counting in duplicate and was expressed in CFU per milliliter. The spore suspension aliquots were activated by thermal shock (80°C for 10 min in a water bath), diluted, plated on BAT plates (Inlab, Interlab, São Paulo, Brazil) by streaking, and incubated at 45°C for 24 to 48 h.

Experimental conditions. Square pieces of AISI 304 stainless steel and PVC (1.0 by 1.0 by 0.1 cm) and strands of nylon (1.0 by 0.1 by 0.1 cm) were used as test surfaces. The squares were individually washed, sanitized, and sterilized using the procedure published by Marques et al. (12). Three chemical agents were selected and tested on vegetative *A. acidoterrestris* cells at concentrations previously determined using the MIC technique (5) and a contact time of 10 min. A 500-ppm peracetic acid solution was prepared from 17% peracetic acid (Ecoper, Mairiporá, Brazil), a 1,000-ppm sodium hypochlorite solution was prepared from 15% sodium hypochlorite (Quibras, Maringá, Brazil), and a 15.62-ppm solution of quaternary ammonia–benzalkonium chloride was prepared (Acrros Organics, Fair Lawn, NJ).

Cell adhesion and quantification. The test surfaces were placed in sterile 24-well plates (TPP, Trasadingen, Switzerland) containing 900 µl of BAT broth at pH 4.0 (8) and 100 µl of vegetative *A. acidoterrestris* cell suspension. The plates were incubated for 5 days at 45°C, during which the BAT medium was changed every 24 h. The squares were then treated with the sanitizers for 10 min and then neutralized as follows: 0.2% sodium thiosulfate (Synth, Diadema, Brazil) was used to neutralize peracetic acid and sodium hypochlorite, while Letheen broth (Difco, BD, Sparks, MD) was used for the quaternary ammonia. The squares were subsequently transferred to cryotubes containing 1.0 ml of saline solution and were sonicated for 5 min at 25,000 Hz to disperse the cells. Serial dilutions were then made and plated onto BAT agar via streaking. The plates were incubated at 45°C for 48 h. After incubation, the cells were counted and the results expressed as log CFU per square centimeter.

Scanning microscopy. Prior to analysis by scanning electron microscopy, the squares were treated with sanitizer for 10 min and then neutralized. The squares were washed in saline solution and fixed in 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) in a 0.1 M sodium cacodylate buffer (EMS, Hatfield, PA). They were then dehydrated in ethanol, dried to the critical point in CO2, coated with gold, and examined under a Shimadzu SS-550 (Tokyo, Japan) scanning electron microscope (11).

Determination of the minimum sporicidal concentration for each sanitizer. The sporicidal concentration was determined by microdilution in BAT medium in a 96-well plate (TPP). The sanitizers were serially diluted in the wells, and 5 µl of spore suspension standardized to 4 log was added to each well. Contact times of 15 s and 1, 5, 10, 15, and 30 min of the sanitizer with the spore suspension were also evaluated. After each contact time, 100 µl of the neutralizing solution was added. The plate was then sealed and placed in an 80°C water bath for 10 min. After removal from the water bath, 10 µl from each well was plated on BAT agar in triplicate. The plates were incubated at 45°C for 24 h. The minimum sporicidal concentration is defined as the lowest concentration that results in a negative subculture on the BAT agar.

Flow cytometry. *A. acidoterrestris* spores and vegetative cells at a standardized 4-log concentration were treated with sanitizers at their respective MICs for 10 min and then neutralized. For cases in which the MIC for the spores during the contact time was not found, i.e., peracetic acid and sodium hypochlorite, the maximum concentration evaluated (1,000 ppm) was used. The spore suspensions were then subjected to thermal shock at 80°C for 10 min. The cells were resuspended in phosphate-buffered saline (PBS) buffer, centrifuged, washed, and stained with propidium iodide in PBS (2 µg/ml). The samples were counted in a FACS Calibur (BD Bioscience) flow cytometer, and the bacterial survival rates following sanitizer treatment were determined using fluorescence intensity.

Statistical analysis. All assays were performed in triplicate. The cell counts in the biofilms were compared using Student’s t test. The data were analyzed using the Assistat 7.6 Beta software package. A P value of <0.05 was used to determine significant differences.

RESULTS AND DISCUSSION

The quantification of *A. acidoterrestris* cells adhered to the various surfaces and the counts after the 10-min sanitizer treatments are shown in Table 1. PVC is the material used for mats by food industries. In the processing of oranges, it is used in transporting the oranges to the extractor. The bristles that are used in washing the fruit are nylon, and stainless steel is present throughout the processing, in silos, the extractor, and also, the storage of the juice. Biofilm formation on these surfaces helps the bacteria that are present throughout the industrial environment.

Steel and nylon exhibited greater cell adhesion than PVC under all experimental conditions. Ronner and Wong (18) and Wirtanen et al. (21) demonstrated that a minimum of 5.0 to 6.0 log CFU/cm² is necessary for biofilm formation, and lower counts may indicate adhesion only. Scanning electron microscopy (Fig. 1) confirmed biofilm formation on the steel and nylon surfaces. We also observed cell adhesion and extracellular polysaccharide production on the PVC surface, but the cell counts did not reach 5.0 log CFU/cm² even after 5 days of incubation.

The literature lacks information on biofilms produced by *A. acidoterrestris* on stainless steel, PVC, and nylon, making comparisons of our results with previously published studies difficult.

All of the sanitizer treatments had results that were significantly different from the results for the control, except
for the treatments of PVC and nylon with the MIC of sodium hypochlorite, which did not significantly reduce the number of adhered cells.

Treatment with 2 times the MIC of peracetic acid reduced the cell counts by more than 2 log CFU/cm² on all surfaces. Similar results were obtained by Meira et al. (13), who evaluated the use of peracetic acid and sodium hypochlorite on biofilms produced by *Staphylococcus aureus* on stainless steel and polypropylene.

Moretro et al. (14) tested nine commercial disinfectants on *Salmonella* species biofilms on stainless steel at the concentrations recommended by the manufacturers. These researchers also demonstrated that sodium hypochlorite–based solutions were the least effective, followed by quaternary ammonia–based compounds, while peracetic acid solutions reduced counts by more than 4 log.

Cruz and Fletcher (7) tested seven groups of commercial sanitizers with different active ingredients on *Listeria monocytogenes* biofilms grown on a PVC surface for 48 h. The MIC values for the biofilms were much higher than those for the bacterial suspensions, regardless of the active compound in the solution. Our results were similar; peracetic acid was the only sanitizer with a MIC value lower than that recommended by the manufacturer (500 ppm). The quaternary ammonia–based compound had a MIC value 2 times higher than that recommended

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Steel</th>
<th>PVC</th>
<th>Nylon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.00 ± 0.89 a A</td>
<td>4.85 ± 0.52 b A</td>
<td>6.43 ± 0.08 a A</td>
</tr>
<tr>
<td>MIC of peracetic acid</td>
<td>4.10 ± 0.64 a BC</td>
<td>2.65 ± 0.27 b CD</td>
<td>4.19 ± 0.05 a CD</td>
</tr>
<tr>
<td>2 × MIC of peracetic acid</td>
<td>3.97 ± 0.66 a c</td>
<td>1.88 ± 0.27 b d</td>
<td>3.8 ± 0.04 a d</td>
</tr>
<tr>
<td>MIC of sodium hypochlorite</td>
<td>4.97 ± 0.15 ab b</td>
<td>4.44 ± 0.22 b A</td>
<td>5.72 ± 0.03 a AB</td>
</tr>
<tr>
<td>2 × MIC of sodium hypochlorite</td>
<td>4.26 ± 0.67 ab BC</td>
<td>3.54 ± 0.86 b B</td>
<td>4.86 ± 0.33 a BC</td>
</tr>
<tr>
<td>MIC of quaternary ammonia</td>
<td>4.41 ± 0.13 a BC</td>
<td>3.32 ± 0.06 b BC</td>
<td>4.76 ± 0.02 a C</td>
</tr>
<tr>
<td>2 × MIC of quaternary ammonia</td>
<td>4.17 ± 0.1 a BC</td>
<td>3.14 ± 0.04 b BC</td>
<td>4.64 ± 0.02 a CD</td>
</tr>
</tbody>
</table>

a The differences between surfaces (a and b) and between treatments for each surface (A, B, C, and D) annotated with the same letter are not significant (P > 0.05).
(2,000 ppm), and the sodium hypochlorite–based solution had a MIC value 18 times higher than the recommended concentration (3,600 ppm).

The results in Figure 1 show that peracetic acid removed the polysaccharide matrix from the three surfaces more efficiently, leaving only a small number of adherent cells. Treatment with sodium hypochlorite affected the biofilm matrix only slightly, with the cell resistance being similar to but less intense than that with quaternary ammonia.

According to Abee et al. (1), biofilms provide an ideal environment for bacillus sporulation, and this process is tightly linked to the development of the biofilms, as spores appear in their upper structures.

According to a study conducted by Podolak et al. (17), products based on peracetic acid and sodium hypochlorite were more effective than chlorine dioxide against spores of *A. acidoterrestris* when tested at temperatures of 40 to 90°C in apple juice.

The apparent efficacy of the peracetic acid–based products on the biofilms is likely based on several factors, including the ability of the small molecule to penetrate the extracellular matrix of the biofilm, the mode of activity of the antimicrobial agent, and its tolerance of moderate quantities of organic material (2).

In another study, by Friedrich et al. (10), sodium hypochlorite also did not have a good result on *Alicyclobacillus* species spores under the conditions tested. The authors tested the effectiveness of chlorine dioxide and sodium hypochlorite on spores on three surfaces, stainless steel, wood, and rubber, and the most effective concentration-and-time regimen applied was 100 ppm of chlorine dioxide for 10 min.

The performance of a sanitizer can be affected by the biofilm stage and by the structure of the extracellular polysaccharide matrix. Cruz and Fletcher (7) demonstrated that the ability of *L. monocytogenes* strains to survive exposure to sanitizers may be more closely linked to the quantity of extracellular polysaccharide produced by the cells in the biofilm than to the number of cells in the biofilm or, even, other factors, such as the genetic subtype.

In this study, we found that the reduced numbers of cells attached in the *A. acidoterrestris* biofilms on PVC did not interfere with our analysis of the sanitizers, as the biofilms behaved similarly to those grown on steel and nylon with higher cell counts. This finding indicates that the quantity of cells in the biofilm does not directly affect the sanitizer function.

The results of scanning electron microscopy (Fig. 1) indicated that the surface type contributed to the cell adhesion. Steel provided a rougher surface, which facilitated adhesion. This result was also observed on the nylon strands, which provided pockets for biofilm development due to the space between the strands. The microscopy results are consistent with the cell survival assays, which also demonstrated reduced adhesion and biofilm formation on the PVC surface.

The presence of *A. acidoterrestris* spores in orange juice causes serious problems after its reconstitution and pasteurization, as the spore encounters conditions that are ideal for germination and multiplies. Spores that contaminate the final product may originate from biofilms that formed on the surfaces during industrial processing.
The sporicidal activities of the sanitizers, shown in the graphs in Figure 2, indicate that peracetic acid was less effective against the spores than against the biofilm. Peracetic acid at a concentration of 1,000 ppm (2 × MIC for the vegetative cells) and a 10-min contact time reduced the cell counts by 1 log CFU/ml and the biofilm by more than 2 log CFU/cm² on all surfaces. After 30 min of contact, this concentration of peracetic acid reduced the spore concentration by only 1.5 log CFU/ml and did not completely inactivate the spore germination at any of the concentrations tested in the experiment.

The sanitizer that demonstrated the best sporicidal activity was quaternary ammonia, which completely inactivated the spores after 5 min of contact at a concentration of 40.35 ppm. Quaternary ammonia inactivated both the spores and the vegetative cells at low concentrations (15.62 ppm), but these low concentrations did not effectively inactivate the A. acidoterrestris biofilms.

Sodium hypochlorite exhibited low activity on the biofilm and spores, although it was able to inactivate the spores at a concentration of 700 ppm after 30 min of contact. However, this combination of time and concentration is impractical.

The results presented in the flow cytometry charts in Figure 3 suggest that the principal mechanism for these sanitizers on both the A. acidoterrestris vegetative cells and the spores does not rely on membrane lysis, because propidium iodide did not strongly stain the microorganisms after sanitizer treatment.

However, little information is available in the literature on the mode of action for the sanitizers against Alicyclobacillus spp., and the present study is a pioneering work on A. acidoterrestris. Most previous studies suggest that the mode of action is similar to that reported for Bacillus spp. and Clostridium spp.

Cortezzo et al. (6) reported that the targets of the sanitizers may be the fatty acids and proteins in the spore, but their results obtained using Bacillus subtilis indicated that oxidation of the unsaturated fatty acids did not play a significant role in spore inactivation. Thus, the target may
be the proteins, as their membranes are rich in fatty acids and are not significantly affected by the sanitizers in the present study.

Although the exact nature of the damage inflicted by the sanitizers on Alicyclobacillus species spores is unknown, Cortezzo et al. (6) found that the damaged spore could not germinate. If it did germinate, the membrane would have been so damaged that the cell would rapidly die.

The cell count and scanning electron microscopy results indicate that A. acidoterrestris adheres to and forms biofilms on stainless steel, PVC, and nylon. The sanitizers tested did not effectively remove A. acidoterrestris in biofilms under the given conditions. The sanitizer that best reduced the cell numbers was peracetic acid. For all sanitizers, doubling the bactericidal concentration for vegetative cells only reduced the cell concentrations by 2 log CFU/cm² after adhesion and biofilm formation on all surfaces.

Quaternary ammonia exhibited the best sporidical activity, as it inactivated the spores at low concentrations and short contact times. Sanitization using peracetic acid and quaternary ammonia is a good approach to control A. acidoterrestris in industrial settings, but these sanitizers are difficult to apply together, due to their cost and practicality.

Considering the need to reduce or eliminate the spores in the industrial environment, 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 get good results.

For better results, the recommended concentrations of the sanitizers used in industry should be based on more-stringent conditions that consider the specific resistance mechanisms for each bacterium, such as biofilm and spore formation.

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