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Effect of ultraviolet (UV-C) radiation on spores and biofilms of *Alicyclobacillus* spp. in industrialized orange juice

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ABSTRACT

Bacteria of the genus *Alicyclobacillus* pose serious quality problems for the juice processing industries that have sought effective alternatives for its control. The present study evaluated the effect of UV-C radiation on the reduction of spores and biofilm formation of *Alicyclobacillus* spp. on stainless steel and rubber surfaces using industrialized orange juice as a culture medium. Four reference *Alicyclobacillus* spp. species and different UV-C dosages were investigated. After exposed for 20 min (16.8 kJ/m^2) to UV-C, the spores of *Alicyclobacillus acidoterrestris*, *Alicyclobacillus herbarius*, and *Alicyclobacillus cycloheptanicus* decreased drastically more of 4 log CFU/mL, with counts below the detection limit of the method (< 1.7 log CFU/mL), while the *Alicyclobacillus acidocaldarius* spores were more sensitive to UV-C, once this spore reduction was observed within 15 min (12.6 kJ/m^2). Morphological changes in the *Alicyclobacillus acidoterrestris* spores were observed by scanning electron microscopy. A reduction of biofilm formation was observed for all UV-C treatments, and the higher reductions (approximately 2 log CFU/mL) were found for the *Alicyclobacillus acidocaldarius* species after 30 min (26.2 kJ/m^2), on the stainless steel and rubber surfaces. The results suggest that UV-C can be used to reduce the biofilm formation and could be a promising alternative for controlling *Alicyclobacillus* spp. spores in industrialized orange juice.

1. Introduction

Alicyclobacillus spp. are characterized as Gram-positive, thermophilic, acidophilic bacilli, spore former, containing cyclic fatty acids as the main component of the cell membrane (Goto et al., 2007; Lee et al., 2010). The spores can survive the conventional heat treatments used in food processing of acid products and produce compounds such as guaiacol (2 - methoxyphenol) and 2,6 - dibromophenol, both responsible for the formation of phenolic or antiseptic odor in acidic beverages (Durak et al., 2010; Goto et al., 2008; Wang et al., 2013).

This genus comprises 20 *Alicyclobacillus* species, two subspecies and two genomic species (Nakano et al., 2015). *Alicyclobacillus acidoterrestris* is the predominant species responsible for most of the deterioration related to *Alicyclobacillus* and has been considered crucial in quality control for fruit juices and beverages (Smit et al., 2011).

Orange juice is the most consumed fruit juice in the world, with a growing market share. Brazil is the largest producer of concentrated and frozen orange juice in the world, exporting > 1 million and 400 thousand tons in 2017 (CitrusBr, 2018). The deterioration caused by *Alicyclobacillus* sp. in this product is generally imperceptible and remains undetected until consumer complaints. This can be very detrimental to juice manufacturers, as it also results in the return of the product with consequent economic losses causing damage to the image of the manufacturer (Gobbi et al., 2010; Zhang et al., 2013).

Alicyclobacillus can be found in the form of biofilms on surfaces within the food processing facilities (Anjos et al., 2013) and are able to

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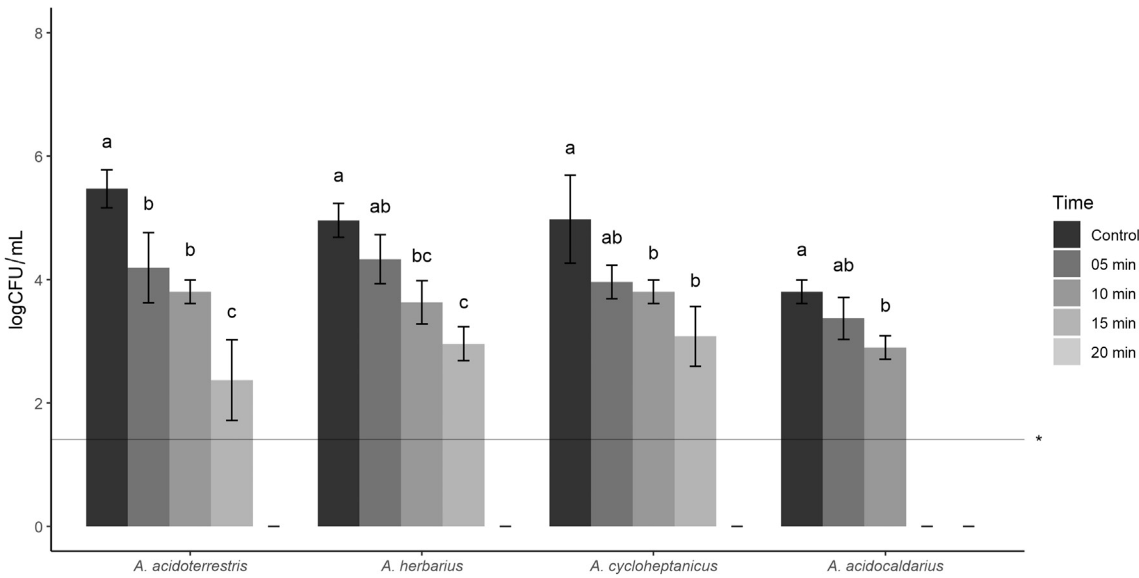

Fig. 1. Reduction of spores of *Alicyclobacillus* spp. (*A. acidoterrestris* 0244^T; *A. herbarius* 0246^T; *A. cycloheptanicus* 0297^T; *A. acidocaldarius* 0299^T) exposed to UV-C for 5, 10, 15, and 20 min. Controls groups were not exposed to UV-C. Different lowercase letters represent significant differences ($p < 0.05$) by the Tukey's test. * Detection limit = 1.7 log CFU/mL. Standard deviation not established.

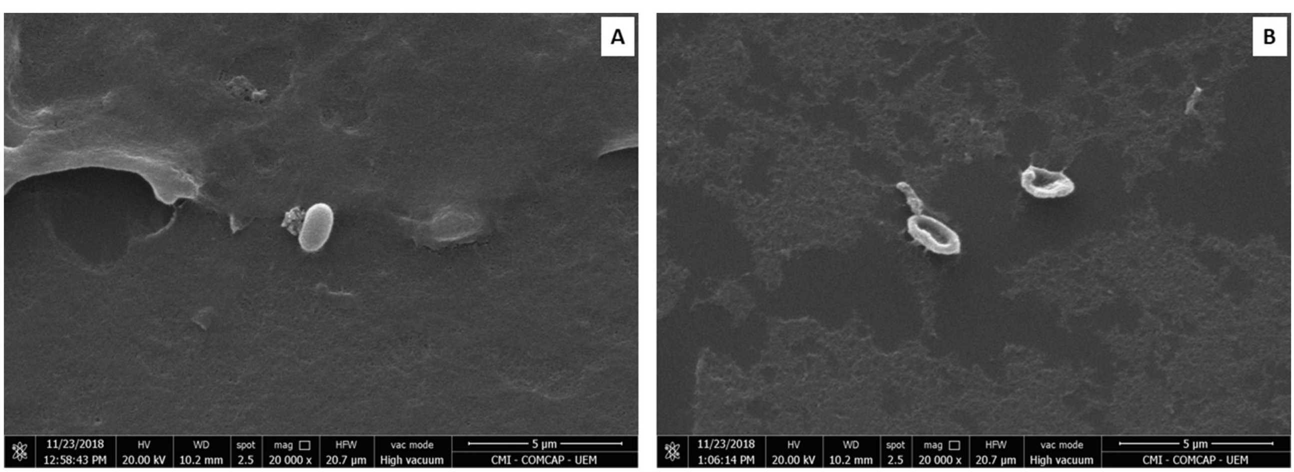

Fig. 2. Scanning Electron Microscopy of *Alicyclobacillus acidoterrestris* spores: A) Control, magnification: 20,000×; B) Treatment exposed to UV-C for 15 min: 20,000×.

survive on abiotic surfaces for prolonged periods (Prado et al., 2018).

The combination of detergents and sanitizers (*e.g.* ammonium quaternary or sodium hypochlorite plus peracetic acid) is one of the main strategies used to control the biofilm formation. However, these traditional methods are often inefficient in biofilm removal. In this context, ultraviolet (UV) radiation appears as an alternative disinfection mechanism, with advantages over the existing sanitation methods, once it does not require the use of heat or chemicals (Kim et al., 2016).

The wavelength of UV radiation for food processing ranges from 100 to 400 nm and is categorized as UV-A (320–400 nm), UV-B (280–320 nm), and UV-C (200–280 nm). Thus, UV-C radiation is considered the lethal germicidal region for most microorganisms (Gayàn et al., 2012).

UV-C irradiation is a technology approved by the US Food and Drug Administration (FDA) as a safe method to control microorganisms in liquid foods, water, and food contact surfaces (US FDA, 2000). However, Koutchma (2009) reported that several factors might influence the action of UV dosage, due to the interaction of UV light with a complex food matrix, which can often be referred to as a radiative transfer in a semi-transparent or cloudy medium.

Recently, Tremarin et al. (2017) found that ultraviolet radiation eliminated *A. acidoterrestris* spores from apple juice. However, there are no studies on the effect of UV-C on the *Alicyclobacillus* spp. spores using industrialized orange juice as a culture medium, and on the role of UV-C in the control of biofilm formation of these species on stainless steel and rubber surfaces.

In this search for control alternatives, it is important to emphasize the importance of clean and refreshing sources with no impacts on the environment, such as UV-C, thus contributing to the sustainability and food safety.

The objective of the present study was to evaluate the effect of UV-C radiation on the reduction of spores and biofilm formation of *Alicyclobacillus* spp. on stainless steel and rubber surfaces using orange juice as a culture medium.

2. Material and methods

2.1. Obtaining the microbial strains

Alicyclobacillus spp. were obtained from the Brazilian Collection of

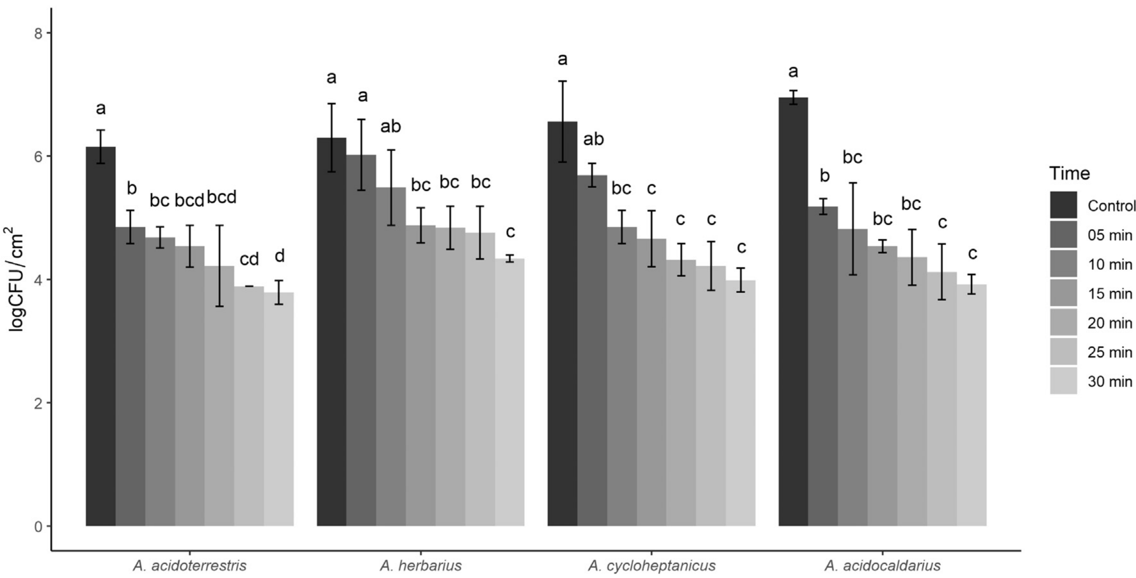

Fig. 3. Reduction of biofilms of *Alicyclobacillus* spp. (*A. acidoterrestris* 0244^T; 0 *A. herbarius* 0246^T; *A. cycloheptanicus* 0297^T; *A. acidocaldarius* 0299^T) on stainless steel surfaces exposed to UV-C for 5, 10, 15, 20, 25, and 30 min. Controls groups were not exposed to UV-C. Different lowercase letters represent significant differences ($p < 0.05$) by the Tukey's test.

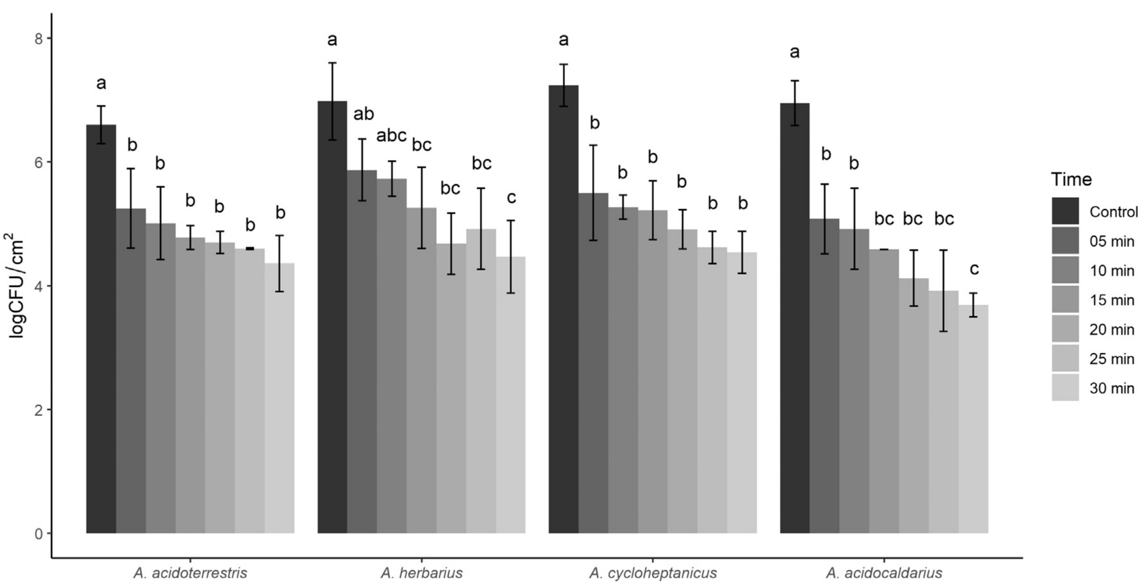

Fig. 4. Reduction of biofilms of *Alicyclobacillus* spp. (*A. acidoterrestris* 0244^T; 0 *A. herbarius* 0246^T; *A. cycloheptanicus* 0297^T; *A. acidocaldarius* 0299^T) on the rubber surfaces exposed to UV-C for 5, 10, 15, 20, 25, and 30 min. Controls groups were not exposed to UV-C. Different lowercase letters represent significant differences ($p < 0.05$) by the Tukey's test.

Environmental and Industrial Microorganisms (CBMAI), located at the Chemical, Biological and Agricultural Multidisciplinary Research Center (CPQBA/UNICAMP), which is composed of the following reference species: *A. acidoterrestris* 0244^T; *A. herbarius* 0246^T; *A. cycloheptanicus* 0297^T, and *A. acidocaldarius* 0299^T. The strains were stored in 30% glycerol at −20 °C and cultivated in 3 mL of *Bacillus acidoterrestris* medium - BAT broth (Deinhard et al., 1987) at 45 °C for 24 h.

2.2. Spore suspension

The bacterial suspension of *Alicyclobacillus* sp. vegetative cells was cultured in BAT broth, at pH 4 and 45 °C for the species *A. acidoterrestris* 0244^T, *A. herbarius* 0246^T, *A. cycloheptanicus* 0297^T, and 60 °C for *A. acidocaldarius* 0299^T for 120 h or until 80% sporulation was reached, determined by phase contrast microscopy according to Anjos et al. (2013) with adaptations. The culture was then transferred to 1 mL microtubes and centrifuged at 3000 rpm (2856 $\times g$) for 3 min and the pellet was washed 3 times with sterile distilled water. The pellet was resuspended in 1 mL of sterile distilled water and stored at 4 °C. The viability of the spore suspension was determined by dilution in saline (0.85%) and plate counted on BAT medium in duplicate, expressed in CFU/mL. The spore suspension aliquots were activated by heat shock (80 °C for 10 min in a water bath), diluted, streak plated on BAT agar, and incubated at 45 °C for the species *A. acidoterrestris* 0244^T, *A. herbarius* 0246^T, *A. cycloheptanicus* 0297^T, and 60 °C for *A. acidocaldarius* 0299^T for 24 h.

2.3. Preparation of stainless steel and rubber coupons

Bacterial biofilms formed on the AISI 304 stainless steel coupons

(8 mm × 8 mm × 1 mm) and rubber coupons (8 mm × 8 mm × 3 mm), consisting of a non-toxic rubber, commonly used as a fruit conveyor belt in the food industry. Before each assay, the coupons were rinsed with neutral detergent and distilled water, immersed in 70% (v/v) alcohol for 1 h at room temperature, rinsed again in distilled water and sterilized at 121 °C for 15–20 min.

2.4. Preparation of the culture medium (reconstituted orange juice)

Concentrated orange juice (66° Brix) was reconstituted (11° Brix) using a digital refractometer (Pocket, PAL-1, brand ATAGO) and was used as a culture medium. The concentrated orange juice was obtained from a juice industry located in Paranavaí (Paraná, Brazil) and was previously certified of the absence of vegetative cells and spores of *Alicyclobacillus* spp. through of plating method and common optic microscopy of spore staining with malachite green.

The UV-C transmittance at 254 nm was measured in the reconstituted orange juice using a spectrophotometer (T90 + UV/VIS, PG Instruments Ltd., Australia). It was observed that 98% of the UV-C is absorbed in a 1 mm thick sample.

2.5. Biofilm formation

The bacterial suspension of vegetative cells was prepared in 0.85% saline solution to match the turbidity of the McFarland scale at 10^8 CFU/mL, followed by serial dilutions. The coupons (stainless steel and rubber) were immersed in 24 well cell culture plates, containing 900 μL and 100 μL of reconstituted juice and culture medium in each well, respectively, with an initial inoculum of 10^4 CFU/mL. The assays were performed individually for each species. The plates were incubated for 72 h at 45 °C for the species *A. acidoterrestris* 0244^T, *A. herbarius* 0246^T, *A. cycloheptanicus* 0297^T, and 60 °C for *A. acidocaldarius* 0299^T. The treatment with UV-C was applied to the biofilm formed after 72 h of incubation. At each inoculation, a control was performed by counting *Alicyclobacillus* cells on BAT agar surface to confirm the concentration of the microorganism inoculated.

2.6. UV-C device

The treatments using UV-C radiation were performed in a chamber ($75 \times 70 \times 45\ cm^3$) manufactured at the State University of Maringá, Paraná-Brazil, as reported by Tremarin et al. (2017) with modifications. Three monochromatic UV-C emitting lamps at 254 nm were used (Germicide 15 W T8 - OSRAM). The intensity of UV-C was obtained using a radiometer (Gooch & Housego, OL 756) coupled to an optical fiber (Gooch & Housego, OL 730 7q-1.0) and a 50.8 mm diameter integrating sphere (Gooch & Housego, IS-270) to provide a power density of 14 W/m^2. All measurements were taken at a distance of 24 cm from the lamp. The UV-C fluence (kJ/m^2) was calculated as the power density applied (W/m^2) x time (s)/1000. The UV-C lamp was switch on for at least 30 min prior to the experiments to ensure the light stability of UV-C.

2.6.1. Treatment of spores with UV-C radiation

The spore suspension (100 μL) was immersed in 900 μL of reconstituted juice in 24 well cell culture plates with a final spore concentration of approximately 10^4–10^6 CFU/mL, according to the species. The assays were performed individually for each species. The samples were subjected to constant stirring during the UV-C treatment to ensure uniform distribution of the radiation. UV-C exposure times of 0, 5, 10, 15, and 20 min were evaluated after the heat shock (80 °C for 10 min in a water bath). Then, dilution and drop-plating on BAT agar was performed, which was incubated at 45 °C for *A. acidoterrestris* 0244^T, *A. herbarius* 0246^T, *A. cycloheptanicus* 0297^T, and 60 °C for *A. acidocaldarius* 0299^T species for 24 h. The spores of the control group were not submitted to UV-C exposure.

2.6.2. Scanning electron microscopy of spores

The spores were subjected to scanning electron microscopy according to the protocol proposed by De Pascoli et al. (2018). Spores were exposed to UV-C for 15 min (12.6 kJ/m^2) and an untreated control was also performed. The samples were washed in saline and fixed in 2.5% gluteraldehyde (Sigma-Aldrich, St. Louis, MO) in 0.1 M sodium cacodylate buffer (SEM, Hatfield, PA). Then, they were washed in 0.1 M sodium cacodylate buffer and placed on the coverslip with poly-L-lysine. After that, the samples were dehydrated in ethanol, critical point dried, coated with gold and observed in a Scanning Electron Microscope (Quanta 250, FEI Company).

2.6.3. Treatment of biofilm with UV-C radiation

After the incubation period, the coupons were removed from the orange juice and transferred to microtubes containing 1.0 mL of 0.85% saline solution, in which they were immersed for 1 min at rest to release the weakly adhered cells. Each vial was transferred to 24-well cell culture plates with 1 mL of 0.85% saline solution to simulate a moist industrial condition, such as after the conventional sanitizing process for example, and different UV-C exposure times were applied (0, 5, 10, 15, 20, 25, and 30 min). After the treatment time, the coupons were submitted to the ultrasound bath at 25 kHz for 5 min to remove the sessile cells (Prado et al., 2018). Then, dilution and drop-plating on BAT agar and incubation at 45 °C was performed for *A. acidoterrestris* 0244^T, *A. herbarius* 0246^T, *A. cycloheptanicus* 0297^T, and 60 °C for *A. acidocaldarius* 0299^T for 24 h.

2.7. Statistical analysis

Data analysis was performed using the R software (R Development Core Team, 2010). The results were expressed as mean ± standard deviation (SD), analysis of variance (ANOVA) and Tukey's test was used, considering a level of significance of 5% (Montgomery, 2005).

3. Results and discussion

3.1. Reductions of Alicyclobacillus spp. spores from orange juice

Fig. 1 shows the spore reductions (log CFU/mL) in the orange juice samples. After 20 min exposure to the UV-C light (16.8 kJ/m^2), the *A. acidoterrestris*, *A. herbarius*, and *A. cycloheptanicus* spores showed counts below the detection limit of the method, i.e. < 1.7 log CFU/mL, according to the plating method used.

Significant reductions of until 3 log CFU/mL ($p < 0.05$) in the spore counts of *A. acidoterrestris* were observed from 5 to 15 min (4.2 kJ/m^2 to 12.6 kJ/m^2) of UV-C exposure when compared to the control group. Significant ($p < 0.05$) reductions of *A. herbarius*, *A. cycloheptanicus* and *A. acidocaldarius* spores were observed for all treatment times, except for the treatment exposed for 5 min (4.2 kJ/m^2).

The *A. acidocaldarius* spores were more sensitive to UV-C for 15 min (12.6 kJ/m^2) when compared to the other *Alicyclobacillus* species, with counts below the detection limit of the method, < 1.7 log CFU/mL.

Fig. 2 shows the results of scanning electron microscopy of A. acidoterrestris spores. The untreated control spores were intact and exhibited regular contours in its shape and structure (Fig. 2-A). In contrast, the treated spores (Fig. 2-B) subjected to 12.6 kJ/m^2 exhibited visible changes such as morphological distortions, central depression and expressive roughness. Scanning electron microscopy contributed to the understanding of the effective action of UV-C against spores, according to the results demonstrated in this study and dynamizes the ability to evaluate the use of UV-C as a control of microbial growth in the industrial axis.

Previous studies have used UV-C for inactivation of *A. acidoterrestris* spores in fruit juice (*i.e.*, apple juice and grape) (Baysal et al., 2013; Tremarin et al., 2017; Usaga et al., 2015). Tremarin et al. (2017)

reported that the highest UV-C intensity (13.44 W/m^2) at a dose of 6.45 kJ/m^2 in apple juice allowed to reduce 5 log cycles of contamination within 8 min of treatment.

Usaga et al. (2015) reported that different UV dosages, microorganisms and the nature of the liquid subjected to the UV treatment can affect the inactivation kinetics. The authors found that a mixture of vegetative cells and spores of *A. acidoterrestris* in apple juice was sensitive to UV (4.96 log CFU/mL, UV radiation dosages of 14 mJ/cm^2), which corroborates with the present study.

Several studies have evaluated the effect of UV incidence on orange juice for microbial reduction (Char et al., 2010; Gayàn et al., 2012; Taze et al., 2015). Char et al. (2010) reported the survival of *Escherichia coli and Saccharomyces cerevisiae* in orange juice (pH 3.5, 9° Brix) and apple juice (pH 3.1, 12° Brix) using ultrasound and/or UV-C treatment. The efficiency of UV-C was highly dependent on the nature of the juice, and the UV-C radiation in orange juice was more effective when combined with ultrasound ($p < 0.05$). Gayàn et al. (2012) demonstrated the efficacy of the UV treatment for the inactivation of *E. coli* in orange juice using a dosage of 27.10 J/mL combined with a temperature of 55 °C, which allowed a reduction of 5 log CFU/mL. Taze et al. (2015) showed that the UV exposure time was a very important factor ($p < 0.0001$) in the number of yeasts survival, and the UV-C radiation has proven to be an effective alternative to reach a required level of microbial reduction in orange juice.

The present results show that UV-C can be promising to treat *Alicyclobacillus* spp. spores from orange juice. In addition, Tran and Farid (2004) showed that the color and pH of orange juice were not significantly influenced by the UV-C treatment, while Keyser et al. (2008) reported that the flavor was also not affected by UV-C. The authors confirmed that higher UV-C dosages are required to achieve an effective microbial reduction in lighter-colored fruit juices, such as apple juice.

3.2. Effects of UV-C radiation on Alicyclobacillus spp. biofilms formed on stainless steel and rubber surfaces

A greater reduction in *Alicyclobacillus* spp. biofilms formed on the stainless steel surface was observed with the increase in the UV-C exposure time, as presented in Fig. 3.

After the treatment 30 min (26.2 kJ/m^2), the reduction of *A. acidoterrestris* sessile cell were approximately 2 log CFU/cm^2. In addition, a significant reduction ($p < 0.05$) was observed for all treatments when compared to the control group, while no changes ($p > 0.05$) were observed in the *A. herbarius* biofilms at the times of 5 and 10 min (4.2 kJ/m^2 and 8.4 kJ/m^2) in comparison to the control group.

The maximum biofilm reduction was observed for the *A. acidocaldarius* species, with approximately 3 log CFU/cm^2 within 30 min (26.2 kJ/m^2). In addition, significant differences ($p < 0.05$) were observed for the exposure time of 5 min (4.2 kJ/m^2) when compared to the exposure for 25 min (21 kJ/m^2) and 30 min (26.2 kJ/m^2).

Studies with other microorganisms and the use of UV-C on stainless steel surfaces used in the food industry have been reported (Jahid et al., 2014; Kim et al., 2016). Jahid et al. (2014) reported an interaction between a mixed culture of *Salmonella Typhimurium* and microorganisms isolated from lettuce leaf on the resistance of biofilms to UV-C radiation. Regarding the mixed culture conditions, a UV-C dosage of 35 mJ/cm^2 was required to achieve a biofilm reduction of 5.0 log CFU/cm^2 on the stainless steel surface, while a dose > 360 mJ/cm^2 reduced the number of biofilm cells by approximately 2.0 log CFU/cm^2 in the lettuce, showing that the biofilm resistance differs according to the surface treated.

Kim et al. (2016) demonstrated the synergistic effect of the combination of UV-C at dosages of (300, 600, 1200, and 1800 mJ/cm^2) and sodium hypochlorite (NaOCl) (50, 100, 150, and 200 ppm) on *Listeria monocytogenes* biofilms formed on stainless steel surfaces and eggshell. The highest reduction was observed at a dosage of 1800 mJ/cm^2 (1.47 CFU/cm^2), while the best synergistic effect was observed at 1800 mJ/cm^2 and 200 ppm NaOCl (3.68 log CFU/cm^2) on a stainless steel surface.

The results of the treatment with UV-C radiation on *Alicyclobacillus* spp. biofilms on the rubber surfaces are shown in Fig. 4.

The results have shown similar results between the *A. acidoterrestris* and *A. cycloheptanicus* biofilms treated with UV-C, with significant reductions ($p < 0.05$) in relation to the control group. However, there were no significant differences ($p > 0.05$) for the different exposure times.

No significant reductions ($p > 0.05$) were found for *A. herbarius* between the samples treated for 5 and 10 min (4.2 kJ/m^2 and 8.4 kJ/m^2) when compared to the control group.

When comparing the stainless steel and rubber surfaces, both had the highest biofilm reduction of *A. acidocaldarius* species at 30 min of exposure (26.2 kJ/m^2), with reductions of 3.03 and 3.26 log CFU/cm^2, respectively. As expected, the treated biofilms presented significant reductions when compared to the control group.

The use of UV-C reduced the spore counts and biofilm formation on the treated species, being a promising alternative for the control of *Alicyclobacillus* spp., especially the *A. acidocaldarius* species, for which the best results were found. In addition, combined treatments can be used as an alternative to improve the efficiency of UV-C in orange juice, including the treatment of other microorganisms present in the food processing environment. Thus further studies on the combination between UV-C and other methods are required.

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