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Effect of sanitizers combined with ultrasound on adhesion of microorganisms isolated from fish from the Amazon region

Evelyn Ivana Trindade Damasceno*, Hamilton Mendes Figueiredo and Antonio Manoel Da Cruz Rodrigues

Food Science and Technology Program, Federal Institute of Pará (IFPA), Brazil.

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Attachment of undesirable microorganisms to surfaces that contact food is a source of concern, since it can result in product contamination leading to serious economic and health problems. Bacteria aggregated to form biofilms are more resistant to environmental stress than planktonic cells. The objective of this paper was to evaluate the bactericidal effect of sodium hypochlorite and peracetic acid associated with ultrasound (40 Hz) to control the adhesion of *Staphylococcus aureus*, *Staphylococcus hominis,* and *Pseudomonas aeruginosa* isolated from two fish species from the Amazon region: butterfly peacock (*Cichla ocellaris*) and piramutaba (*Brachyplatystoma vailantii*). After incubation at 30°C for 24 h, stainless steel coupons were treated for 10 min by different concentrations of sodium hypochlorite (50, 100 and 150 mg/L) and peracetic acid (40, 60 and 80 mg/L) at 25°C. The sodium hypochlorite (150 mg/L) and peracetic acid (80 mg/L) treatments were also combined with ultrasound (40 Hz) for 10 min at 25°C. The results showed that the recommended treatment based on this study was the use of peracetic acid combined with ultrasound.

Key words: Sanitizer, adhesion, *Staphylococcus aureus*, *Staphylococcus hominis, Pseudomonas aeruginosa.*

INTRODUCTION

The surfaces that come into contact with foods are important sources of microbial contamination in food-processing plants, which may be associated with food quality and safety (Vogel-Fonnesbech et al., 2001). This happens because some pathogenic bacteria are able to adhere to food-contact surfaces and remain viable even after cleaning and disinfection (Ammor et al., 2004). One of the most common ways for bacteria to live is by adhering to surfaces and forming biofilms in which they are embedded in an organic extracellular polymeric matrix (Chae and Schraft, 2000).

The surface characteristics of the microorganisms themselves and the various environmental conditions encountered in agri-food industries (organic materials, pH, temperature, water activity, etc.) influence microbial attachment to inert surfaces (Giovannacci et al., 2000; Gross et al., 2001). Adhesion of undesirable microorganisms to these surfaces is a source of concern, since it can result in product contamination leading to serious economic and health problems.

*Corresponding author. E-mail: evelyn.damasceno@ifpa.edu.br.

According to Costerton et al. (1999), biofilms are cell aggregates embedded in an organic extracellular polymeric matrix that confers resistance to the microorganisms involved. Bacteria aggregated to form biofilms are more resistant to environmental stress than their planktonic counterparts, including sensitivity to sanitizers (Fux et al., 2004; Spoering and Lewis, (2001).

To remove biofilm organisms, the sanitizing solution must penetrate the exopolymer matrix and gain access to the microbial cells, which causes biofilm inactivation and removal. Chlorinated products such as hypochlorite salts (Meyer, 2003; Srey et al., 2012) constitute the most widely used group of sanitizing compounds. However, there has been some concern regarding the use of hypochlorite and other chlorine salts considered precursors in the formation of organic chloramines, which are harmful to health due to their high carcinogenic potential (Andrade, 2008).

In order to reduce the incidence of microorganisms in foods, the industry has used several sanitizers such as chlorates, peracetic acid (PAA), and quaternary ammonium, among others.

The most widely used chlorate compounds are: sodium hypochlorite (NaClO), lithium hypochlorite, calcium hypochlorite, and chlorine dioxide (inorganic) and chloramine-T, dichloramine-T, dichloroisocyanuric acid, and dichloro dimethyl hydantoin (organic) (Srebernich, 2007).

NaClO in aqueous medium dissociates into hypochlorous acid and hypochlorite. The bactericidal power of the chlorate compounds is usually based on the release of hypochlorous acid in its non-dissociated form when in aqueous solution, except for chlorine dioxide, which does not hydrolyze in aqueous solution and the whole molecule is considered the active agent (Andrade, 2008).

The use of hypochlorite and of the other chlorine salts considered precursors in the formation of organic chloramines has raised a lot of concern since they are harmful to health due to their high carcinogenic potential (Andrade, 2008). Thus, several sanitizing agents have been proposed to replace NaClO.

The use of PAA has many advantages when compared with NaClO (Kunigk and Almeida, 2001). One important advantage is that it does not produce toxic residues when decomposed and therefore does not affect the final product or the waste treatment process. PAA can be used over a wide temperature spectrum (0 to 40°C) in clean-in-place (CIP) processes (Leaper, 1984). PAA can also be used with hard water and protein residues do not affect its efficiency. Up until now, no microbial resistance to PAA has been reported and it is efficient over a wide pH range (3.0 to 7.5) (Block, 1991; Lenahan, 1992).

Ultrasound (US) was adopted by the electronic industry to decontaminate surfaces and its use has recently been recommended as an alternative sanitization step in the food industry (Nascimento et al., 2008; Adekunte et al., 2010; Cao et al., 2010; Sagong et al., 2011). When applied to liquids, ultrasonic waves promote cavitation, which consists on the formation, growth, and collapse of air bubbles. These bubbles generate localized mechanical and chemical energies that are capable of inactivating microorganisms such bacteria as virus (Valero et al., 2007; Gogate and Kabadi, 2009; Adekunte et al., 2010). Bubble collapse causes pressure changes, which is considered the main cause of microbial cell disruption (Patil et al., 2009). US has been frequently studied in research aimed at interrupting the biofilm or even at inactivating microorganisms.

The objective of this paper was to evaluate the bactericidal effect of NaClO (50, 100, and 150 mg/L) and PAA (40, 60, and 80 mg/L) associated with US (40 Hz) to control the surface adhesion of *Staphylococcus aureus*, *Staphylococcus hominis*, and *Pseudomonas aeruginosa* isolated from fish species from the Amazon region.

MATERIALS AND METHODS

Bacterial strains

The pure cultures were isolated from fish species butterfly peacock [*Cichla ocellaris*] and piramutaba [*Brachyplatystoma vailantii*] from the Amazon region. The bacteria were isolated through seeding in Agar surface using violet red bile glucose (VRBG) (Kasvi, Spain) for *P. aeruginosa* strains and Baird-Parker with egg-yolk tellurite (Kasvi, Spain) for *S. aureus* and *S. hominis*, both with incubation at 36°C/48 h. Next, colonies were selected to be striated in VRBG or Baird-Parker agar plates to obtain pure cultures. After another incubation at 36°C/48 h, these colonies were transferred to BHI (brain-heart infusion) with 10% glycerol (Kasvi, Spain) and stored in a freezer to be used in further tests.

The bacteria isolated were previously identified with Gram stain tests. Next, *P. aeruginosa* strains were identified using the API 20E kit (Enterobacteria) while *S. aureus* and *S. hominis* strains, with the API Staph kit (Staphylococci). This procedure was in accordance with the manufacturer's recommendations (Biomérieux, France) (Harrigan, 1998).

Surface

Stainless steel coupons (6 cm^2) were used as test surfaces. The coupons were individually cleaned and sterilized according to Marques et al. (2007).

Adhesion to surfaces, quantification of adhered cells and sanitizers application

Strains were reactivated in nutrient broth for 72 h at 36°C and replicated to another nutrient broth (Himedia, India) for 24 h at 36°C. Then, 2 mL of activated contents were transferred to 300 ml of a new nutrient broth, in which the stainless-steel coupons were immersed and incubated at 30°C/24 h. After this last incubation period, the population density (planktonic cells) in the bacterial suspension was estimated using nutrient agar (Himedia, India). Next, all coupons were aseptically removed, rinsed three times with sterile distilled water to remove unattached cells, and dried in a laminar flow cabinet (DELEQ MA1500/90) for 30 min.

Afterwards, the coupons were immersed in sterile distilled water at 25°C (control group) for 10 min. Finally, microorganisms were

Table 1. Population density of planktonic and sessile cells of the tested organisms in nutrient broth with stainless steel coupons.

Bacteria	Planktonic cells (log CFU/mL)	Sessile cells (log CFU/cm^2)
S. aureus	7.09±0.05^a	4.11±0.09^a
S. hominis	6.82±0.78^a	4.95±0.85^b
P. aeruginosa	6.99±0.17^a	4.14±0.17^a

Means followed by the same letter in the column did not differ by Tukey's test at 5% probability. Values represent the mean of three repetitions.

quantified using a swab rubbed 20 times onto two coupons and then immersed in 0.1% peptone water for subsequent plating.

The remaining coupons were immersed for 10 min in sanitizer solutions at 25°C at three different concentrations: commercial NaClO (50, 100, and 150 mg/L) and PAA (40, 60, and 80 mg/L). Two coupons were used for each sanitizer concentration. The sanitizing effect was neutralized with the aid of a 0.1% sodium thiosulfate solution. The microorganisms were quantified using the swab technique.

All coupons were plated in duplicate on nutrient agar and incubated at 37°C for 48 h. Next, the NaClO and PAA concentrations that obtained the greatest decimal reduction in the microorganism population were associated with the US treatment in an ultrasound bath (QSONICA Q700) for 10 min following the same procedures mentioned earlier.

The effectiveness of the disinfectant agent expressed as germicidal effect or decimal reduction (DR), was determined by the equation:

$$DR = \log N_i - \log N_f \quad (1)$$

where N_i is the cell count in the control group (no sanitizer treatment) (CFU/cm^2) in nutrient agar and N_f is the count after exposure to sanitizer.

Reproducibility and statistical analysis

All analyses were carried out in duplicate with three repetitions on separate occasions, and the results are expressed as the average of the assays. Counts were converted into decimal logarithmic values (log CFU/cm^2). The test results before and after sanitizer application were compared using Tukey's test. Data were analyzed using the software Statistica 7.0. A probability value $p<0.05$ was accepted as indicating significant differences.

RESULTS

Bacterial adherence to surfaces

The population density of the bacterial suspensions in nutrient broth is not significantly different ($p>0.05$) after 24 h/30°C. However, the number of *S. hominis* cells adhering to stainless steel coupons was higher ($p<0.05$) compared to the species *S. aureus* and *P. aeruginosa*, which shows their greater adhesion capacity in the test conditions (Table 1).

Effect of sanitizers

Counts of *S. aureus, S. hominis*, and *P. aeruginosa* cells adhered to stainless steel surfaces after application of PAA (40, 60, and 80 mg/L) and NaClO (50, 100, and 150 mg/L) are as shown in Figures 1 to 3. These results are significantly different ($p<0.05$) when submitted to analysis of variance (ANOVA) (Table 2).

The different NaClO concentrations (50 to 150 mg/L) yielded a significant difference ($p<0.05$) when applied to adhered cells of *S. aureus* and *S. hominis*, reaching reductions between 1.57-2.20 and 1.52-2.35 log cycles, respectively. The application of PAA (40-80 mg/L) yielded reduction values between 2.09-2.64 log cycles for *S. aureus* and 3.22-4.34 for *S. hominis*. These values differed among themselves at a 95% significance level.

DISCUSSION

All species evaluated were able to adhere to stainless steel surfaces, reaching values between 4.11 and 4.95 log CFU/cm^2 (Table 1). Parizzi et al. (2014) found results of approximately 5.0 log CFU/cm^2 for *S. aureus* on stainless steel after 12 h of contact at 30°C. Another study showed that the adhesion of *S. aureus* reached 6.10 log CFU/cm^2 (Silva et al., 2009). Also, Jeromino et al. (2012) showed adhesion of 6.9 log CFU/cm^2 for *S. aureus* on stainless steel after 24 h at 28°C. Krolasik et al. (2010) observed the adhesion of *S. hominis* in stainless steel after incubation for 4 h at 20°C, while Vanhaecke et al. (1990), Cloete and Jacobs (2001), Figueiredo et al. (2009), and Caixeta et al. (2012) also observed the adhesion capacity of *P. aeruginosa* to stainless steel.

The different NaClO concentrations (50 to 150 mg/L) reached reductions between 1.57-2.20 and 1.52-2.35 log cycles (*S. aureus* and *S. hominis*). The application of PAA (40-80 mg/L) yielded reduction values between 2.09-2.64 log cycles for *S. aureus* and 3.22-4.34 for *S. hominis*.

The United States Environmental Protection Agency's (EPA) Scientific Advisory panel has stated that any treatment which can reduce microbial contamination by 2 log cycles is significant (Michaels et al., 2003). That shows that PAA was more efficient than NaClO in reducing *S. aureus* and *S. hominis* populations since lower PAA concentrations (40 mg/L) yielded population reductions equivalent to those observed for the highest

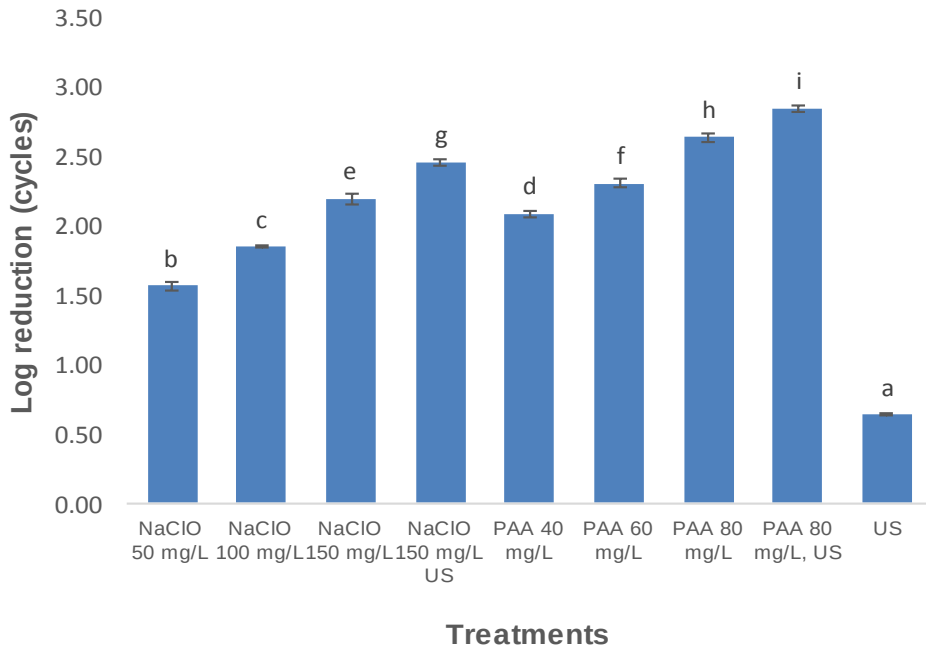

Figure 1. Effect of applying peracetic acid (PAA) and sodium hypochlorite (NaClO) combined with ultrasound (US) to control *S. aureus* adhesion. Treatments indicated with the same letter did not differ (p>0.05) among themselves.

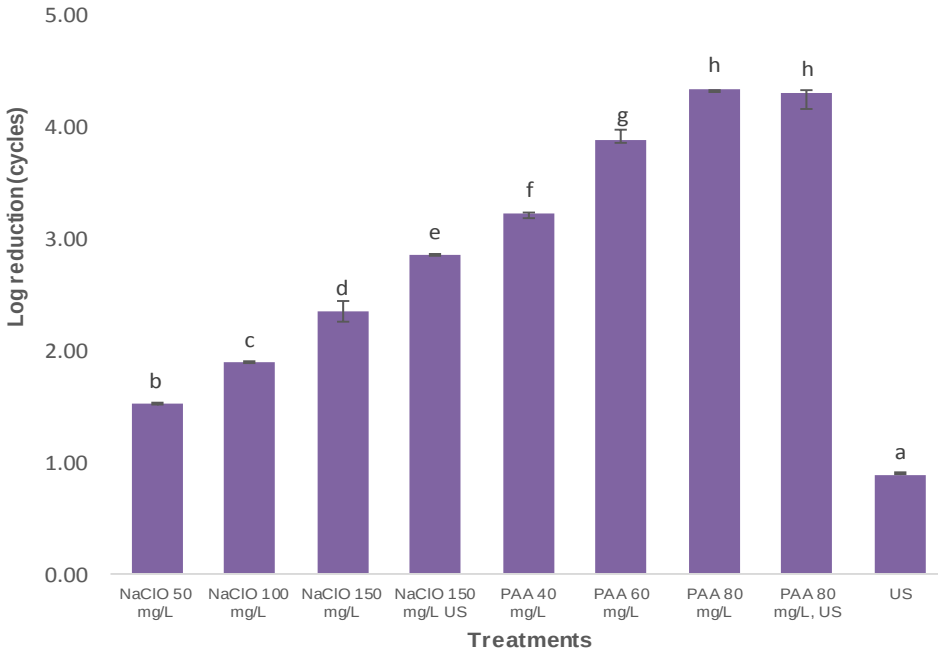

Figure 2. Effect of applying peracetic acid (PAA) and sodium hypochlorite (NaClO) combined with ultrasound (US) to control *S. hominis* adhesion. Treatments indicated with the same letter did not differ (p>0.05) among themselves.

NaClO concentrations (150 mg/L).

When associated with US (40 Hz, 10 min, NaClO (150 mg/L) and PAA (80 mg/L)) allowed the reduction of 2.46 and 2.85 cycles, respectively, of adhered *S. aureus* cells. For *S. hominis*, this association yielded reductions of 2.85 and 4.30 cycles, respectively. In other words, PAA at 80

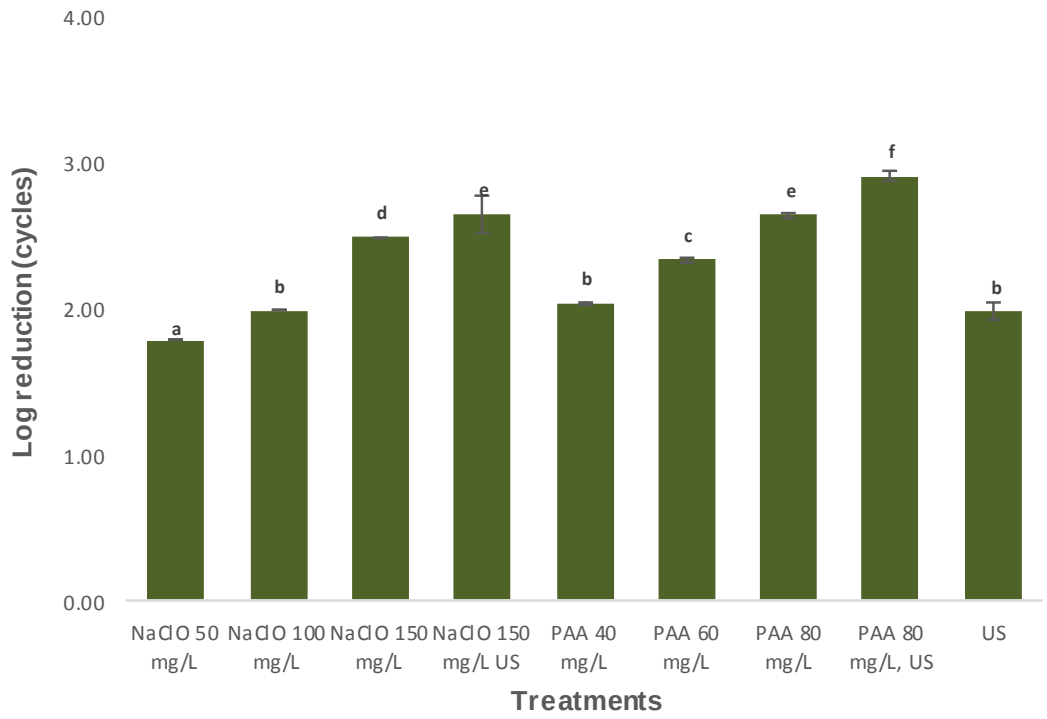

Figure 3. Effect of applying peracetic acid (PAA) and sodium hypochlorite (NaClO) combined with ultrasound (US) to control *P. aeruginosa* adhesion. Treatments indicated with the same letter did not differ (p>0.05) among themselves.

Table 2. ANOVA for sanitizer treatments applied to *S. aureus*, *S. hominis*, and *P. aeruginosa*.

Bacteria/Treatment	SS	S	F	p
S. aureus	10.4888	1,311	1,913.50	0.00
S. hominis	36.6267	4,578	1,334.94	0.00
P. aeruginosa	3.5450	0,433	168.51	0.00

mg/L and NaClO at 150 mg/L associated with US yield significant values (at a 95% level) in the adhesion of *S. aureus* and *S. hominis* compared to the application of these sanitizers alone.

However, US (40 Hz, 10 min) applied alone yielded a reduction of only 0.64 and 0.89 cycles for *S. aureus* and *S. hominis*, respectively. These results suggest that no synergistic or additive effect occurred between the sanitizers (NaClO and PAA) and US in the conditions studied.

Therefore, US might help aqueous sanitizers penetrate inaccessible sites (hydrophobic pockets and folds in leaf surfaces on fruits and vegetables), which makes such sanitizers more effective (Seymour et al., 2002; Gogate and Kabadi, 2009; Sagong et al., 2011).

Studies have combined US with other sanitizers such as organic acids (Sagong et al., 2011), hydrogen peroxide (São José and Vanetti, 2012), and chlorine dioxide (Huang et al., 2006) and have found an additive or even synergistic bactericidal effect compared to the individual treatments (Ding et al., 2015). São José and Vanetti (2015) observed no synergistic effect of applying US with sodium dichlorocyanurate (50 and 200 mg/L) and PAA (40 mg/L) to remove *Salmonella* from cherry tomato surfaces.

In fact, US is a clean technology (Rahman et al., 2010) with potential to be used in bacteria inactivation. However, it is not very effective alone in killing microorganisms in food at ambient or sub-lethal temperatures (Sengül et al., 2011). Microorganism reduction by US is mainly due to the physical phenomenon called cavitation (Alegria et al., 2009; Piyasena et al., 2003; Seymour et al., 2002).

Lee et al. (2014) suggested that the treatment with US alone may not be effective for application in the food industry. Piaysena et al. (2003) reported that bactericidal effects on food treated with US alone is localized and does not affect a large area.

Others studies have examined the inactivation of

pathogenic bacteria by chemical disinfection treatments such as NaClO *in vitro*. Ha and Ha (2012) reported strong resistance of *S. aureus* against NaClO. NaClO has also been reported to be a potential antimicrobial agent against *S. aureus* in biofilm (Toté et al., 2010). Bodur and Cagri-Mehmetoglu (2012) noted that NaClO (250 mg/L) was not efficient in completely removing *S. aureus* cells adhered to stainless steel surfaces. Meira et al. (2012) found similar results when studying *S. aureus* biofilm formation on stainless steel surfaces.

Rossini and Gaylarde (2000) stated PAA has an important advantage because this compound does not pose an environmental risk and does not produce toxic compounds after reaction with organic materials. Marques et al. (2007) confirmed that PAA was the most effective in removing adhered *S. aureus* cells. Meira et al. (2012) reported that PAA (30 mg/L) was more effective than NaClO (250 mg/L) in reducing the viable cell count of *S. aureus* in the biofilm matrix. Vázquez-Sánchez et al. (2014) noted PAA (100-750 mg/L) was more effective against *S. aureus* biofilms and planktonic cells when compared with NaClO (500-1,000 mg/L) treatment.

Nonetheless, more studies on the inactivation of *S. hominis* by the application sanitizers are required given the scarce literature data on the subject.

The different NaClO (50 to 150 mg/L) and PAA (40-80 mg/L) concentrations yielded a significant difference ($p<0.05$) when applied to adhered *P. aeruginosa* cells, reaching reductions between 1.78-2.49 and 2.04-2.64 log cycles, respectively. When associated with US, only PAA yielded reductions (2.91 log cycles) that are significantly different at a 95% level when compared with the treatment with PA alone.

The individual application of US yielded a reduction of 1.98 cycles and can be compared to the efficiency of NaClO (100 mg/L). Moreover, it can be considered an efficient treatment to control the adhesion of this microorganism according to the EPA since it alone yielded a reduction of approximately 2 log cycles.

A synergistic effect ($p<0.05$) was also observed between the treatments with NaClO (150 mg/L) and PAA (80 mg/L) when combined with US.

Herceg et al. (2012) noted that Gram-negative bacteria are more susceptible to the US treatment than Gram-positive ones. Gram-positive bacteria, especially *S. aureus*, usually have a thicker and more tightly adherent layer of peptidoglycan than Gram-negative bacteria, and this morphological feature did seem to be a differentiating factor in ranking the microorganisms according to the percentage of bacteria killed by US treatment.

Conclusion

The results in the present study showed that *S. hominis* is quite sensitive to the treatment with PAA and may reach reductions of up to 4 log cycles. Furthermore, the results showed that the best treatment combination both for *S. aureus* and *S. hominis* and *P. aeruginosa* was PAA at 80 mg/L associated with US. The use of US at 40 Hz to remove adherent *P. aeruginosa* can be considered efficient and has an effect comparable to that of NaClO (100 mg/L).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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