Effect of peracetic acid on biofilms formed by *Staphylococcus aureus* and *Listeria monocytogenes* isolated from dairy plants

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ABSTRACT

This research investigated the removal of adherent cells of 4 strains of *Staphylococcus aureus* and 1 *Listeria monocytogenes* strain (previously isolated from dairy plants) from polystyrene microtiter plates using peracetic acid (PAA, 0.5%) for 15, 30, 60, and 120 s, and the inactivation of biofilms formed by those strains on stainless steel coupons using the same treatment times. In the microtiter plates, PAA removed all *S. aureus* at 15 s compared with control (no PAA treatment). However, *L. monocytogenes* biofilm was not affected by any PAA treatment. On the stainless steel surface, epifluorescence microscopy using LIVE/DEAD staining (BacLight, Molecular Probes/Thermo Fisher Scientific, Eugene, OR) showed that all strains were damaged within 15 s, with almost 100% of cells inactivated after 30 s. Results of this trial indicate that, although PAA was able to inactivate both *S. aureus* and *L. monocytogenes* monospecies biofilms on stainless steel, it was only able to remove adherent cells of *S. aureus* from polystyrene microplates. The correct use of PAA is critical for eliminating biofilms formed by *S. aureus* strains found in dairy plants, although further studies are necessary to determine the optimal PAA treatment for removing biofilms of *L. monocytogenes*.

Key words: *Staphylococcus aureus*, *Listeria monocytogenes*, biofilm, peracetic acid

INTRODUCTION

In natural and manufactured ecosystems, bacteria cells have a tendency to live attached to surfaces and to form a complex structure called a biofilm (Abdallah et al., 2014). Once biofilms are formed on a contact surface, they become quite resistant to antimicrobial

agents because of the slimy layer formed by bacteria (Martins and Germano, 2011). The persistence of biofilm on food contact surfaces and equipment may constitute a continuous source of contamination (Abdallah et al., 2014). Moreover, several reports have already shown the ability of bacteria species to produce biofilms on materials commonly used in the food industry, such as stainless steel, glass, rubber, and polystyrene (Simões et al., 2010). Bacterial cells in biofilms are considered difficult or even impossible to eradicate (Cos et al., 2010; Römling and Balsalobre, 2012). Additionally, biofilm formation on dairy equipment can lead to economic loss due the deterioration of food and equipment (Bremer et al., 2006).

Pathogenic microorganisms including *Listeria monocytogenes* and *Staphylococcus aureus* are of great concern in the food processing industry (Cappitelli et al., 2014) and have demonstrated high capacity to produce biofilms on surfaces, especially in the environment of dairy plants (Sasidharan et al., 2011). In the food processing industry, the development of biofilm will depend on the frequency of sanitation regimens (Cappitelli et al., 2014). Usually, the efficacy of disinfectants against biofilms increases with increasing biocide concentration and time of treatment (Surdeau et al., 2006; Belessi et al., 2011). However, the resistance of pathogens to sanitizers widely used in food industry can be one of the factors contributing to the involvement of specific microorganisms in foodborne outbreaks.

In dairy plants, one of most used sanitizers is peracetic acid (PAA), which has a wide spectrum of antimicrobial activity (Falsanisi et al., 2006) and is active at low temperatures (0–25°C; Ölmez and Kretzschmar, 2009). Also, PAA decomposes into safe and environmental friendly residues (acetic acid and hydrogen peroxide), and its efficacy is not affected by protein residues, hence it can be applied without risk of contaminating food with toxic residues (Souza et al., 2014). However, little information exists on the effectiveness of PAA on biofilms formed by foodborne pathogens, except for the study of Marques et al. (2007), who found that PAA

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had higher efficiency in removing adhered cells of *S. aureus* compared with hydrogen peroxide and sodium dichloroisocyanurate. The aim of this study was to investigate the effect of PAA treatment for different times on removal and inactivation of monospecies biofilms formed on microtiter plate and stainless steel coupons by 4 strains of *S. aureus* and 1 *L. monocytogenes* strain isolated from dairy plants, using a biofilm-forming index (BFI) according to Srey et al. (2014), and the visualization of biofilms by epifluorescence microscopy.

MATERIALS AND METHODS

Bacterial Strains

Four *S. aureus* strains (SA1, SA2, SA3, and SA4) and one *L. monocytogenes* (LM) strain were used in the study. The LM strain was isolated from brine in a dairy plant located in São Paulo State, Brazil (Barancelli et al., 2011). Strains SA1 and SA2 were isolated from bulk tank milk in São Paulo State, Brazil, as described by Lee et al. (2012). Strains SA3 and SA4 strains were isolated from brine and glove swabs collected in dairy plants located in São Paulo State. After 10-fold dilution of samples, 0.1 mL of each dilution was surface plated on Baird Parker agar (Oxoid, Basingstoke, UK) supplemented with egg-yolk emulsion (Oxoid) and incubated at 37°C for 24 to 48 h. Isolates were identified on the basis of culture characteristics (typical black, convex colonies, with or without light halo), Gram stain reaction, and the results of catalase and tube coagulase tests. Up to 3 characteristic colonies of putative *S. aureus* were purified on tryptone soy agar (Oxoid) with yeast extract and incubated at 35°C for 24 to 48 h. All isolates were kept in tryptone soy broth (TSB; Oxoid) with 15% glycerol, and stored at –80°C for further biofilm assays with PAA.

PAA Treatment of Biofilms on Microtiter Plates

First, one loopful of each bacterial strain was added to 5 mL of TSB (Oxoid) and incubated statically at 37°C for 24 h. After incubation, the culture broths were diluted until reaching 0.5 on the MacFarland scale ($\sim 10^8$ cells/mL). Biofilms were formed in a microtiter plate following the method described by Stepanović et al. (2003) with some modifications. Briefly, each bacterial strain was transferred to 3 wells of triplicate aliquots of 200 μ L of TSB in a 96-well, flat-bottomed plastic microplate and incubated at 37°C for 48 h. After incubation, the microplates were washed with sterile PBS (pH 7.2), fixed with methanol (Synth, Diadema,

Brazil), stained using crystal violet 0.1% (Synth) for 15 min, dried, and resolubilized with 33% (vol/vol) glacial acetic acid (Synth). The optical density (OD) of each well was measured at 570 nm using a microtiter plate reader (MultiSkan, Labsystems/Thermo, Waltham, MA). Triplicate negative controls with only sterile TSB were prepared as previously described for bacterial strains, and used as reference for determination of the ability of *S. aureus* and *L. monocytogenes* strains to produce biofilms. The mean OD value of negative controls (OD_{NC}) was 0.141 ± 0.02 . Additionally, the strains were classified as weak ($OD_{NC} < OD \leq 2 \times OD_{NC}$), moderate ($2 \times OD_{NC} < OD \leq 4 \times OD_{NC}$), or strong ($OD > 4 \times OD_{NC}$) biofilm producers, according to Stepanović et al. (2003).

Experiments using BFI were performed in triplicate according to Srey et al. (2014) with some modifications. After a 48-h incubation, the OD value of the total bacteria in the microtiter plate was measured at a wavelength of 600 nm with a microtiter plate reader (MultiSkan, Labsystems/Thermo). The planktonic cells and medium were removed, and each well was rinsed 3 times with 250 μ L of PBS to remove the loosely attached cells. Then, 250 μ L of PAA acid (0.5%; Dinâmica, Diadema, Brazil) were added for different treatment times (15, 30, 60, and 120 s); instead of disinfectant, PBS was used for treating positive control (well with a bacterial biofilm not subjected to disinfectant challenge) and negative control (well with only TSB). The disinfectant was removed by pipetting immediately after each treatment time, and then 250 μ L of sodium thiosulfate (Chemco, Hortolandia, Brazil) was introduced into each well, including the control wells, for 5 min to stop the reaction. Finally, the wells were rinsed 3 times with 250 μ L of PBS. The biofilms were fixed with 250 μ L of methanol (Synth) for 15 min; after pouring out the methanol, the plates were dried in the inverted position for 30 min. Then, 250 μ L of crystal violet dye (0.1%, Synth) was used to stain biofilms and positive and negative control wells for 15 min and removed by pipetting. The plate was rinsed with distilled water until the washing water was dye free, and air-dried for at least 2 h. The bound dye was resolubilized in 95% ethanol (Synth) for 30 min and transferred into a new well plate. The OD of the dye solution was measured at 570 nm (OD_{570nm}).

The biofilm-removing efficacy of the different treatment times using PAA was compared using the BFI calculated with the following formula (Niu and Gilbert, 2004):

$$BFI = \frac{(OD_{570nm} - OD_{C570nm})}{(OD_{600nm} - OD_{C600nm})},$$

where OD_{570nm} is obtained from the disinfected (treated) or positive control (biofilm treated with PBS) wells after staining; OD_{C570nm} is obtained from negative control wells (TSB wells treated with PBS) after staining; OD_{600nm} is obtained from the disinfected or positive control wells; and OD_{C600nm} is obtained from negative control wells after 48-h biofilm formation.

PAA Treatment of Biofilms on Stainless Steel

The production of biofilms on stainless steel was performed according to LIVE/DEAD *BacLight* (Molecular Probes/Thermo Fisher Scientific, Eugene, OR) manufacturer's protocol, with modifications. A stainless steel coupon (1.0 × 1.0 cm) was placed at the bottom of a tube in an inclined position. Next, 2 mL of each TSB bacterial suspension (~10⁸ cells/mL, or 0.5 on MacFarland scale) was pipetted into each tube and incubated at 35°C for 48 h without stirring. After 24 h, an additional 2 mL of TSB was added to each tube. After incubation, the stainless steel coupons were removed from each tube with sterile forceps, rinsed with sterile NaCl (0.85%), and placed in a sterile tube. Immediately before treatment, 2% PAA was diluted to 0.5% with distilled sterile water. Treatment times chosen were 15, 30, 60, and 120 s. In each treatment time, 2 mL of PAA was added, and after each treatment time had elapsed, 6 mL of sodium thiosulfate was added and left for 5 min to stop the reaction. After treatment, the tubes were emptied, and the stainless steel coupons were removed from each tube with sterile forceps, placed on a glass slide, and allowed to dry at room temperature.

The viability of surface-bound bacteria was examined using kit L7007 LIVE/DEAD *BacLight*, which contains Syto-9 and propidium iodide dyes that are usually applied to suspended bacteria. Therefore, 30 µL of LIVE/DEAD *BacLight* solution was applied directly to the adhered bacteria on stainless steel. After 10 min of incubation, the effects of the PAA treatments on biofilms on stainless steel coupons were examined using an epifluorescence microscope (Nikon Eclipse N1-U 80i with camera software NIS Elements AR 4.13.01 64 bit, 4.0; Nikon, Tokyo, Japan). All treatments were conducted in duplicate, and results were compared using the negative control (biofilm formation without any treatment of each bacterium on stainless steel). Syto-9 is a green fluorescent (maximum emission 498 nm) nucleic acid stain that is able to penetrate both healthy and damaged bacterial cells. In contrast, propidium iodide has a maximum emission of 617 nm and is unable to penetrate healthy bacteria (Karkashan et al., 2015). Therefore, viable cells with intact cell walls allow uptake of only the Syto-9 dye and display green fluorescence, whereas

nonviable cells (with damaged cell walls permitting the penetration of propidium iodide) exhibit red fluorescence (Alkawareek et al., 2014).

Statistical Analysis

All treatments were conducted in independent trials, each having duplicate (stainless steel assays) or triplicate (polystyrene microplates) samples. The BFI values were analyzed by one-way ANOVA using SAS software (SAS Institute, 2002). The means for treatments showing significant differences were compared using the Tukey test. Statistical significance was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the ability of the *S. aureus* and *L. monocytogenes* strains to produce biofilms in the polystyrene microplate assays. The 4 *S. aureus* strains and 1 *L. monocytogenes* strain were classified as moderate and weak biofilm producers, respectively, according to Stepanović et al. (2003). Biofilm production is recognized as an important virulence factor for bacteria of the genera *Staphylococcus* and *Listeria* (Bremer et al., 2006). Hence, formation of biofilms by those pathogens is of major concern for the dairy industry, which may be associated with failure to monitor the operational cleaning standards established for processing foods (Abdallah et al., 2014).

The BFI values for biofilms produced by the 5 bacterial strains in microtiter plates after challenging with PAA (0.5%) at different treatment times are presented in Table 2. Peracetic acid removed ($P < 0.05$) adhered cells of *S. aureus* but not *L. monocytogenes*. The *L. monocytogenes* strain used in our experiment was clas-

Table 1. Biofilm formation by *Staphylococcus aureus* and *Listeria monocytogenes* strains in the microplate assay

Strain	OD ¹	Category ²
Control (tryptone soy broth)	0.14 ± 0.02	—
<i>L. monocytogenes</i> strain	0.24 ± 0.01	Weak
<i>S. aureus</i> strains		
SA1	0.40 ± 0.01	Moderate
SA2	0.36 ± 0.01	Moderate
SA3	0.37 ± 0.01	Moderate
SA4	0.29 ± 0.01	Moderate

¹OD = optical density; values are expressed as mean ± standard deviation of 3 replicates of each strain.

²Classified by comparison of each strain's OD with the mean OD value of negative controls (OD_{NC} = 0.141 ± 0.02), according to Stepanović et al. (2003), as follows: weak (OD_{NC} < OD ≤ 2×OD_{NC}) or moderate (2×OD_{NC} < OD ≤ 4×OD_{NC}) biofilm producers.

sified as a weak biofilm producer, and the fact that it was not removed by PAA raises concern about the incidence of this pathogen in dairy plants (Välimaa et al., 2015). Importantly, the BFI method allows an estimation of the number of cells that attach to a surface, and therefore an indirect evaluation of the ability of the disinfectant to remove the biofilm (Srey et al., 2014). It does not provide information on the biological state of the cells.

Our results for *S. aureus* biofilms agree with those described by Marques et al. (2007), who found that, compared with hydrogen peroxide and sodium dichloroisocyanurate, PAA was the most efficient in removing adhered cells of *S. aureus*. Those authors obtained 5.26 and 4.5 decimal reductions for adhered cells on stainless steel and glass surfaces, respectively. In the present study, we detected no significant difference ($P > 0.05$) between the BFI for the different treatment times, except for 2 *S. aureus* strains (SA2 and SA3). For SA1 and SA4, 15 s of treatment with PAA was sufficient to remove the cells, leading to 40 to 90% reduction of the bacterial load in the microtiter plates. For SA2 and SA3, lower BFI values ($P < 0.05$) were observed after treatment times of 30 and 60 s, respectively. However, those differences in BFI values were not consistent with the reduction values obtained for SA2 and SA3 at 120 s, which did not differ ($P > 0.05$) from those at other treatment times.

On stainless steel biofilms, all bacterial strains were damaged by PAA (0.5%) after 15 s, with almost 100% of cells damaged after 30 s. Figure 1 presents photomicrographs of the LM and SA1 strains after different treatment times with PAA. Results for SA2, SA3, and SA4 were similar to those for SA1. Previous works used crystal violet staining as a colorimetric method to measure biofilm formation by means of the biomass of viable or possibly lysed bacteria adherent to a polystyrene surface (Niu and Gilbert, 2004), as described for the assays with microtiter plate. However, for biofilms prepared on stainless steel, we used the LIVE/DEAD BacLight method to evaluate biofilm formation and the effects of PAA because this staining method does not interfere with cellular viability (Stocks, 2004).

Arias-Moliz et al. (2015) observed that the effect of PAA on *Enterococcus faecalis* biofilms was lower than that of sodium hypochlorite (NaClO). Those authors postulated that, although PAA was able to diffuse inside the biofilm clusters, its lower antimicrobial effect compared with that of NaClO could be explained by the resistance of *Enterococcus faecalis* to PAA oxidative stress. In contrast, the 5 bacterial strains tested in the present study were damaged by PAA after 15 s, with almost 100% of cells damaged after 30 s (*L. monocytogenes*) or 60 s (*S. aureus*). After contaminating stainless

Table 2. Biofilm-forming index (BFI) of 5 bacterial strains (4 *Staphylococcus aureus* strains, SA1 to SA4, and 1 *Listeria monocytogenes* strain, LM) in microtiter plates after challenging with peracetic acid (0.5%) for different times

Time (s)	SA1			SA2			SA3			SA4			LM		
	BFI	Reduction		BFI	Reduction		BFI	Reduction		BFI	Reduction		BFI	Reduction	
0	0.41 ± 0.11 ^a	—		0.43 ± 0.12 ^a	—		0.33 ± 0.13 ^a	—		0.20 ± 0.09 ^a	—		0.12 ± 0.03 ^a	—	
15	0.16 ± 0.03 ^b	0.25 ± 0.06		0.23 ± 0.05 ^{bc}	0.20 ± 0.06		0.16 ± 0.03 ^b	0.17 ± 0.05		0.07 ± 0.01 ^b	0.13 ± 0.01		0.10 ± 0.01 ^a	0.01 ± 0.01	
30	0.18 ± 0.06 ^b	0.26 ± 0.06		0.17 ± 0.04 ^c	0.26 ± 0.04		0.11 ± 0.02 ^{bc}	0.22 ± 0.02		0.03 ± 0.02 ^b	0.17 ± 0.02		0.07 ± 0.03 ^a	0.05 ± 0.01	
60	0.20 ± 0.09 ^b	0.21 ± 0.09		0.28 ± 0.05 ^b	0.15 ± 0.06		0.06 ± 0.02 ^c	0.27 ± 0.03		0.05 ± 0.01 ^b	0.15 ± 0.01		0.06 ± 0.09 ^a	0.06 ± 0.09	
120	0.14 ± 0.07 ^b	0.27 ± 0.07		0.19 ± 0.08 ^{bc}	0.24 ± 0.11		0.07 ± 0.02 ^{bc}	0.26 ± 0.03		0.02 ± 0.01 ^b	0.18 ± 0.01		0.10 ± 0.04 ^a	0.02 ± 0.05	

^{a-c}Mean ± standard deviation values denoted by different superscript letters in the same column are significantly different ($P < 0.05$).

Figure 1. Photomicrographs of biofilms formed by *Staphylococcus aureus* SA1 (A) and *Listeria monocytogenes* (B) strains stained with LIVE/DEAD BacLight (Molecular Probes/Thermo Fisher Scientific, Eugene, OR) after treatment with peracetic acid (0.5%) for 15, 30, 60, and 120 s. Damaged and normal cells are dark (red) and bright (green), respectively. Magnification: 1,000 $\times$; bar = 10 μ m. Color version available online.

steel with *S. aureus*, *Escherichia coli*, and *Candida albicans*, Costa et al. (2015) immersed the specimens in 2% and 0.25% PAA for 30 min, and both concentrations significantly eliminated the microorganisms, a result that corroborates the findings of the present study. Moreover, *S. aureus* and *L. monocytogenes* are gram-positive bacteria with a permeable cell wall (Schäffer and Messner, 2005), which is not considered a barrier to the entrance of antibacterial compounds through the cytoplasmic membrane (Júnior et al., 2014).

The hygiene of the dairy environment is a crucial aspect to be monitored by dairy processors (Cusato et al., 2014). Our findings emphasize the importance of strictly following sanitation procedures, particularly the correct use of PAA for eliminating biofilms formed by *S. aureus*. However, the fact that PAA was not effective in removing adherent cells of *L. monocytogenes* on polystyrene microplates raises concern about its effectiveness against biofilms formed by this pathogen, because the PAA concentration (0.5%) used in our study was in the normal range used by dairy processors.

CONCLUSIONS

Although 0.5% PAA was able to inactivate *S. aureus* and *L. monocytogenes* monospecies biofilms on stainless steel, it was able to remove only adherent cells of *S. aureus* on polystyrene microplates. Further studies are necessary to determine the ideal PAA treatment for removing biofilms of *L. monocytogenes*.

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