J. Dairy Sci. 99:1–7
http://dx.doi.org/10.3168/jds.2015-10007
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Effect of peracetic acid on biofilms formed by *Staphylococcus aureus* and *Listeria monocytogenes* isolated from dairy plants

S. H. I. Lee,* L. P. Cappato,† C. H. Corassin,* A. G. Cruz,‡ and C. A. F. Oliveira*[1]
*University of São Paulo, School of Animal Science and Food Engineering, Department of Food Engineering, Av. Duque de Caxias Norte, 225, CEP 13635-900 Pirassununga, SP, Brazil
†Federal University Rural of Rio de Janeiro (UFRRJ), Food Technology Department, CEP 23890-000, Seropédica, RJ, Brazil
‡Federal Institute of Rio de Janeiro (IFRJ), Food Department, CEP 20270-021, Rio de Janeiro, RJ, Brazil

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 (*Bac*Light, 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

Received June 24, 2015.
Accepted November 6, 2015.
[1]Corresponding author: carlosaf@usp.br

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study of Marques et al. (2007), who found that PAA 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 μL 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 ($\mathbf{OD_{NC}}$) 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 μL of PBS to remove the loosely attached cells. Then, 250 μL 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 μL 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 μL of PBS. The biofilms were fixed with 250 μL of methanol (Synth) for 15 min; after pouring out the methanol, the plates were dried in the inverted position for 30 min. Then, 250 μL 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):

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

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