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Research Article

Individual and Combined Effects of Alpha-amylase and Biocides on Biofilms Formed by *Staphylococcus aureus* Strains Isolated from Brazilian Dairy Farms

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Abstract

This work aimed at evaluating the effect of alpha-amylase (AA, 100 mg/mL), sodium hypochlorite (SH, 0.5%), peracetic acid (PAA, 0.3%), and enzyme-biocide combinations (SH or PAA and AA) on mono-species biofilms formed by four *Staphylococcus aureus* strains (P01F2T1, P01F5T2, P16 and P24) previously isolated from dairy farms in São Paulo state, Brazil. Biofilm formation index (BFI) and culturable cell counts were evaluated for biofilms formed on polystyrene microplates for 72 h at 25ºC. The BFI of isolates P16 and P24 significantly decreased ($P < 0.05$) after treatment with PAA or SH combined with AA, compared with the biocides alone. The biofilms formed by isolates P01F2T1 and P01F5T2 had lower BFI values ($P < 0.05$) after treatment with combinations of PAA + AA and SH + AA, respectively. However, significant reductions in the biofilm culturable counts were observed only for isolates P01F2T1 treated with SH + AA and PAA + AA, P01F5T2 treated with SH + AA, and P24 treated with PAA + AA. Further studies are required to define the best combinations of AA and SH or PAA to completely remove *S. aureus* biofilms formed on plastic surfaces in processing dairy environments.

Keywords: *S. aureus;* Bacterial Adhesion; Peracetic Acid; Sodium Hypochlorite; Alpha-amylase

Abbreviations

AA: Alpha-amylase; BFI: Biofilm Formation Index; BHI: Brain Heart Infusion Broth; CFU: Colony-forming Unit; CIP: Cleaning in Place; EPS: Extracellular Polymeric Substances; *L. monocytogenes: Listeria monocytogenes;* nm: Nanometer; OD: Optical Density; PAA: Peracetic Acid; PBS: Phosphate Buffered Saline; *P. aeruginosa: Pseudomonas aeruginosa;* SH: Sodium Hypochlorite; *S. aureus: Staphylococcus aureus;* **µL**: Microliter.

Introduction

Staphylococcus aureus is a spherical-shaped, coagulase and catalase-positive bacterium with facultative anaerobic behavior [1]. *S. aureus* has been recognized as one of the main bacterial agents that cause foodborne diseases, due to the ingestion of its enterotoxins produced during growth on foods [2]. The disease caused by *S. aureus* enterotoxins are characterized by unpleasant symptoms such as nausea, vomiting, colic and diarrhea [3]. In dairy production systems, *S. aureus* is among the most important microrganims that require attention [4]. The contamination of milk may occur in dairy farms, from animals infected with clinical or subclinical mastitis [5], and at the industrial level during processing in dairy plants. In both dairy farms and dairy plants, the surfaces of equipment, utensils and other milk-contact surfaces such hands are considered the major sources of contamination of milk and dairy products [6].

Several bacterial species including *S. aureus* are able to adhere to surfaces to guarantee the survival in an inhospitable environment, forming complex communities that are called biofilms [7]. Biofilms are defined as communities of sessile microbial life, which adhere to solid supports and produce extracellular polymeric substances (EPS) [8] that may contain polysaccharides, proteins, phospholipids and extracelular DNA (eDNA) [9]. After the initial adhesion on surfaces, the formation of mature biofilms occurs from three to six days, through an increase in the population density and the EPS content of biofilm [10]. This process increases the thickness of the adhered biofilm, thus providing a potential source of contamination by foodborne pathogens during food processing [11].

The control or eradication of biofilms in the food industry has been explored with the application of biocides such as sodium hypochlorite (SH) and peracetic acid (PAA) [12-14]. SH is a common biocide used in dairy farms, since it has a quick action, easy application and effective microbiological control [15]. In addition, SH also has an anti-microbial potential against *S. aureus* biofilms [16]. PAA is a broad-spectrum biocide that is widely used in dairy industries because it does not produce toxic waste and is considered environmentally friendly, when compared to other biocides [17]. However, microorganisms adhered to surfaces after biofilm formation are more resistant to biocides, such as SH and PAA, than non-adherent microorganisms [17,18].

Enzymes are often used as complementary cleaning agents for biocides [19] being natural catalysts capable of accelerating chemical reactions [20]. Alpha-amylase (AA, α-1,4-glucan-4-glucan-hydrolase) is an enzyme found in humans, plants and microorganisms, being able to catalyze the hydrolysis reactions in the α-1,4-glicosidic bonds in starch to the production of glucose and maltose [21], having action against *S. aureus* biofilms in inhibiting its formation [22]. Fleming., *et al.* [23] observed that the use of AA and cellulase solution against biofilms from *S. aureus* and *P. aeruginosa* significantly reduced the biomass of biofilms. In this context, it can be hypothesized that enzyme-biocide combinations may increase the effectiveness in removing the biofilms formed by pathogenic bacteria on surfaces. Thus, the present study aimed to evaluate the effect of SH, PAA, AA and enzyme-biocide combinations (SH or PAA and AA) on mono-species biofilms produced in polystyrene microplates by *S. aureus* strains previously isolated from dairy farms in São Paulo state, Brazil.

Material and Methods

Staphylococcus aureus Isolates

Four strains of *S. aureus* (P01F2T1, P01F5T2, P16 and P24) previously isolated and classified as strong biofilm producers by Lee., *et al.* [18] were used in this work. The isolates P01F2T1 and P01F5T2 were obtained from milk tanks of a dairy farm located in the area of Franca, state of São Paulo, while strains P16 and P24 were isolated from cow's milk and milking system respectively, both from farms located in Pirassununga, state of São Paulo, Brazil [18]. All strains were preserved in Trypticase Soy Broth (TSB, Oxoid) with 15% glycerol (v/v) and stored at -80 ºC until biofilm formation and subsequent treatments.

Effect of Biocides and Alpha-Amylase on Biofilms Formed on Polystyrene Microplates

Each strain of *S. aureus* was inoculated in 5 mL of Brain Heart Infusion broth (BHI, Oxoid) and incubated at 37 ºC for 24 h. Subsequently, the culture was adjusted on a McFarland scale to 0.5 (10^8 cells/mL) using uncultured BHI broth as blank. The production of biofilms followed the methodology described by Stepanovíc., *et al.* [24]. The bacterial isolates were transferred to 96-wells polystyrene microplates in triplicate volumes of 200 µL for each well, and incubated at 25 ºC for 72 h. After the incubation period, the optical density (OD) of the total bacteria in the microtiter plate was measured at 600 nm (OD_{600nm}) in an microplate reader (Labsystems, MultiSkan, USA). The broth and weakly adhered planktonic cells were removed, and each well was rinsed three times with 200 **µ**L of phosphate buffer saline (PBS) (pH 7.2). Next, 200 **µ**L of SH (Dinâmica, Diadema, Brazil) at 0.5% (v/v), or PAA (Dinâmica, Diadema, Brazil) at 0.3% (v/v), or AA (Sigma-Aldrich, Saint Luis, MO) at 100 mg/mL were transferred to the wells and kept for 15 min for the removal of biofilms. The combined treatment of biocide-enzyme (SH and AA, PAA and AA) were carried out with the introduction of AA (200 **µ**L) in the wells for 5 min, then discharging the enzyme and finally treating with 200 **µ**L of SH or PAA solution for 10 min. After treatment, the wells were washed again with 200 **µ**L of PBS to insert methanol (200 **µ**L) for 15 min. The solvent was discharde and the wells were dried at room temperature. Two-hundred mL of violet crystal solution (0.1%, m/v) was inserted in each well for staining the adhered biofilms for 15 min, followed by three subsequent washes with sterile distilled water. The microplates were dryed at room temperature for 15 min, and the stained

biofilms adhered to the wells were re-solubilized by adding 200 µL of glacial acetic acid. After 15 min. incubation, the volumes were transferred to a new microplate and read in a microplate reader (Labsystems, MultiSkan, USA) at 570 nm (OD_{570nm}). The calculation of the biofilm formation index (BFI) was performed using the formula of Niu and Gilbert [25]:

Where OD_{570nm} is obtained from disinfected (treated) or positive control wells (biofilm treated with PBS) after coloring; OD_{C570nm} is obtained from negative control wells (treated with PBS) after coloring; OD_{600nm} is obtained from the disinfected or positive control wells; and OD_{C600nm} is obtained from negative control wells after 72 h of biofilm formation.

The efficiency of SH, PAA and AA and enzyme-biocide treatment was also evaluated by culturable cell counts in each well of the polystyrene microplates, following the methodology described by Srey., *et al.* [26]. Each *S. aureus* isolate in BHI broth was transferred in 200 **µ**L triplicates to 96-well polystyrene microplates and incubated for 72 h. After this period, all planktonic cells and the broth were removed, and the wells were washed three times with 200 **µ**L of PBS (pH 7.2). Each well was treated with the addition of 200 **µ**L of SH (0.5%, v/v), PAA (0.3%, v/v) and AA (100 mg/mL) for 15 min, while the combined treatments SH and AA, PAA and AA were performed with the insertion of AA in the wells for 5 min, followed by treatment with SH or PAA (200 **µ**L) for 10 min. After the contact times with biocides, enzyme, or enzyme-biocide, the wells were emptied and soon thereafter, a sterile cotton swab was pressed at the bottom of the well and rotated 50 times clockwise and another 50 times counterclockwise. The swabs were placed in test tubes containing sterile PBS and left to rest for 5 min, with each tube being shaken for 30 s. Then, the contents of the tubes were subjected to serial dilutions to inoculate on Baird-Parker agar (Merck KGaA, Darmstadt, Germany) supplemented with egg yolk emulsion and tellurite (Oxid), about 0.1 mL of each dilution was incubated at 37 ºC for 48 h before counting. The results were expressed as colony forming units per well (CFU/well).

Statistical analysis

The BFI values and colony counts obtained in the tests on polystyrene microplates with SH, PAA, AA, SH + AA, and PAA + AA were analyzed by unilateral analysis of variance [27]. The means of the treatments that showed significant differences were compared using the Tukey test at 5% probability ($P < 0.05$).

Results and Discussion

Table 1 presents the BFI values and their respective percentage reductions of biofilm-producing *S. aureus* isolates on polystyrene microplates after treatment with SH (0.5%), PAA (0.3%) and AA (100 mg/mL) and combinations of enzyme-biocides (SH + AA, PAA + AA). Compared with controls, biofilms formed by all *S. aureus* isolates had lower ($P < 0.05$) BFI, with values of 0.33 ± 0.05 to 0.48 ± 0.09 and percent reductions varying from 31.7 ± 5.2 to 52.9 ± 3.4%.

No differences ($P > 0.05$) were observed between the BFI values or percent reductions of biofilms produced by the four isolates of *S. aureus* treated with individual SH and PAA, except for isolate P24, which BFI for SH was higher than PAA. These results are consistent with those reported by Lee., *et al*. [18], who described that the use of PAA in the concentration of 0.5% efficiently removed the adhered cells of *S. aureus* from plastic materials. In another study, Chino., *et al*. [28] reported that PAA at 0.3% was efficient against *S. aureus* strains, although the authors observed that *S. aureus* biofilms were resistant to treatment with SH at a concentration of 0.1%.

In our study, the treatment with AA only had BFI values similar to controls ($P > 0.05$) and mild reductions (up to 10.0 ± 1.2%) in biofilms, when compared to the other individual treatments (SH and PAA). However, the combination of AA with SH or PAA resulted in higher reductions ($P < 0.05$) of BFI for biofilms formed by isolates P16 and P24, which values were 68.3 ± 6.6 and 66.3 ± 9.0%, respectively. The highest ($P < 0.05$) BFI reduction (79.8 ± 8.4%) was achieved with the combination of PAA and AA on biofilms formed by the isolate P01F2T1, while isolate P01F5T2 had greater BFI reduction ($P < 0.05$) after treatment with SH and AA (70.7 ± 9.1%). Importantly, no treatment was able to completely reduce the BFI of biofilms formed by the *S. aureus* isolates evaluated in this work.

S. aureus biofilms have greater resistance to the sanitization process, resulting in less inhibition and removal on surfaces such as stainless steel and glass [29], but especially in polystyrene and polypropylene [30]. Souza., *et al.* [31] observed that PAA (30 mg/L) and SH (250 mg/L) were not efficient in removing adhered *S. aureus* cells on polypropylene and stainless steel surfaces incubated

Treatment	Isolate P01F2T1		Isolate P01F5T2		Isolate P16		Isolate P24	
	BFI[1]	Reduction[2] (%)	BFI[1]	Reduction[2] (%)	BFI[1]	Reduction[2] (%)	BFI[1]	Reduction[2] (%)
Control	0.70 ± 0.09 [a]	-	0.65 ± 0.02 [a]	-	0.63 ± 0.04 [a]	-	0.70 ± 0.01 [a]	-
SH	0.35 ± 0.06 [b]	50.4 ± 5.5 [b]	0.39 ± 0.12 [b]	44.8 ± 6.1 [c]	0.33 ± 0.17 [b]	52.4 ± 9.6 [b]	0.48 ± 0.09 [b]	31.7 ± 5.2 [b]
PAA	0.37 ± 0.06 [b]	46.7 ± 4.7 [b]	0.37 ± 0.16 [b]	46.5 ± 7.3 [c]	0.33 ± 0.05 [b]	52.9 ± 3.4 [b]	0.39 ± 0.05 [c]	44.4 ± 5.4 [b]
AA	0.70 ± 0.09 [a]	0	0.63 ± 0.03 [a]	10.0 ± 1.2 [d]	0.73 ± 0.14 [a]	0	0.68 ± 0.02 [a]	3.1 ± 0.1 [c]
SH + AA	0.35 ± 0.07 [b]	50.5 ± 4.9 [b]	0.21 ± 0.13 [c]	70.7 ± 9.1 [a]	0.24 ± 0.12[bc]	65.9 ± 8.5 [a]	0.31 ± 0.14 [bc]	55.1 ± 8.9 [a]
PAA + AA	0.14 ± 0.08 [c]	79.8 ± 8.4 [a]	0.31 ± 0.08 [bc]	55.9 ± 3.4 [b]	0.22 ± 0.05 [c]	68.3 ± 6.6 [a]	0.24 ± 0.11 [c]	66.3 ± 9.0 [a]

Table 1: Biofilm formation index (BFI) and percentage reduction of monospecies biofilms formed by *Staphylococcus aureus* isolates on polystyrene microplates, after treatment with sodium hypochlorite (SH, 0.5%, v/v), peracetic acid (PAA, 0.3%, v/v) and alpha-amylase (AA, 100 mg/mL), alone or in combination.

[1]Results are reported as mean ± standard deviation of triplicate assays.

[2]Percentage calculated in relation to the control (biofilm treated with phosphate buffer solution).

[a-d] In the same column, means followed by different letters differ significantly ($P < 0.05$).

for 72 h, conditions similar to the present study in the production of mature biofilms, where the biocides were not able to completely remove the adhered biofilms. In this context, the insertion of enzymes in combination with biocides offers an alternative for the removal of biofilms on surfaces, through mechanisms such as the degradation of the biofilm matrix components, which facilitates the inactivation and removal of cells during cleaning procedures [22,32].

The greater effect of combined treatments with enzyme and biocide compared with individual treatments on biofilms of *S. aureus* on polystyrene microplates cultivated for 72 h was confirmed by culturable cell count, as illustrated in table 2.

Individual SH or PAA treatments of all *S. aureus* isolates tested resulted in lower counts ($P<0.05$) of their respective planktonic cells (range: 2.00 ± 0.4 to 3.60 ± 0.4 CFU/well), when compared with the controls (range: 4.37 ± 0.6 to 5.74 ± 0.7 CFU/well). However, lower counts ($P < 0.05$) were observed for the combined treatments (SH + AA, PAA + AA). The counts for the treatment of combined SH and AA ranged from 1.95 ± 0.2 to 2.51 ± 0.7 log CFU/well, while the values for biofilms treated with PAA and AA varied from 1.30 ± 0.4 to 2.41 ± 0.4 log CFU/well. These results are coherent with the BFI obtained for the treated biofilms (Table 1), in which the PAA and AA treatment demonstrated higher reductions in biofilms. Araújo., *et al.* [33] also observed that the associated use of protease and cetyl trimethyl ammonium bromide (CTAB) against biofilms of *Pseudomonas fluorescens* increased the reduction in about 1.93 log CFU/cm^2, thus corroborating our findings on the use of enzymes as potential complementing agents to commercial biocides for reducing biofilms on surfaces.

Compared with controls, AA (100 mg/mL) alone had non-significant effects on the culturable cell counts of *S. aureus*, resulting in smaller reductions of biofilms varying from 2.41 ± 0.4 to 12.4 ± 2.0%. Accordingly, Mnif., *et al.* [34] found that in high concentrations (500 mg/mL) of amylases were not effective against *Macrococcus caseolyticus* biofilms. In contrast, Craigen., *et al.* [35] observed that the use of AA on biofilms of *S. aureus* formed during 18 h led to a reduction of 79%. In the present study, the mild effect of AA on mature biofilms after 72 h may be explained by the higher degree of biofilm formation, which provides greater protection for the biofilm and hamper its removal from surfaces due to the polymeric matrix composed by polysaccharides, eDNA and proteins [10,36]. Oliveira., *et al.* [37] observed that maturation of *L. monocytogenes* ATCC 19111 biofilms was completed 72 to 144 hours after the initial adhesion to surfaces, during which the growth of the cell population density resulted in and increased thickness of the biofilm. Consequently, mature biofilms are more resistant to individual SH or PAA treatments, when compared with planktonic microbial cells [17,18].

Treatment	Isolate P01F2T1		Isolate P01F5T2		Isolate P16		Isolate P24	
	Count (Log CFU/well)[1]	Reduction[2] (%)	Count (Log CFU/well)[1]	Reduction[2] (%)	Count (Log CFU/well)[1]	Reduction[2] (%)	Count (Log CFU/well)[1]	Reduction[2] (%)
Control	4.46 ± 0.3 [a]	-	4.37 ± 0.6 [a]	-	5.74 ± 0.7 [a]	-	4.99 ± 0.8 [a]	-
SH	2.70 ± 0.1 [b]	39.5 ± 2.0 [b]	3.60 ± 0.4 [b]	19.2 ± 1.8 [d]	2.00 ± 0.4 [b]	65.2 ± 1.3 [a]	2.32 ± 0.2 [c]	53.5 ± 4.8 [b]
PAA	2.45 ± 0.2 [b]	45.2 ± 2.4 [c]	2.65 ± 0.5 [bc]	40.6 ± 1.6 [c]	2.95 ± 0.6 [b]	48.6 ± 1.6 [c]	2.97 ± 0.1 [b]	40.45 ± 2.5 [c]
AA	4.00 ± 0.5 [a]	10.3 ± 0.7 [d]	4.36 ± 0.7 [a]	2.1 ± 0.2 [e]	5.03 ± 0.5 [a]	12.4 ± 2.0 [d]	4.52 ± 0.3 [a]	9.4 ± 1.0 [d]
SH + AA	2.00 ± 0.8 [bc]	55.2 ± 1.7 [a]	1.95 ± 0.2 [d]	56.2 ± 1.3 [a]	2.51 ± 0.7 [b]	56.3 ± 1.9 [b]	2.00 ± 0.6 [cd]	59.9 ± 6.7 [b]
PAA + AA	2.00 ± 0.2 [c]	55.2 ± 1.3 [a]	2.41 ± 0.3 [c]	45.9 ± 1.7 [b]	2.41 ± 0.4 [b]	56.4 ± 1.8 [b]	1.30 ± 0.4 [d]	73.9 ± 4.1 [a]

Table 2: Culturable cell counts and respective reductions of monospecies biofilms formed by *Staphylococcus aureus* isolates on polystyrene microplates, after treatment with sodium hypochlorite (SH, 0.5%, v/v), peracetic acid (PAA, 0.3%, v/v) and alpha-amylase (AA, 100 mg/mL), alone or in combination.

[1]Results are reported as mean ± standard deviation of triplicate assays.

[2]Percentage calculated in relation to the control (biofilm treated with phosphate buffer solution).

[a-d] In the same column, means followed by different letters differ significantly ($P<0.05$).

The formation and survival of *S. aureus* biofilms in dairy environments are directly related to the physical-chemical characteristics of milk, since compounds such as vitamins, minerals and calcium participate directly in the formation of residues on surfaces and pipes, contributing to microbial growth on the spot [38]. In this context, in order to prevent biofilms from reaching the mature stage, unit operations such as cleaning in place (CIP) are considered as the best control strategy in the food industry [16]. *S. aureus* cells have great capacity to form biofilms on polystyrene and stainless steel surfaces, due to crucial factors in microbial adhesion such as hydrophobicity on the surface [18]. This adhesion and subsequent biofilm formation provide the conditions for survival of the bacterial cells in the environment, also decreasing the efficacy of CIP procedures in the food processing environment [39]. In the present study, a partial reduction of mature biofilms of *S. aureus* isolates was observed after treatments with SH (0.5%) or PAA (0.3%). The fact that these commercial biocides widely used in food industries were not effective against *S. aureus* strains isolated from dairy farms warrants concern on their persistance in dairy industries. Although the combined enzyme-biocide treatments did not achieve complete reduction of *S. aureus* mature biofilms, the increased reduction provided by SH + AA or PAA + AA indicated a promising strategy that could be useful for cleaning equipment, utensils and other surfaces in dairy processing environments.

Conclusion

Mature biofilms formed on polystyrene plates by four *S. aureus* isolated from dairy farms were partially reduced after treatments with SH (0.5%, v/v) or PAA (0.3%, v/v), while AA (100 mg/mL) was not effective for biofilm reduction. However, higher reductions in the BFI and in biofilm culturable counts were observed in biofilms treated with SH+ AA or PAA + AA. Although any treatment studied was able to completely remove *S. aureus* biofilms on polystyrene, the biocide-enzyme combinations evaluated in this study offer a promising strategy to increase the efficiency of CIP procedures, especially when applied on surfaces that come into direct contact with food. Further studies on the enzyme-biocide combination on mature biofilms are required to define efficient treatments for the complete removal of pathogens such as *S. aureus*.

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Competing Interests

The authors declare that there is no conflict of interest.

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