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Antiobiofilm activity of commercial detergents and disinfectants in the removal of *Salmonella* Heidelberg biofilms

Atividade antibiofilme de detergentes e desinfetantes comerciais na remoção de biofilmes de *Salmonella* Heidelberg

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Salmonella Heidelberg is highly prevalent in poultry. This pathogen is associated with multidrug-resistant and biofilm-producing isolates, both of which are associated with increased persistence in food-processing facilities. Cleaning and disinfection processes are essential for guaranteeing hygienic and sanitary conditions. Several traditional compounds used in poultry facilities have shown decreased biofilm removal efficiency. This study aimed to evaluate the antibiofilm activity of four commercially available products (three detergents and one disinfectant) against *S.* Heidelberg biofilms formed on polypropylene surfaces. All *S.* Heidelberg strains produced biofilms on polypropylene surfaces at 28 °C and approximately 70% formed biofilms at 37 °C. The Biofilm and biomass production by *S.* Heidelberg was significantly lower ($p<0.05$) after using the alkaline detergent, than after the control and other treatments. There were no significant differences ($p>0.05$) between the other treatments. The alkaline detergent resulted in a reduction of more than 90% in the biofilm-forming strains at 28 °C.
Keywords: bacteria, biofilm production, poultry facilities.

Salmonella Heidelberg é altamente prevalente em aves. Este patógeno está associado a isolados multirresistentes e produtores de biofilme, ambos associados a uma maior persistência em instalações de processamento de alimentos. Os processos de limpeza e desinfecção são essenciais para garantir as condições higiênicas e sanitárias. Vários compostos tradicionais usados em instalações avícolas mostraram diminuição da eficiência de remoção de biofilme. Este estudo teve como objetivo avaliar a atividade antibiofilme de quatro produtos disponíveis comercialmente (três detergentes e um desinfetante) contra biofilmes de *S.* Heidelberg formados em superfícies de polipropileno. Todas as cepas de *S.* Heidelberg produziram biofilmes em superfícies de polipropileno a 28 °C e aproximadamente 70% formaram biofilmes a 37 °C. A produção de biofilme e biomassa por *S.* Heidelberg foi significativamente menor ($p<0,05$) após o uso do detergente alcalino, do que após o controle e outros tratamentos. Não houve diferenças significativas ($p>0,05$) entre os demais tratamentos. O detergente alcalino resultou em uma redução de mais de 90% nas cepas formadoras de biofilme a 28 °C.
Palavras-chave: bactéria, produção de biofilme, instalações avícolas.

1. INTRODUCTION

To ensure high quality chicken meat, control and prevention measures are essential to inhibit the main pathogens responsible for foodborne disease outbreaks in humans [1]. Salmonellosis is one of the main foodborne diseases worldwide, and poultry products are the main cause of outbreaks [2, 3]. Although it is not frequently isolated from human cases of salmonellosis, *Salmonella enterica* serotype Heidelberg is reported as highly prevalent in poultry, especially in North America and Brazil [4, 5]. Imported chicken meat from these regions is associated with infections in Europe [6].

Salmonella Heidelberg can multiply in macrophages and reach several organs via the bloodstream or lymphatic system [7]. Additionally, its higher invasive potential may cause more severe infections than other serotypes [8]. This pathogen is associated with multidrug-resistance and biofilm formation, which are both increasingly persistent in food-processing facilities [6, 9-14].

The attachment of pathogens to food surfaces increases their survival in hostile environments, such as poultry facilities [15, 16]. Rupturing of these structures can lead to the cross-contamination of consumer products. Thus, reducing foodborne pathogens before processing is important for mitigating cross-contamination [11]. Cleaning and disinfection processes such as removing the biofilms and preventing new biofilm formation are essential for guaranteeing hygienic and sanitary conditions of these environments [11].

The sanitizers generally used in poultry facilities are peroxygens, alkalis, acids, and quaternary ammonium compounds [17]. The choice of compounds to be used should consider their antimicrobial activity and their anti-biofilm capacity. Several traditional products have shown decreased biofilm removal efficiency due to their properties, including reduced diffusion, which promotes the extracellular polymeric matrix, enzyme-mediated resistance, physiological changes, and genetic adaptation [18, 19]. Several studies have attempted to develop a standardized system to prevent and remove biofilms but these structures are diverse and unique [18]. However, there are still many challenges to be understood regarding the formation of biofilms and the best strategy to remove them [20].

The aim of this study was to evaluate the antibiofilm activity of four commercially available products (three detergents and one disinfectant) against *S*. Heidelberg biofilms formed on polypropylene surfaces.

2. MATERIALS AND METHODS

2.1 *Salmonella* Heidelberg strains

In this study, 53 *S*. Heidelberg strains were selected. The strains were isolated using drag swabs from poultry farms in southern Brazil between 2018 and 2019. They were previously identified and serotyped by two private laboratories accredited by the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) (Brazil). The bacterial isolates were stored at –20 °C in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK), supplemented with 15% glycerin (Synth, Diadema, Brazil). The strains were reactivated in BHI for 18–24 h at 37 °C, then on xylose lysine deoxycholate (XLD) agar (Oxoid) for 18–24 h at 37 °C.

2.2 Inoculum preparation

Salmonella Heidelberg strains were reactivated in BHI, seeded on tryptone soy agar without glucose (TSA; Oxoid), then incubated for 24 h at 37 °C. One colony of each strain was inoculated into 5 mL of tryptone soy broth without glucose (TSB; Oxoid) and incubated for 24 h at 37 °C. McFarland standard No. 1 (Probac do Brasil, Brazil) was used as a reference to adjust the turbidity of the bacterial suspension in TSB to 3×10^8 CFU/mL. A spectrophotometer SP-22 (Biospectro, Brazil) was used to measure turbidity at 620 nm, which ranged from 0.224 to 0.300.

2.3 Biofilm formation assay

Biofilm formation was evaluated as described by Borges et al. (2018) [21], with some modifications. An aliquot (200 µL) of the inoculum of each strain was added in triplicate to each well of sterile 96-well flat-bottomed polystyrene plates (Kasvi, Curitiba, Brazil). The plates were incubated for 24, 48, and 72 h at 28 °C (the optimum temperature for the expression of extracellular matrix components in some *Salmonella* serotypes and the ideal temperature at

poultry farms for broilers) and 37 ºC (the optimum temperature for *Salmonella* growth). A biofilm-producing strain of *Salmonella* Enteritidis was selected from our stock collection as a positive control. Non-inoculated TSB was added to six wells as a negative control.

After incubation, the contents of the microtiter plate were removed and the wells were washed three times with 250 µL of sterile distilled water. The bacteria attached were fixed with 200 µL of methanol (Nuclear, Diadema, Brazil) for 15 min. The plates were then emptied, air-dried, and stained with 200 µL of 2% Hucker crystal violet for 15 min. The stain was removed, and the plates were washed and air-dried. A total of 200 µL of 33% glacial acetic acid (Êxodo Científica, Sumaré, Brazil) was added to each well. Optical density (OD) was measured at 550 nm using an ELx800 Absorbance Reader (Biotek, Winooski, VT, USA). The OD of each strain (ODs) was obtained using the arithmetic mean value of the three respective wells. The cut-off OD (ODnc) for the microtiter plate test was defined as three standard deviations above the mean OD of the negative control. Biofilm production was determined using two parameters: (1) non-biofilm producers (ODs ≤ ODnc) and (2) biofilm producers (ODnc < ODs). Biological and technical triplicates were performed, and strains that demonstrated adhesion capacity in the three assays were considered biofilm producers.

2.4 Biofilm removal with commercial sanitizers

For this assay, one disinfectant and three detergents that are commercially available and commonly used in the process of washing and disinfecting poultry environments were selected (Table 1). The concentration of the products adhered to the manufacturer's recommendations.

Table 1 – Treatment used for the biofilm removal assay: products, composition, and concentrations.

Treatment	Product	Composition	Concentration (%)
1	non-treated control	sodium chloride solution	0.9
2	chlorinated alkaline detergent	7% sodium hydroxide, oxidant, alkalizing, sequestering agent and vehicle	2
3	neutral detergent	dodecyl benzene sulfonic acid; anionic surfactant; stabilizer; opacifying; neutralizing; thickener; dye; preservative and vehicle	4
4	acid detergent	20% phosphoric acid, 2% hydroacetic acid	2
5	disinfectant	dimethyl ammonium didecyl chloride; vehicle; acidifiers and humectant	1

The technique described by Carvalho et al. (2022) [16] was used to evaluate biofilm removal. An aliquot (200 µL) of each bacterial suspension was inoculated in triplicate into each well of the sterile 96-well flat-bottomed polystyrene microplates, followed by incubation for 72 h at 28 °C. These conditions were selected for this assay because they presented the highest number of biofilm-forming isolates in the previous experiment. After incubation, the cell suspension was removed, and the microplates were washed with 0.9% sodium chloride solution. Next, 200 µL of each compound per well was inoculated at their respective concentrations and maintained for 30 (detergents) and 60 min (disinfectant) of contact, simulating the times commonly used during cleaning and disinfection processes in poultry facilities. A microplate treated with 0.9% sodium chloride solution was used as the non-treated control. After contact, the wells were washed with distilled water. Subsequently, the microplates were fixed with methanol, stained with 2% Hucker crystal violet, washed with water, air-dried, resuspended in 33% glacial acetic acid, and the OD measured at 550 nm in a spectrophotometer, as previously described.

2.5 Statistical analysis

All statistical analyses were performed using PASW Statistics software with a 5% significance level. Descriptive statistics were used to evaluate biofilm formation and removal. Fisher's exact test was used to compare biofilm formation between temperatures and incubation periods, and biofilm removal among products. The Bonferroni correction was applied to adjust the confidence intervals for multiple hypothesis testing. Comparisons of the mean OD according to the treatment was carried out using the non-parametric Mann–Whitney test.

3. RESULTS

3.1 Statistical analysis

The biofilm formation of *Salmonella* Heidelberg according to the temperature and incubation time is described in Table 2.

Table 2 – Relative (%) and absolute (n) frequencies of biofilm formation of Salmonella Heidelberg for 24, 48, and 72 h at 28 and 37 ºC.

Incubation time (h)	Biofilm production % (n=53)	
	Temperature (ºC)	
	28	37
24	88.7 (47)^{aA}	53.6 (30)^{bA}
48	75.5 (40)^{aA}	54.7 (29)^{bA}
72	100 (53)^{aB}	69.8 (37)^{bA}

Different lowercase letters in the same row indicate statistically significant differences ($p<0.05$) between the temperatures for the same incubation time. Different capital letters in the same column indicate statistically significant differences ($p<0.05$) among incubation times at the same temperature.

There was a significant difference ($p<0.05$) in the ability of *S.* Heidelberg to produce biofilms between the two temperatures (28 °C and 37 °C) evaluated during the three incubation periods. Biofilm production was higher at 28 °C, regardless of the incubation period. There was no significant difference ($p>0.05$) in the ability of *S.* Heidelberg to produce biofilms during the incubation period at 37 °C. However, at 28 °C, biofilm production was the highest after 72 h of incubation.

3.2 Biofilm removal

The biofilm production and OD obtained after treatment to remove biofilms at 72 h and 28 °C (conditions with the highest number of biofilm-forming isolates in the previous experiment) are described in Table 3.

Table 3 – Relative (%) and absolute (n) frequencies of biofilm production and optical density of Salmonella Heidelberg after 72 h at 28 ºC.

Treatment	**Product concentration (%)**	**Time of contact (min)**	**Biofilm production % (n=53)**	**Optical density**
Control	0.9	30	92.5 (49)^{a}	0.300 ± 0.163^{a}
alkaline detergent	2	30	7.5 (4)^{b}	0.104 ± 0.022^{b}
neutral detergent	4	30	98.1 (52)^{a}	0.316 ± 0.141^{a}
acid detergent	2	30	100 (53)^{a}	0.297 ± 0.120^{ac}
disinfectant	1	60	100 (53)^{a}	0.243 ± 0.129^{c}

Different lowercase letters in the same column indicate significant differences ($p<0.05$) among treatments and between treatments and the control.

4. DISCUSSION

Biofilm formation depends on the growth conditions, contact surfaces, serotypes, and intrinsic characteristics of each isolate [11, 21]. Thus, the biofilm-forming ability of *S*. Heidelberg isolates was investigated at two temperatures (28 °C and 37 °C) and three incubation times (24, 48, and 72 h). The conditions used for this study, such as incubation in TSB without glucose and polystyrene microplates, allowed for the detection of biofilm-producing isolates. TSB is a low nutrient availability broth that favors biofilm formation, and polystyrene mimics some plastics used in poultry production and food processing plants [21-23].

The influence of temperature on biofilm formation was observed in the analyzed isolates. A significantly higher number of isolates produced biofilms at 28 °C than at 37 °C, regardless of the incubation period. A reduction in the incubation temperature below 30 °C favors biofilm production because isolates can express the major components of the biofilm extracellular matrix (curli fimbriae and cellulose), which are not produced at higher temperatures. These components are usually produced between 25 °C and 28 °C [24-27]. Similarly, the incubation time significantly influenced biofilm production at 28 °C. After 72 h of incubation at 28 °C, all the isolates produced biofilms. Thus, these conditions were selected to test the biofilm removal ability of the commercially available products.

Salmonella Heidelberg is a foodborne pathogen that can persist for long periods in poultry production environments [28]. Thus, the use of compounds capable of interrupting the microbial contamination cycle is essential [29]. Despite presenting different mechanisms of action and purposes in poultry facilities, the antibiofilm activity of one disinfectant and three detergents was evaluated against preformed biofilms of *S*. Heidelberg. Detergents are used to remove soil from surfaces, while disinfectants are used to reduce bacterial loads on the surfaces. However, both can be used to remove biofilms [17].

Various methods have been used to evaluate the removal of preformed biofilms. Crystal violet staining of polystyrene plates is suggested for in vitro tests because it is a simple, fast, and reliable method [21, 30]. To determine the antimicrobial activity of the products, two criteria were used in this study: the classification of biofilm and non-biofilm producers, and the mean OD. The classification method does not consider the reduction in the number of adhered bacteria and only indicates the presence of a biofilm. In contrast, the mean OD considers the number of attached bacteria [31, 32]. These different criteria resulted in different outcomes. In the classification method, alkaline detergent was the only product that reduced the number of biofilm-producing isolates. When OD was considered, alkaline detergent presented the lowest bacterial load. However, disinfectants also reduced the OD.

These results may be explained by the biofilm and product compositions. Biofilms are multicellular complexes surrounded by an extracellular matrix composed mainly of exopolysaccharides. The major components of the extracellular matrix are curli fimbriae and cellulose [33].

Acid detergents act mainly on minerals, which are not present in large amounts in biofilms [34]. Although phosphoric and hydroxyacetic acids have antimicrobial activities, the concentrations of these compounds in detergents are insufficient for biofilm elimination. For example, the antimicrobial action of phosphoric acid requires concentrations above 30% [35]. Similarly, the compounds present in the neutral detergent (dodecyl benzene sulfonic acid: anionic surfactant) are mostly associated with the dissolution of surface tension [36]. The alkaline detergent is composed of sodium hydroxide (NaOH), which releases hydroxyl ions (OH-). These ions are associated with changes in cellular metabolism, phospholipid destruction, protein denaturation, enzymatic inactivation, and lipid and fatty acid degradation [17, 37, 38]. Furthermore, the pH of the solution plays an important role in the removal of biofilms using detergents. Previous studies have demonstrated that a higher pH is more efficient for biofilm removal [39, 40]. The disinfectant selected for this study was a quaternary ammonium compound (QAC)—a broad-spectrum biocide and cationic surfactant. QAC easily integrates into cell membranes, disrupting intermolecular interactions, and dissociating lipid bilayers [41]. This mechanism of action is likely to promote the detachment of bacterial cells from surfaces.

The Brazilian legislation requires tests with planktonic cells for the approval of sanitizers produced industrially and used according to the conditions indicated by the manufacturer. However, bacteria in biofilms can be up to 1,000 times more resistant to antimicrobials than planktonic cells [42]. Thus, the evaluation of sessile cells is essential for food production [43].

5. CONCLUSION

All *S.* Heidelberg strains produced biofilms on polypropylene surfaces at 28 °C. Approximately 70% produced biofilms at 37 °C. At 28 °C, the alkaline detergent resulted in a reduction of more than 90% in the biofilm-forming strains, unlike the other sanitizers which did not differ from the control group. To obtain an even better performance and result, it is recommended to evaluate the complete procedure. This includes cleaning with an alkaline detergent followed by a disinfectant.

Biofilm production by *S.* Heidelberg was significantly lower ($p<0.05$) after using the alkaline detergent than after using the control and the other treatments. In the other cases, there were no significant differences ($p>0.05$) between the treatment and control groups. The biomass was significantly lower ($p<0.05$) after the use of alkaline detergent than after using the control and the other treatments. A significantly lower biomass ($p<0.05$) was observed for the disinfectant than for the control and neutral detergents. In the other cases, there were no significant differences ($p>0.05$) between the treatments.

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