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Biofilm-producing ability and tolerance to industrial sanitizers in *Salmonella* spp. isolated from Brazilian poultry processing plants

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ABSTRACT

The aims of this study were to analyze the biofilm-producing ability of 98 strains isolated from different surface materials in poultry cutting rooms; to assess the presence of the most important to *Salmonella* biofilm formation genes *adrA* and *csgD* in these strains; and to evaluate the tolerance biofilms formed in polypropylene and polyurethane slides to sanitizers commonly used in the industry. Viable cells were removed from the slides soon after treatment with sanitizers, and then submitted to reincubation for a new count. Only one strain was a strong biofilm-producer in polystyrene; 70% of strains were weak, and 29% were moderate producers. Both genes were found in all strains. There were differences in adhesion to polypropylene and polyurethane, and scanning electron microscopy showed that polyurethane surface was more irregular. No viable cells were recovered in polypropylene slides treated with sanitizers; in polyurethane, reduction in viable cell counts soon after sanitizer treatment was enough to consider that sanitizers were efficient. On the other hand, treatment with peracetic acid was not considered efficient. Results of this study should be considered a food safety warning, due to the importance of the biofilm-producing ability both *in vitro* and in real poultry processing plants.

1. Introduction

Salmonella spp. is one of the most important foodborne pathogens worldwide (Nguyen, Yang, & Yuk, 2014). In Brazil, in spite of the underreporting of foodborne diseases, data of the Ministry of Health indicate that, in recent years, *Salmonella* was the most frequent agent identified in outbreaks of foodborne diseases (Brazil, 2014). Surfaces with *Salmonella* can serve as a source of food contamination by cross-contamination. Biofilm formation may allow *Salmonella* spp. to survive on surfaces and persist in food processing environments for long periods (Corcoran et al., 2013; Simões, Simões, & Vieira, 2010; Vestby, Møretrø, Langsrud, Heir, & Nesse, 2009). Besides, biofilms are related to increased tolerance to biocides (Lejeune, 2003), given the organization of bacterial cells inside the polymer matrix, which reduces the penetration of the biocide agent (Gilbert, Allison, & McBain, 2002).

Most sanitizers are efficient against *Salmonella* in suspension tests. However, sanitizer effect is weaker against adhered cells (Møretrø, Heir, Nesse, Vestby, & Langsrud, 2012). In order to be considered efficient, a sanitizer used in suspension has to reduce the bacterial population in 5 $\log_{10}$ (Riazi & Matthews, 2011). In cells adhered to a surface, Møretrø et al. (2009) observed that reduction

Abbreviations: CA, chlorinated alkaline detergent; PA, peracetic acid; PP, polypropylene; PU, polyurethane.

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should be no less than 4 $\log_{10}$. In Brazil, the current regulation to assess sanitizers (Brazil, 1993) considers efficiency only in relation to planktonic microorganisms, and not on biofilms.

The objectives of this study were to assess biofilm production in polystyrene microplates, polyurethane and polypropylene slides by strains of *Salmonella* spp. isolated from poultry processing plants; to evaluate the viability of bacterial cells in the biofilm after treatment with industrial sanitizers; and to study the effect of slide reincubation in increasing the recovery of viable cells that were injured by sanitizer treatment.

2. Material and methods

2.1. Salmonella isolation and identification

Salmonella strains were obtained from cutting rooms of poultry processing plants that slaughtered more than 160 thousand broilers/day. Isolation of *Salmonella* from the surface of polypropylene and polyurethane conveyor belts was carried out with sponges (Nasco Whirl-Pak™) pre-moistened with 10 mL of peptone saline (peptone 0.1%, NaCl 0.85%) on a 400-cm^2 area. *Salmonella* detection was carried out according to the USA Food and Drug Administration method, published in the Bacteriological Analytical Manual (Andrews & Hammack, 2007). After these tests, *Salmonella* spp. isolates were confirmed by genus identification by polymerase chain reaction (PCR) for the *sifB* gene according to the protocol by Almeida, Silva, and Nero (2014).

2.2. Biofilm production in polystyrene plates

For biofilm production in polystyrene plates, all the strains were diluted to 10^8 CFU/mL (0.5 in MacFarland scale) using Luria–Bertani broth (LB, Difco™). Aliquots of 200 μL of each strain were cultured in four wells of a polystyrene microplate with 96 flat-bottom wells (Nest®). Additionally, four positive controls (*Salmonella* Typhimurium ATCC 14028), and four negative controls (non-inoculated culture medium) were placed in each plate. Microplates were incubated for 96 h at 35 °C. After that, plates were washed three times with phosphate buffered saline (PBS, pH 7.2), dried at room temperature, and stained with crystal violet 1% for 15 min. Then, plates were washed three times with distilled water and dried at room temperature, to be read in a microplate reader (Babsystems, MultiSkan EX) at 540 nm. In order to evaluate absorbance results according to Stepanović et al. (2000), mean optical density (OD) of four wells of each sample was compared with the mean absorbance of negative controls. Strains were then classified as non-adherent, weak adherent, moderate adherent, and strong adherent.

2.3. Biofilm production in polyurethane and polypropylene

As polyurethane (PU) (PosiClean®) and polypropylene (PP) (Tecnoplástico Belfano®) are the materials that made up the conveyor belts where the strains were isolated, they were chosen to be used in the biofilm production assay. PU (1 × 1 × 0.2 cm) and PP (1 × 1 × 0.1 cm) slides were cut, washed, and sterilized in autoclave in flasks with 10 mL of LB broth (Difco™). Three strains were selected for this procedure, one weak adherent, one moderate adherent, and one strong adherent. Fifteen mL of LB broth with 10^8 CFU/mL (0.5 in MacFarland scale) were added to the flasks containing the sterile slides. For biofilm production, flasks were kept for 96 h at 37 °C under stirring at 100 rpm in an Orbital Shaker (BIOSAN®). A non-inoculated flask with sterile slides was incubated in the same conditions as a negative control.

2.4. Sanitizer treatment

After biofilms were formed in PU and PP, slides were transferred to a polystyrene plate with 24 wells (NEST®) and washed with PBS to remove planktonic cells. Slides were treated with sanitizers as follows:

- CA treatment: Chlorinated alkaline detergent Sanifoam® (Sodium hypochlorite 5–10%; Sodium hydroxide more than 5%; Dimethyl cocamine oxide 1–5%) (A&B Bioquímica Latino Americana S/A) at 4%;
- PA treatment: Peracetic acid (PubChem CID: 6585), Peracid® (A&B Bioquímica Latino Americana S/A), 0.2%;
- CA+PA treatment: Initial use of Sanifoam® (A&B Bioquímica Latino Americana S/A) at 4%, followed by rinsing and treatment with Peracid® (A&B Bioquímica Latino Americana S/A) 0.2%.

Contact times analyzed were 5, 10, and 15 min. PU slides were also kept in contact with the sanitizers for 30 min. Sanitizer concentrations were based on the manufacturer's recommendations. Each plate was made in duplicate, one for bacterial recovery on the day of the treatment, and the other to be reincubated for 96 h (added to the initial time, full 192 h) at 37 °C after addition of 1 mL of LB broth (Difco™). Each plate had six control wells, three negative ones non-inoculated (one per treatment) and three positive ones inoculated (one per strain).

2.5. Viable microorganism counts

Removal of viable cells from the slides was based on the methodology adapted from Nguyen and Yuk (2013). Both untreated (positive control) and treated slides were transferred to test tubes (180 × 20 mm) containing 5 mL of saline solution and 20 to 25 sterile glass beads (0.4–0.5 mm in diameter). Tubes were kept in a vortex for 3 min in order to remove adherent *Salmonella* cells. After vortexing, 100 μL of the tubes with the slides treated with sanitizers and controls were cultured in TSA (Difco™) spread plates. The same method was used for slides incubated for extra 96 h.

After vortexing, 100 μL of each tube were also transferred to a 96-well polystyrene plates (NEST®) for later colorimetric assay with 50 μL XTT sodium salt − ≥90% (Sigma–Aldrich®) (PubChem CID: 14195569) at 5 mg/mL, and 4 μL Menadione (Sigma–Aldrich®) (PubChem CID: 4055) 1 mM incubated under stirring at 70 rpm in an orbital shaker (BIOSAN®) for 4 h at 35 °C. Dilution of XTT and Menadione were carried out according to Chandra, Mukherjee, and Ghannoum (2008); reading was carried out in a Polaris (Celer®) microplate reader at 492 nm.

2.6. PCR assay

For duplex detection of the *csgD* and *adrA* genes, polymerase chain reaction amplifications were performed in a final volume of 25 μL, as follows: 2.5 μL buffer 10X, 2.5 mM magnesium chloride, 200 mM each dNTP (Ludwig Biotec), 1.25 U Taq DNA polymerase (Ludwig Biotec), 10 pmol each primer, ultrapure distilled water qsp (Invitrogen™), and 3 μL DNA. PCR was carried out in Veriti 384-well Thermal Cycler (Applied Biosystems). Bacterial DNA was extracted by boiling. Cycles were as follows: initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94°C/30 s, 60°C/30 s, and 72°C/30 s. Final extension was carried out at 72°C/4 min. Ultrapure distilled water was used as the negative control, and reference strain *Salmonella* Typhimurium ATCC 14028 was the positive control. Primers for genes *csgD* and *adrA* were designed by Oliveira et al. (2014).

PCR products were visualized in an electrophoresis chamber