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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

(Electrophoresis Power Supply Model EPD 600 – Amersham-Pharmacia Biotech Inc.) with agarose gel (2% Ludwig Biotec), tris-borate-EDTA buffer (TBE), stained with Sybr Safe DNA Gel (Invitrogen™). DNA fragments were compared with 50-bp molecular weight markers (Ludwig Biotec), and images were captured by an image analyzer (AlphaImager®).

2.7. Scanning electron microscopy

Scanning electron microscopy was carried out in slides of each of the materials used in biofilm production (PP and PU) without inoculation, and in the biofilm formed in polystyrene by a strong adherent strain.

Slides were fixated in glutaraldehyde 3% diluted in PBS (pH 7.2) and sent to the Electron Microscopy Center at the Biological Sciences Sector of UFPR – Curitiba. Topographic characterization of the surfaces was carried out in a scanning electron microscope model VEGA 3 (Tescan®) at 15 kV.

2.8. Statistical analysis

Three repetitions were carried out for each of the *Salmonella* strains tested in the analysis of biofilm formation in PP and PU. Each assessment was carried out in duplicate for each contact time, 5, 10, 15, 30 min in PU, and 5, 10, 15 min in PP.

Mean plate counts in $\log_{10}$ (CFU/cm^2) and OD readings were submitted to analysis of variance, triple factorial design (biofilm classification, material and time), and means were compared by Tukey test at 5% probability in the Genes software (Cruz, 2006).

3. Results and discussion

From the 98 *Salmonella* spp. strains tested, all were positive for genes *csgD* and *adrA* (Fig. 1). These results are similar to those Oliveira et al. (2014) who also found genes *csgD* and *adrA* in all strains of *Salmonella* tested by them. Although there are results consistent with the existence of genes specific for biofilm formation and signaling, such as *csgD* and *adrA*, it is not possible to state that a given strain is able to produce biofilms only based on molecular analysis, without any observation of the environmental conditions (Monds & O'Toole, 2009). In the present study, all strains showed that they had the ability for form biofilms in 96-well polystyrene plates. Only one strain, which came from a polyurethane conveyor belt, was strong adherent in polystyrene plates according to the classification suggested by Stepanović et al. (2000). Oliveira et al. (2014) assessed the ability of *Salmonella* to produce biofilms in different materials and at different temperatures. They observed that 98.3% of the strains produced biofilms in some of the materials at least in one of the temperatures tested, and none of the strains was strong adherent. Most of the strains evaluated in the present study (69) were weak adherent, and 28 were moderate adherent.

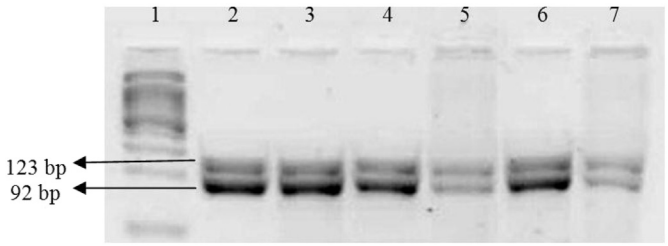

Fig. 1. Polymerase chain reaction amplification for detection of *csgD* (123 bp) and *adrA* (92 bp) genes in *Salmonella* spp. 1. 50-bp molecular weight marker; 2. *Salmonella* Typhimurium ATCC 14028; 3–7. Positive strains (negative control not shown).

The greater or lesser ability that microbial cultures have to adhere to the surfaces is directly related to the type of substrate (Shi & Zhu, 2009), and most of the *in vitro* studies on biofilms use only polystyrene plates to evaluate this ability (Díez-García, Capita, & Calleja, 2012; Lianou & Koutsoumanis, 2012; Rodrigues et al., 2009; Wang et al., 2013). Together with using polystyrene plates in the biofilm-producing assay, the present study also showed that *Salmonella* spp. strains were able to form biofilms in polyurethane (PU) and polypropylene (PP), which are the materials that made up the conveyor belts in cutting rooms of poultry processing plants where the strains were initially isolated.

The statistical interaction of the classification of *Salmonella* spp. strains according to adherence to polystyrene, and the production of biofilms in PU and PP may be seen in Fig. 2. It was observed that the three strains of *Salmonella* selected according to the adherence to polystyrene (weak, moderate, and strong) did not show any statistical interaction with the materials, as there were no statistical differences ($p > 0.05$) in plate counts (96 and 192 h).

Means of the type of material after 192 h of incubation (Fig. 2) show that counts in $\log_{10}$ in PP were statistically different ($p < 0.05$) in Tukey test, and were greater than counts in PU. This finding indicates that there are important differences in *Salmonella* spp. adhesion to polystyrene, the material commonly used to estimate *in vitro* biofilm production, compared with the materials used in poultry processing plants, PU and PP. Given these results, caution is recommended when results are compared or extrapolated for different materials. Individual situations should be carefully analyzed.

There was no statistical interaction for the materials in relation to optical density at 96 h of incubation, either. Mean OD per material was 0.215 for PP and 0.179 for PU. When strains were classified as weak, moderate and strong adherent, means were 0.200, 0.190 and 0.199, respectively. With 192 h of incubation, there was a statistical interaction between the classification of the strain in adherence to polystyrene and the other materials tested (Table 1). In PU, the three strains tested showed similar mean OD, without any statistically significant variation. Mean OD for PP was lower for the weak adherent strains in polystyrene, and greater in moderate and strong adherent strains. Therefore, it may be inferred that PP is similar to polystyrene in *in vitro* adhesion tests. In some cases, it was possible to correlate *in vitro* biofilm production in polystyrene microplates and in other surfaces commonly found in food plants

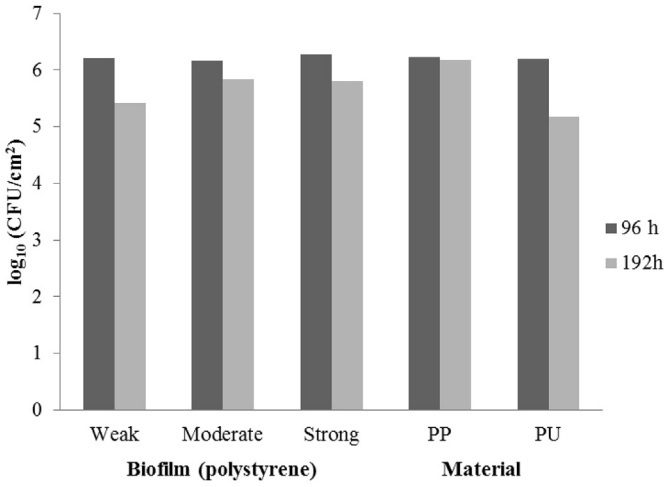

Fig. 2. Means in $\log_{10}$ (CFU/cm^2), according to the adherence strength of *Salmonella* spp. to polystyrene and to conveyor belt materials tested after 96 and 192 h of incubation. Coefficient of variation after 96 h was 4.23%, and after 192 h was 8.31%.

Table 1
Mean optical density (OD) of *Salmonella* spp. strains according to the adherence strength to polystyrene, and statistical interaction of this factor with PU and PP after 192 h of incubation.

Material	Biofilm		
	Weak adherent	Moderate adherent	Strong adherent
PU (OD)	0.170^{Aa}	0.179^{Ab}	0.185^{Ab}
PP (OD)	0.209^{Ba}	0.364^{Aa}	0.380^{Aa}

Different uppercase letters in the lines and different lowercase letters in the rows indicate statistical difference in the comparison of the means by Tukey test $P < 0.05$. Coefficient of variation: 38.62%.

(Patel & Sharma, 2010; Vestby et al., 2009).

In order to evaluate sanitizer efficiency in food processing plants, Møretrø et al. (2012) recommends that methods should simulate real conditions. In order to make the *in vitro* study similar to real conditions of sanitizer use in the industry, the same materials that made up the conveyor belts where *Salmonella* spp. strains were isolated were used. Additionally, strains isolated in poultry processing plants were used instead of reference strains, and sanitizers analyzed were those commonly used in food processing plants.

Table 2 shows mean residual counts ($\log_{10}$ CFU/cm^2) of *Salmonella* spp. strains in PU without treatment (control) and treated with sanitizers (CA, PA, and CA+PA) according to the contact time. Recovery of viable cells was carried out soon after the treatment with sanitizers in biofilms incubated for 96 h and after reincubation for other 96 h. Viable cells were recovered only from PU slides. One of the reasons for these results is the fact that PU surface is rougher than that of PP (Fig. 3A). It may be noted that, in spite of the recovery of viable cells from biofilms formed in PU slides (96 h) and treated with PA, compared with the control, reduction was greater than 4 $\log_{10}$. According to Møretrø et al. (2009), this result is adequate to consider that a sanitizer is efficient against adhered cells. Residual cell counts of biofilms formed for 96 h and treated with CA and CA+PA were not detectable. Therefore, in spite of the recovery of viable cells soon after the treatment, it may be observed that all sanitizers were efficient. Mean counts, in $\log_{10}$, after the treatment of biofilms formed in PU and after slides were reincubated for other 96 h were statistically different ($p < 0.05$). Means of the PA treatment were greater and, consequently, closer to the positive control. The greatest reduction was observed in CA and CA+PA treatments, which were not statistically different from each other ($p < 0.05$).

In the analysis of the means of sanitizer treatments, it was evident that CA and CA+PA treatments were statistically similar and reduced *Salmonella* counts in more than 4 $\log_{10}$. Even in the reincubation conditions of the present study, chlorinated alkaline sanitizers demonstrated to be effective against some adhered bacterial cells, different from peracetic acid, whose counts were reduced in only 1.87 $\log_{10}$, which is lower than the reduction necessary for this sanitizer to be considered efficient (Møretrø et al., 2009). However, in the present study, the only concentration of peracetic acid use analyzed was 0.2%. Recommended usage ranges from 0.1 to 1.5%.

Table 2
Effect of the treatments on the $\log_{10}$ means of plate counts for biofilms formed by *Salmonella* spp. in PU after 96 h of incubation and after reincubation (192 h, total), according to the contact time. Coefficient of variation after 96 h was 61.67%, and after 192 h, 65.83%. CA: chlorinated alkaline, PA: peracetic acid, CA+PA: chlorinated alkaline and peracetic acid, n/d: undetectable by our microbiological procedures (<1.25 log (CFU/cm^2).

Time (min)	log (CFU/cm^2)							
	Control		CA		PA		CA+PA	
	96 h	192 h	96 h	192 h	96 h	192 h	96 h	192 h
5	6.12	4.62	n/d	1.57	1.42	4.80	n/d	2.21
10	6.17	5.20	n/d	1.56	0.68	2.73	n/d	1.24
15	6.16	5.16	n/d	n/d	0.45	3.77	n/d	1.12
30	6.00	5.32	n/d	0.54	1.31	1.57	n/d	n/d
Mean	6.11^{a}	5.08^{a}	n/d	0.92^{c}	0.97^{b}	3.21^{b}	n/d	1.14^{c}

Different letters indicate statistical difference in the comparison of the means at each incubation time by Tukey test $P < 0.05$.

Variation in counts observed in the slides analyzed soon after sanitizer treatment and in reincubated slides may be due to the stress produced by the sanitizers in *Salmonella* cells, making it difficult for the microorganism to form colonies on the slides after the treatment. However, when these cells were placed again in optimal incubation conditions, they became viable and formed colonies. This finding should be a warning for the need for adequate Standard Operating Procedures (SOP) for food safety and hygiene in the food plants. It should be emphasized that SOP in food processing plants is based on a combination of sanitizer use and rinsing water at high temperatures, and the latter was not analyzed in the present study.

Plate counts of PP slides were undetectable, either after the treatment with sanitizers (96 h) or after reincubation (192 h), for all contact times and treatments. One of the reasons for these results was the fact that PP surface is smoother than PU (Fig. 3), making removal of adhered cells easier. It is important to emphasize that this conclusion is based on the analysis of slides and cannot be completely extrapolated to real conditions, although it is known that the surface in contact with the food interferes with bacterial adhesion and may affect cleaning, either positive or negatively.

In this study, visual inspection of the plates after addition of XTT, and assessment of the orange color by naked eye exactly reflected the results of the treatments in which viable cells were recovered in plates. OD readings, on the other hand, could not be correlated with counts. Final OD was influenced by the OD of the sanitizers tested. Although Tsukatani et al. (2008) found linear relationships between OD and plate counts of viable colonies and Martín-Espada, D'ors, Bartolomé, Pereira, and Sánchez-Fortún (2014) demonstrated that OD may replace plate counts in sanitizer efficiency tests.

In order to isolate factors that interfered with OD, mean OD readings were compared with readings of negative controls for the three repetitions of each strain (weak, moderate and strong) by contact time. After reincubation, the difference between OD readings of the treatments increased compared with negative controls. It was expected that the viability marker XTT and OD readings were more sensitive in the detection of cells that remained viable even after treatment with the sanitizers. However, some negative control readings were identical to readings of wells that yielded colonies in plates. This finding make it difficult to draw inferences based on the results, and it is a warning for caution when OD results are evaluated.

Most of the sanitizers used in the concentrations recommended by the manufacturers are efficient against *Salmonella* in suspension tests (Møretrø et al., 2012). Some of the most important sanitizers used in the food industry are chlorinated alkaline ones. In these agents, chlorine aids the removal of organic matter, specially protein, and has disinfecting properties (Aarnisalo, Lundén, Korkeala, & Wirtanen, 2007). The best results of *Salmonella* spp. inactivation in biofilms, in the present study, were obtained with the use of CA.

Peracetic acid is also widely used in the food industry, and it is greatly appreciated, as it may be used in low concentrations (Wessels & Ingmer, 2013). Peracetic acid showed good efficiency against planktonic cells (Colla et al., 2012). In the present study, after the treatment with PA 0.2% and reincubation, viable cells were

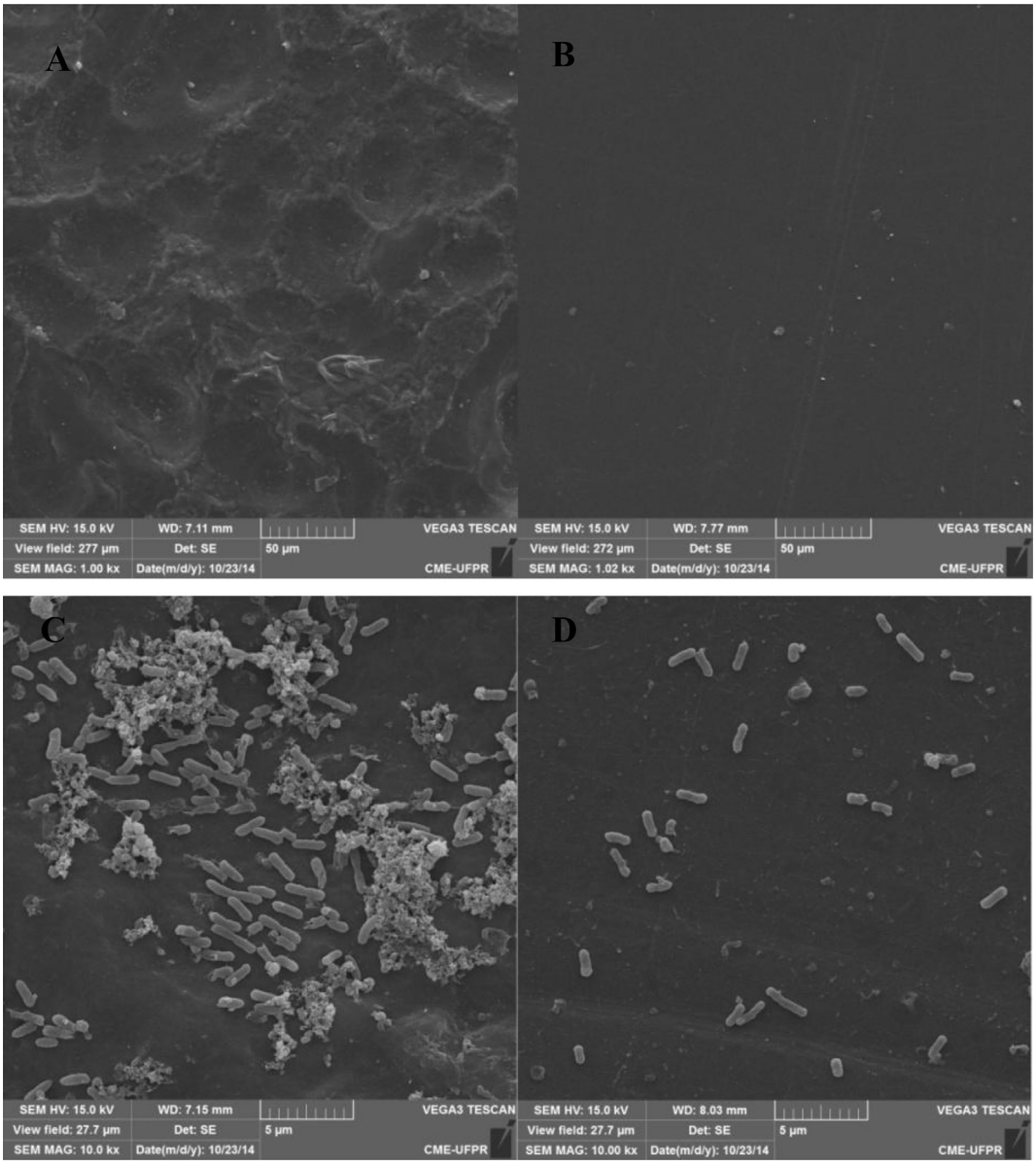

Fig. 3. PU and PP analysis by scanning electron microscopy at 1,000× magnification (A and B). C and D show *Salmonella* spp. adhesion at 10,000× magnification.

recovered, demonstrating that this concentration was not enough for complete elimination of *Salmonella* spp. in PU. Machado, Malheiros, Brandelli, and Tondo (2010) analyzed *S.* Typhimurium and *S.* Enteritidis strains in suspension, which are known to be more sensitive than sessile ones, and observed tolerance to concentrations equal to 0.2% and 0.3%, as well.

4. Conclusion

Our results point out to the need for more studies in order to find out concentrations of peracetic acid that are efficient against biofilms formed by *Salmonella* spp. *in vitro* and *in situ*. Additionally, they are a warning for the need for a review of the official Brazilian tests used to assess sanitizer efficiency, for them to be applicable to bacterial species that are biofilm-producers.

These results should be a warning for the implementation of efficient control programs that ensure the use of adequate concentrations of sanitizers, in a way that they uniformly reach all the surfaces to be cleaned, and to prevent the transfer of potentially pathogenic, biofilm-producing microorganisms to food products.

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