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**Communication**

[*Comunicação*]

# Sanitizer resistance of biofilm-forming *Salmonella* isolated from meat products

[*Resistência de* Salmonella *formadora de biofilme isolada de produtos cárneos a sanitizantes*]

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Biofilm is a community of sessile microorganisms characterized by cells embedded in an extracellular matrix formed by exopolysaccharides that adhere to a surface (Donlan and Costerton, 2002). Its occurrence in the food industry is of great importance due to its potential as a chronic source of microbial contamination in food, in addition to contributing to an increased resistance to cleaning (Stepanovic *et al.*, 2004). For these reasons, the presence of biofilm-forming microorganisms in the food industry, such as *Salmonella*, is a cause for concern on the part of animal product inspection and public health agencies.

Hygiene-health care during food processing is an important tool to ensure that the microbiological quality of food is appropriate for human consumption. For this purpose, two widely used sanitizers in the food industry are sodium hypochlorite, which acts by altering cell permeability and by interfering with enzymatic processes, and iodine, which presents high cell penetration ability, denaturing bacterial proteins (Evangelista, 2000).

This study aimed to evaluate the ability of *Salmonella enterica* subsp. *enterica* strains isolated from meat products to form biofilm on the surface of different equipment and tools which are used by the food processing industry, as well as to test their resistance to sanitizers.

Twenty *Salmonella enterica* subsp. *enterica* strains (Table 1) isolated from meat products, according to U.S. Food and Drug Administration recommendations (Andrews and Hammack, 2011), from a previous study (Conceição *et al.*, 2007) were used. The strains were recovered in Brain Heart Infusion broth (BHI, Acumedia, Michigan, USA) and evaluated as to their biofilm-producing ability in U-shaped bottom microtiter plates (Nunclon, Nune, Roskkilde, Denmark), following the technique described by Møretrø *et al.* (2003), adapted. Overnight cultures in BHI at 37ºC were standardized by spectrophotometry at 600 nm for value 1. Following, a 1:10 dilution in BHI added with 1% glucose (w/v) was performed. Two hundred µL cell suspension aliquots of each strain were distributed into plate wells and incubated at 37ºC for 24 hours. The plates were then washed three times in 200µL 0.1% saline (w/v) to remove cells that had not adhered to the plate and dried by inversion. Adhered cells were stained with 200µL 0.1% crystal violet (w/v) for 15 minutes. The stain was removed and absorbance at 630nm was determined after a 2-hour dry. Strains showing $A_{630} \geq 0.1$ were defined as biofilm-forming. A *Staphylococcus aureus* strain known to produce biofilm, kindly provided by the microbiology laboratory from the Instituto de Biologia of the Universidade Federal de Pelotas, was used as positive control.

In a second step, strains thought to be film-forming in microtiter plates were tested as to their ability to form biofilm on different surfaces. Four $cm^2$ plastic (high density polyethylene) stainless steel and glass flat surface plates were used. The sterile plates were attached to a metallic rod and vertically put into bottles containing 150mL BHI added with 1% glucose. Three mL overnight culture at 37ºC in BHI of each biofilm-forming strain was then added and

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incubated at 37ºC. Every 48 hours of incubation, the plates were gently washed in saline to remove non-adherent cells, and once more put into bottles added with 150mL BHI, without, however, inoculum addition. After five replications of the experiment, sterile swabs were rubbed on each plate surface and then transferred to test tubes containing 9mL saline. From this, serial dilutions for microorganism count in Standard Plate Count Agar (PCA, Acumedia) were done. The LIPOA 2018 strain that did not form a biofilm in a prior step was used as negative control.

Table 1. *Salmonella enterica* subsp. *enterica* strains used in the experiment

| Identification | Serotype | Source |
|---|---|---|
| LIPOA 2001 | Enteritidis | ground chicken meat |
| LIPOA 2002 | Enteritidis | ground chicken meat |
| LIPOA 2003 | Enteritidis | ground chicken meat |
| LIPOA 2004 | Agona | chicken sausage |
| LIPOA 2005 | Infantis | dried pork sausage |
| LIPOA 2006 | Enteritidis | ground chicken meat |
| LIPOA 2007 | Derby | ground chicken meat |
| LIPOA 2008 | Enteritidis | ground chicken meat |
| LIPOA 2009 | Havana | fresh pork sausage |
| LIPOA 2011 | Infantis | dried pork sausage |
| LIPOA 2017 | Enteritidis | ground chicken meat |
| LIPOA 2018 | Enteritidis | ground chicken meat |
| LIPOA 2023 | Typhimurium | pork sausage |
| LIPOA 2024 | Enteritidis | chicken sausage |
| LIPOA 2034 | Muenchen | ground beef |
| LIPOA 2035 | Derby | ground beef |
| LIPOA 2039 | Infantis | ground beef |
| LIPOA 2040 | Anatum | pork chops |
| LIPOA 2042 | Derby | pork chops |
| LIPOA 2043 | Newport | chicken drumstick/thigh |

Finally, the sodium hypochlorite and iodine effectiveness - both at a 100ppm concentration – against biofilm-forming strains adhered to different surfaces was tested. The same process described above was repeated with biofilm-forming strains and the materials on which there was biofilm formation. After the last wash, plates with biofilm were vertically immersed in bottles containing sanitizers for a 10-minute period. Once the pre-established contact time was reached, the plates were immersed in a sodium thiosulfate neutralizing solution for 30 seconds. After wash in 0.85% saline, a sterile swab was rubbed on the surface of each plate and the PCA count was performed.

All experiments were done in triplicate. Analysis of variance of the *Salmonella* counts was done, and means were evaluated by the Tukey test by using the Statistix® (2003).

Two (10%) *Salmonella* serotype Enteritidis strains from ground chicken meat (LIPOA 2017) and chicken sausage (LIPOA 2024) were defined as biofilm-forming, presenting $A_{630}$ = 0.134 and 0.175 optical density, respectively. The other serotypes used in the experiment presented $A_{630}$ <0.1 and were considered non-biofilm forming. These results differ from those of Oliveira (2011), who found only three (1.7%) non-biofilm forming *Salmonella* strains out of 174 strains tested.

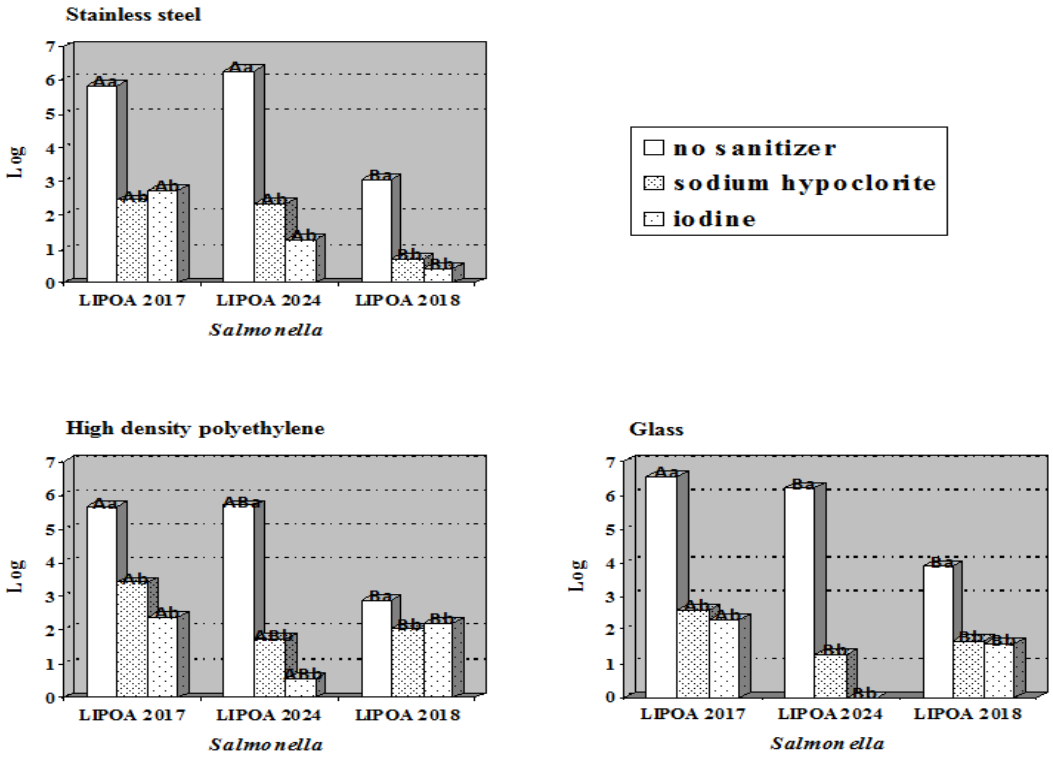
The two *Salmonella* strains that formed biolfilm in the microtiter plates were tested as to their biofilm-forming ability on stainless steel, glass and high density polyethylene surfaces. The biofilm-forming strains showed polymer-forming ability on the three different surfaces tested (Table 2). Strain LIPOA 2018, used as control for being non-biofilm forming, showed a lower count than that of the other strains.

Table 2. Biofilm-producing (LIPOA 2017 and LIPOA 2024) and non-producing (LIPOA 2018) *Salmonella* strains on different surfaces

| Surface | *Salmonella* Enteritidis (CUF/cm$^2$)* | | |
|---|---|---|---|
| | LIPOA 2017 | LIPOA 2024 | LIPOA 2018 |
| Aluminum | 3.2 x $10^6$ a | 5.3 x $10^6$ a | 2.9 x $10^4$ b |
| Polyethylene | 9.8 x $10^6$ a | 1.3 x $10^6$ a | 4.4 x $10^3$ b |
| Glass | 5.3 x $10^6$ a | 4.6 x $10^6$ a | 2.3 x $10^4$ b |

* Average of three replications.
a.b – averages followed by distinct letters differ from Tukey test (P<0.001).

The analysis of variance showed strain and sanitizer effect in the results. Although the LIPOA 2018 strain showed a significant decrease after sanitizer exposure, which was expected, this action was also observed with the two biofilm-forming strains (Figure 1).




A, B - different letters differ by the Turkey test regarding cepa (P<0,05)
a, b - different letters differ by the Turkey test regarding sanitizers (P<0,05)

Figure 1. Counts producing *Salmonella* enteritidis (LIPOA 2017 and LIPOA 2024) and non-producing (LIPOA 2018) biofilm on stainless steel surfaces, glass and high density polyethylene, after exposure to sanitizers.

The biofilm-forming strains identified in this study were serotype Enteritidis. Rodrigues *et al.* (2008) also tested *Salmonella* isolated from chicken meat and confirmed their biofilm-forming ability. However, these authors worked with a serotype Heidelberg strain. In the present study, even though this serotype was not used, nine different serotypes isolated from meat products of three animal species were tested, and only two seroptype Enteritidis strains formed biofilm.

Considering that the physicochemical properties of the surfaces to which microorganisms can adhere greatly influence their adhesion ability (Donlan and Costerton, 2002), the two *Salmonella* strains that formed biofilm in the microtiter plates were tested as to their biofilm-

forming ability on stainless steel, glass and high-density polyethylene surfaces. This experiment showed no *Salmonella* adhesion differences between the tested surfaces. Similar results were also obtained by Manijeh *et al.* (2008), who tested *Salmonella* serotype Enteritidis on polyethylene, glass and stainless steel and found no differences between the surfaces tested.

*Salmonella* Enteritidis biofilm-forming LIPOA 2017 and LIPOA 2014 b strains showed higher counts ($P<0.001$) on aluminum surfaces after sanitizing treatment, as compared to the non-biofilm forming LIPOA 2018 strain. According to Joseph *et al.* (2001), bacteria are more resistant than planktonic cells against the same antimicrobial agents on biofilm. However, in accordance with the results of this study, higher populations of biofilm-forming strains as compared to non-biofilm forming ones seem to occur due to a higher population of the former before sanitizer use rather than to a natural antimicrobial resistance. Therefore, under present industrial processing conditions, a lower efficiency in equipment sanitization regarding contamination by biofilm-forming strains should be expected, not because these strains are resistant to antimicrobials, but rather due to the fact that they occur at higher concentrations provided by their biofilm-forming ability during the sanitization procedure.

*Salmonella* non-biofilm forming LIPOA 2018 population reduction on polyethylene surface by sanitizers was lower than that observed on glass and aluminum surfaces. Although the causes of such differences have not been analyzed in this study, they are likely to be related to inherent characteristics of the materials which facilitate bacterium adherence and persistence on the surface, even without biofilm formation.

Even though there were no statistical differences ($P>0.001$) between the three strains after sanitizer use, LIPOA 2024 seems to have shown higher sensitivity to the action of the antimicrobials used on glass and polyethylene surfaces. Further studies with a larger number of replications could prove this peculiarity and contribute to a better understanding of the characteristics which make this strain more susceptible to hypochlorite and iodine than others.

The processing industry must be on the alert for cases of *Salmonella* isolation from chicken products, once some strains of this bacterium have the ability to form biofilm on the surfaces of the equipment and tools used in food processing, thus preventing their elimination. In addition to this physical barrier, the results obtained in this study indicate that the sanitizing action can also be less effective on biofilm-forming bacteria, being unable to remove bacteria from biofilm on equipment and tool surfaces, and occasionally contaminating food before its shipment, thus increasing the risk of transmission of foodborne diseases.

*Salmonella* Enteritidis which was isolated from chicken products is able to form biofilm on aluminum, polyethylene and glass surfaces, commonly used materials in industry equipment and tools. These strains did not offer a higher resistance to sodium hypochlorite and iodine antimicrobial action; however, due to the fact that they reached larger population concentrations as compared to non-biofilm forming strains, the reduction or elimination of these bacteria in sanitization processes can be less effective.

Keywords: biofilm, *Salmonella*, glass, stainless steel, high-density

## RESUMO

*Este estudo avaliou a capacidade de* Salmonella enterica *subsp.* enterica *isolada de produtos cárneos formar biofilme e testou sua resistência a diferentes sanitizantes. Vinte cepas foram avaliadas quanto à capacidade de formar biofilme em placas de microtitulação. As cepas formadoras de biofilme foram testadas em superfícies de polietileno de alta densidade, aço inoxidável e vidro e tiveram a sensibilidade ao hipoclorito de sódio e ao iodo avaliada. Duas cepas de* Salmonella *Enteritidis isoladas de produtos de frango apresentaram capacidade de formar biofilme nas superfícies testadas. Essas cepas alcançaram maiores populações nas superfícies do que aquelas não formadoras de biofilme, e foram mais difíceis de remover ou reduzir. Devido à ação sanitizante ser menos eficiente sobre bactérias formadoras de biofilme, esses micro-organismos podem persistir no biofilme formado sobre as superfícies de*

*equipamentos e utensílios e ocasionalmente contaminar os alimentos antes da sua expedição, aumentando dessa forma o risco de ocorrência de doenças transmitidas por alimentos.*

*Palavras-chave: biofilme,* Salmonella, *vidro, aço inoxidável, polietileno de alta densidade*

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