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MICROBIOLOGY

Salmonella Enteritidis forms biofilm under low temperatures on different food industry surfaces

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ABSTRACT: *We evaluated the influence of temperature on the ability of Salmonella Enteritidis (SE) to form biofilms on stainless steel, polyethylene, and polyurethane surfaces under different hygiene procedures. These materials were placed on SE culture and incubated at 42±1 ºC, 36±1 ºC, 25±1 ºC, 9±1 ºC, and 3±1 ºC for 4, 8, 12, and 24 h. Hot water at 45 ºC and 85 ºC, 0.5% peracetic acid solution, and 1% quaternary ammonia were used for hygienization. Biofilm formation occurred at all temperatures evaluated, highlighting at 3 ºC which has not been reported as an ideal temperature for the adhesion of SE to these materials. The SE adhered more often to polyethylene surfaces than to polyurethane and stainless steel surfaces (P<0.05). Peracetic acid and water at 85 ºC had similar hygienization efficiency (P<0.05) followed by quaternary ammonia whereas water at 45 ºC was not effective. SE adhered to these materials under low temperatures which to date have been deemed safe for food preservation.*
Key words: *Salmonella Enteritidis, biofilms, surfaces, hygiene procedures, food microbiology.*

Salmonella Enteritidis forma biofilme sob baixas temperaturas em diferentes superfícies da indústria de alimentos

RESUMO: *Avaliou-se o efeito da temperatura na capacidade de Salmonella Enteritidis (SE) formar biofilme em superfícies de aço inoxidável, polietileno e poliuretano e diferentes processos de higienização. Corpos de prova destes materiais foram postos frente a culturas de SE e incubados a 42±1 ºC, 36±1 ºC, 25±1 ºC, 9±1 ºC e 3±1 ºC por 4, 8, 12 e 24 horas. Para a higienização foram testados água aquecida a 45ºC e 85 ºC e soluções de ácido peracético 0,5% e amônia quaternária 1%. Verificou-se a formação de biofilmes em todas as temperaturas avaliadas, ressaltando-se a 3 ºC, ainda não citada como propícia para adesão de SE. Houve maior adesão ao polietileno do que ao poliuretano e ao aço inoxidável (P<0.05). Para higienização, o ácido peracético e a água a 85 ºC tiveram ação semelhante (P<0.05), seguidos por amônia quaternária, enquanto que a água a 45 ºC não foi eficaz. Todos os materiais avaliados propiciaram a aderência de SE, mesmo sob temperaturas baixas, consideradas até então seguras para a conservação dos alimentos.*
Palavras-chave: *Salmonella Enteritidis, biofilmes, superfícies, procedimentos de higienização, microbiologia de alimentos.*

INTRODUCTION

Salmonella Enteritidis (SE) is the most common *Salmonella* serotype that affects humans. It is the main cause of outbreaks of foodborne illness, especially in Europe where it accounts for 85% of cases. According to data published by the EFSA (European Food Safety Authority), more than 100.000 cases of salmonellosis are reported in humans annually, generating more than 3 billion euros in expenses per year (MILJKOVIC-SELIMOVIC et al., 2010; EFSA, 2015; EFSA, 2017).

Based on data released by the World Health Organization (WHO), SE is one of the 15 serovars of *Salmonella* that most often occurs in humans, food, animals, environmental samples, and animal feed (WHO, 2016; WHO, 2017a).

It is considered one of the human enteropathogens most frequently associated with the alimentary tract of chickens, and originates from different poultry sources. Products such as eggs and meat are the most common source of pathogens that may cause infectious gastroenteritis in humans, and are responsible for up to 47% of all infections in people

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(CARDOSO et al., 2000; CDC, 2013). Therefore, the control of SE in poultry abattoirs is essential due to public health concerns (CDC, 2014a) and the economic impact of *Salmonella* to infections which cause losses to the domestic market and exports.

Another concern regarding the hygienic-sanitary conditions in poultry abattoirs is biofilm formation. In this process, microorganisms attach to biotic and abiotic surfaces. These microorganisms manage to grow on these surfaces acting as sources of permanent contamination, releasing biofilms fragments formed by bacterial cells such as those from *Salmonella* spp., and may compromise microbiological quality and safety of food products (FUSTER-VALLS et al., 2008; FLEMMING et al., 2016).

Bacterial growth at refrigeration temperatures should be investigated since it is considered a critical point in food production and conservation (LIMA et al., 2004). Biofilm formation is influenced by a number of environmental parameters including temperature, pH, osmolarity, and atmospheric pressure. This process increases as conditions become less favorable for the microorganism (RODE et al., 2007; REUTER et al., 2010; LIANOU & KOUTSOUMANIS, 2012).

The SE is able to adhere and form biofilm on inert food processing surfaces such as stainless steel, polyethylene and polyurethane surfaces (MANIJEH et al., 2008) under different growth conditions and temperatures. It is reported that *Salmonella* does not grow below 5 °C (GAST, 2008; MOREY & SINGH, 2012). TORTORA et al. (2012) showed that 5 °C is the minimum temperature, whereas 37 ºC is the optimal temperature for *Salmonella* grow.

Sessile bacteria are significantly more resistant to substances used in disinfection procedures (COSTERTON et al., 1995; STEENACKERS et al., 2012). Once the biofilm is formed, it acts as a physical barrier that prevents the action of sanitizing agents (COSTERTON et al., 1995; STEPANOVIC et al., 2004) which makes the elimination of pathogens in food processing facilities difficult. In this context, this study aimed to evaluate the influence of different temperatures and disinfection treatments in the biofilm formation by SE at different surfaces, simulating steps of industrial processing.

MATERIALS AND METHODS

Sources of SE

Two samples of *Salmonella Enteritidis* (SE) were evaluated. These bacterial specimens were cultured from samples of poultry cuts processed for consumers (SE 84) and from a drag swab used to sample a broiler premise (SE 106). Both were submitted to serology and confirmed by DNA microarray technique (Check and Trace, R-Biopharm AG, Darmstadt, Germany), which allows the simultaneous evaluation of the expression of thousands genes of a microorganism.

Specimen preparation

Polyurethane, polyethylene, and AISI 316 stainless steel coupons 1 cm² in diameter and 0.1 cm thick were used. Coupons were manually cleaned with a sponge, water, and neutral liquid detergent, rinsed with distilled water, immersed in ethyl alcohol 70% (v/v) for 1 h at room temperature, rinsed one more time, and sterilized in an autoclave at 121 ºC for 30 minutes.

Biofilm formation tests

For biofilm formation, coupons with bacteria were grown individually on sterile polystyrene 12-well cell culture plates (Nest®Biotech Co. Ltd, Rahway, NJ, USA) 2.75 mL of tryptic soy broth without glucose (TSB, Difco®Laboratories, Sparks, MD, USA) and 250 µL of each SE culture with approximately 10^3 UFC.mL^{-1} were inoculated in each well and verified by culture in Plate Count Agar (PCA, HiMedia®Laboratories, Mumbai, India).

Coupons were immersed in SE 104 and SE 86 cultures and incubated at 42±1 ºC, 36±1 ºC, 25±1 ºC, 9±1 ºC, and 3±1 ºC, reproducing the environmental temperatures for food processing which were optimal for thermo-tolerant microorganisms. These were evaluated at 0, 4, 8, 12, and 24 h, simulating pre-operational and operational hygiene stages in poultry abattoirs (ROSSONI & GAYLARDE, 2000; KUSUMANINGRUM et al., 2003) in triplicate.

At specific times, the coupons were removed from culture medium with sterile tweezers, immersed in 5 mL of Peptone Water 0.1% (AP, HiMedia®Laboratories. Mumbai, India) for 1 minute for removal of planktonic cells, placed in tubes with Peptone Water 0.1%, and sonicated for 10 minutes on ultrasound (40 kHz and 81 W) for the release of sessile cells (SCHERBA et al., 1991). Five 10 µL drops of each dilution were inoculated in PCA agar by drop plate technique and incubated for 24 h at 37±1 ºC.

For calculation of results, the following formula was used: $UFC.cm^{-2}=(V_D/V_A).Av.D/A$ in which V_D was the diluent volume used in rinsing (5 mL), V_A the aliquot volume used in plating (0.05 mL or 0.1 mL), Av the average count recovered from plates (UFC), D

the dilution used in counting, and A the coupon area (2 cm^2) expressed in log^{10}. $UFC.cm^{-2}$ (GIBSON, 1999; ISO, 18593:2012).

Biofilm removal tests

After planktonic cells were removed, coupons were placed in 5 mL of sterile water and heated at 45 ºC or 85 ºC for 3 minutes and in 0.5% peracetic acid solution (Kalykim®, Alvorada, RS, Brazil) or 1% quaternary ammonia (Kalykim®, Alvorada, RS, Brazil) for 5 minutes, and the control it was water at room temperature.

After that, coupons were immersed in 5 mL of peptone water 0.1% with universal neutralizer (composed by 0.2% soy lecithin, 2% tween, 0.25% sodium thiosulphate, 0.1% peptone water and 1 L distillated water) for 1 minute (JOSEPH et al., 2001; ISO, 18593:2012), were placed into tubes with 5 mL of peptone water 0.1%, sonicated, and inoculated by drop plate as described for the biofilm formation in the previous section.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to study surface microtopography. The scanning electron microscopy studies were carried out at the Electron Microscopy Center (CME), UFRGS - Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil (SOUZA, 1998). Coupons were immersed in 10 mL of PBS (KH2PO4, s0.31 M, pH 7.2) for 1 minute for the removal of planktonic cells, and were then fixed in glutaraldehyde 2.5%.

After that they were rinsed with 0.2 M phosphate buffer. Coupons were dehydrated with acetone at 30%, 50%, 70%, 90%, and 100%. After complete dehydration, samples were subjected to critical point drying with CO_2 preserved the microorganisms present and then were placed on stubs for metallization.

Statistical analysis

The average comparison was carried out with the Tukey test at 5% of probability, and results were analyzed by variance (ASSISTAT version 7.7 beta, SILVA, 2016).

RESULTS AND DISCUSSION

Biofilm formation at different temperatures and surfaces

Salmonella Enteritidis samples from poultry origin formed biofilm on stainless steel, polyethylene, and polyurethane surfaces. There was significant adhesion compared other materials ($P<0.05$) of both SE to the polyethylene surface (Figure 1, 2 and 3, Table 1). There was no significant difference in adhesion when SE 84 strain was

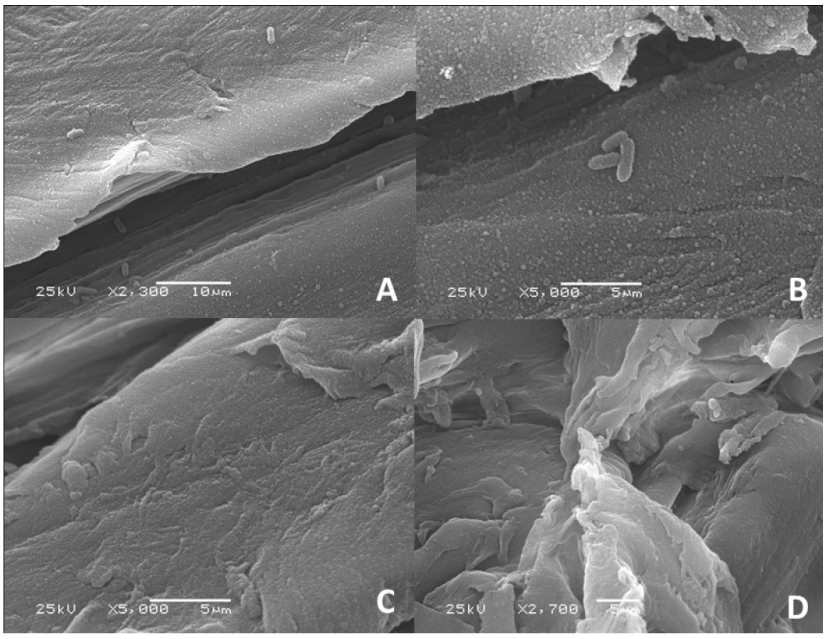

Figure 1 - SE biofilm formation on a polyethylene surface at 3 ºC for 24 h (A and B). Removal treatment with water at 85 ºC in SE preformed biofilm (C and D).

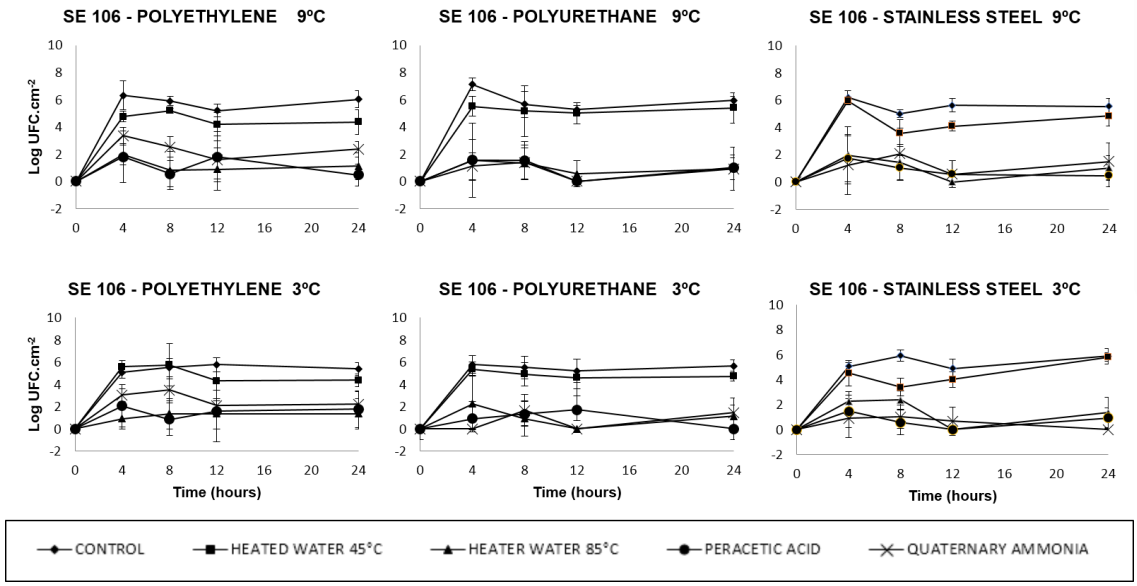

Figure 2 - Biofilm formation by SE 106 on polyethylene, polyurethane, and stainless steel surfaces under low temperatures and sanitizing procedures.

incubated at 3 °C or 9 °C (both of which are refrigeration temperatures) (Figure 2 and 3). Higher adhesion ($P<0.05$) was observed at 25 °C, 36 °C, and 42 °C. SE 106 showed biofilm formation at 3 °C, 9 °C, 25 °C, and 36 °C. There was a higher rate ($P<0.05$) of biofilm formation at 42 °C, which is the selective enrichment temperature for *Salmonella.*

According to RONNER & WONG (1993), at least 10^3 UFC (3 $\log^{10}$. UFC.cm^{-2}) and 10^5 UFC (5 $\log^{10}$.UFC.cm^{-2}) adhered by cm^2 are required for characterization of biofilm formation. Thus, it characterized our findings of SE 84 and SE 106 as biofilm formers, according data presented in table 1 and figure 4.

Virulence genes (*hil*A, *avr*A, *inv*A, *siv*H, *sop*E, *spi*A, *agf*A, *lpf*A, *sef*A, *spv*C) from the bacterial strains used in this study were previously investigated (SILVA et al., 2014). Only SE 84 strain had *spi*A gene, which is involved in both biofilm formation and virulence. It is important to note that the fimbrial *agf*A gene was not detected, whose main function is to promote the initial interaction of the bacterium with the host intestine, which is also related to biofilm formation (DONG et al., 2011). This finding showed that there are significant differences between *Salmonella* spp. serovars on their ability to form biofilms (VESTBY et al., 2009; WANG et al., 2013) as well as the influence of environmental parameters in SE biofilm formation. Biofilm formation tends to increase when conditions for the microorganism are unfavorable (LIANOU & KOUTSOUMANIS, 2012).

Personnel working at these facilities in Brazil should make sure that temperatures above 10 ºC are not used in the cutting room and that cooling temperatures of products do not exceed 4 °C, to ensure the quality of products sold in Brazil and foreign markets (BRAZIL, 1998).

Based on this assumption, in the present study, recommended temperatures were simulated (reproduced in the laboratory) which are 3±1 ºC (cooling temperature), 9±1 ºC (room temperature according to the EU), 25±1 ºC (room temperature), 36±1 ºC (optimal growth temperature for mesophiles) and 42±1 ºC (selective enrichment temperature for *Salmonella*). To the authors' knowledge, this is the first publication on SE biofilm formation at 3 °C on the surfaces evaluated. Our findings emphasized the fact that this specific temperature has not been described in the literature as ideal for the growth of *Salmonella* spp.

REUTER et al. (2010) and RODE et al. (2007) cite that sessile microorganisms are better adapted to the environment. Such ability enables them to survive and multiply under harmful, unfavorable and stressful conditions, which would explain SE

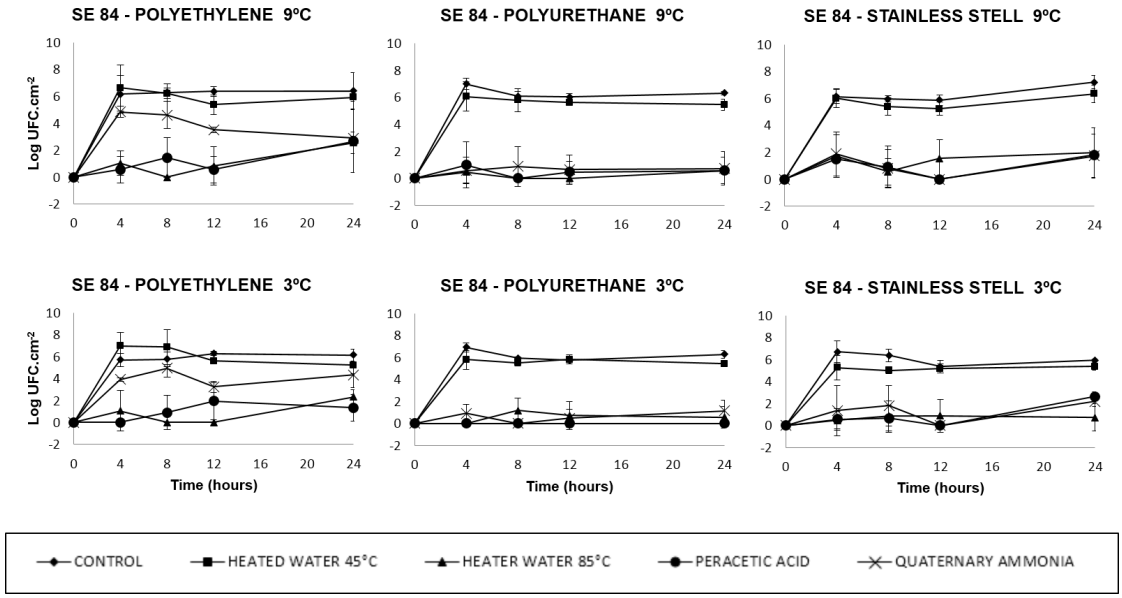

Figure 3 - Biofilm formation by SE 84 on polyethylene, polyurethane, and stainless steel surfaces under low temperatures and sanitizing procedures.

biofilm formation in our study. YANG et al. (2016) also related *Salmonella Enteritidis* as biofilm former at 4 ºC under environmental food-related stress conditions and its resistance to chlorine treatment suggesting that stress conditions encountered in food processing may alter the resistance of *S.* Enteritidis biofilm in the disinfection treatment.

TORTORA et al. (2012) suggested that the minimum temperature for *Salmonella* growth is 5 °C whereas MOREY & SINGH (2012) mentioned that this bacterium does not grow between 4 ºC and 8 ºC. In addition, the pathogen modeling program version 8.0 used by the United States Department of Agriculture (USDA, 2017) established that 5 °C is the lowest temperature possible for the growth of *Salmonella* spp.

Biofilm removal

We noticed that peracetic acid (0.5%) and water heated to 85 °C removed biofilms formed by SE at all temperatures and times with similar efficacy (P>0.05) followed by quaternary ammonia (1%) (Figure 4 and Table 1). Water at 45 ºC had no effect on biofilm removal compared to the control.

According to the studies published by VIALTA et al. (2002), sanitation operation efficiency of an equipment or surface is measured by the number of viable microorganisms adhered to it after this operation. In general, equipment should not contain more than 100 UFC.cm^{-2}. In UE Directive 471/2001, the maximum level accepted for surface testing is 10 UFC.cm^{-2} of mesophilic microorganisms and 1 UFC. cm^{-2} of enterobacteria. Establishments authorized to export avian products to members of the European Union must comply with this directive (EUROPEAN UNION, 2001a).

According to the American Public Health Association (APHA, 2014), physical or chemical sanitizers should reduce the number of microorganisms by up to 2 UFC.cm^{-2} on surfaces so that they can be considered hygienic. According to the European Standard EN 13697:2001 for surface testing in the European Union, there should have a reduction of at least 4 log in adhered surfaces (EUROPEAN UNION, 2001b; MORETRO et al., 2009).

In our study, there was a reduction of 4.23 $\log_{10}$ UFC.mL^{-1} after 5 minutes of contact with peracetic acid (0.5%), 3.562 $\log_{10}$ UFC.mL^{-1} with quaternary ammonia (1%), and 4.26 $\log_{10}$UFC.mL^{-1} with water heated at 85ºC after 3 minutes of contact. Water heated at 45ºC did not meet the surface hygiene recommendations, reducing only 0.511 $\log_{10}$UFC.mL^{-1} (Table 1). This result highlighted the non-use of water pressure in our study, which is recommended by CONTRERAS et al. (2003) in the rinsing step of surfaces, highlighting its importance.

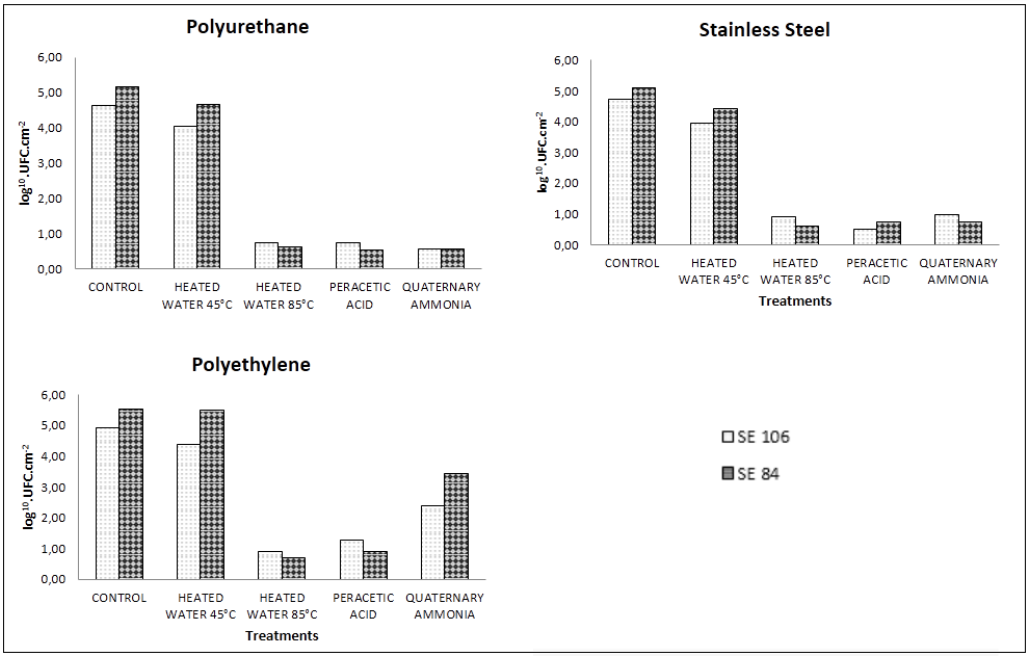

Figure 4 - Removal of biofilm formed by SE of poultry origin on stainless steel, polyethylene and polyurethane surfaces by different hygiene procedures.
Abbreviations: Heat water 45 °C: sterile water heated at 45 °C; Heat water 85 °C: sterile water heated at 85 °C; Peracetic acid (0.5%); Quaternary ammonia (1%).

Considering the existence of several hygienization agents such as chlorine solution, iodine solution and biguanide, we emphasized the use of peracetic acid and quarternary ammonia at these concentrations and times, because were used due to manufacturer's indication and preliminary tests conducted by our research group. In a study performed by Silva et al. (2014) all concentrations of quaternary ammonia (0.3%, 1.0% and 2.0%) and peracetic acid (0.1%, 0.5% and 1.0%) at all times tested (1, 5, 10 and 15 min) were efficient in biofilm removal of *S.* Enteritidis.

According to the results of the present study, the most effective choice for SE biofilm removal on the evaluated surfaces normally used in abattoirs would be the application of peracetic acid as a sanitizing agent and water at 85ºC for the utensils.

On the surfaces assessed, higher biofilm formation was observed in polyethylene coupons (4.917 $\log^{10}$.UFC.cm^{-2}). This is probably due to the

Table 1 - Removal quantification of biofilm formed by SE (*Salmonella Enteritidis*) strains of poultry origin before disinfection procedures. Repetitions average.

Strain	----------Treatments*				
	Control	Water at 45 ºC	Water at 85 ºC	Peracetic acid (0.5%)	Quaternary ammonia (1%)
SE 84	5.258^{Aa}	4.875^{Ba}	0.649^{Ca}	0.725^{Ca}	1.580^{Da}
SE 106	4.765^{Aa}	4.126^{Ba}	0.854^{Ca}	0.836^{Ca}	1.317^{Da}

Means followed by the same letters, uppercase in rows and lowercase in columns do not differ amongst themselves (P≤0.05) by Tukey's test (P<0.05).
*Results expressed in $\log^{10}$.UFC.cm^{-2}.

irregularities that are present on its surface which facilitates deposition of organic materials and also hinders the action of disinfectants (SINDE & CARBALLO, 2000). There is lower biofilm formation on stainless steel coupons (4.745 $\log^{10}$. UFC.cm^{-2}) and polyurethane coupons (4.633 $\log^{10}$. UFC.cm^{-2}) possibly because these two types of surfaces are less irregular than those of polyethylene coupons. However, OLIVEIRA et al. (2006) suggested that the depth of the surface imperfections may result in higher microbial adhesion than surface roughness distance.

CONCLUSION

Our results showed that *Salmonella Enteritidis* formed biofilm at low temperatures and short contact times, which difficult to disinfect food industry surfaces and the possibility of cross-contamination during food processing.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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