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Influence of temperature and surface kind on biofilm formation by *Staphylococcus aureus* from food-contact surfaces and sensitivity to sanitizers

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ABSTRACT

This study aimed to assess the adhesion, detachment kinetic and biofilm formation of *Staphylococcus aureus* isolates from food services surfaces on stainless steel and polypropylene surfaces when cultivated in a vegetable-based broth at 7 and 28 °C, and the efficacy of peracetic acid (30 mg/L) and sodium hypochlorite (250 mg/L) in removing the bacterial cells from the matrix of the preformed biofilm. The isolates adhered over 4 Log cfu/cm^2 regardless the surface kind and incubation temperature. Cell detachment was around 3 Log cfu/cm^2 over the first six contacts with agar characterizing a high persistence of cells on the tested surfaces. Number of cells (5–7 Log cfu/cm^2) needed for biofilm formation was noted at all experimental systems already after 3 days of incubation. A range of 2.0–3.3 and 1.5 to 2.1 Log cfu/cm^2 was observed in the reduction of cells in biofilm matrix caused by peracetic acid and sodium hypochlorite, respectively. The isolates of *S. aureus* revealed high capability to adhere and form biofilm on the tested surfaces in both assayed incubation temperature.

1. Introduction

Worldwide there is a concern about the impact of microbial foodborne diseases on the human behalf (White, Zhao, Simjee, Wagner, & McDermott, 2002). The importance of contaminated surfaces in spreading pathogenic microorganisms to foods is already well established in food processing, catering and domestic environment (Vasseur, Rigaud, Hébraud, & Labadie, 2001; Vautor, Abadie, Pont, & Thiery, 2008). One of the most common ways for bacteria to live is adhering onto surfaces and forming organized communities named biofilms (Jenkinson & Lappin-Scott, 2001; Malheiros, Passos, Casarin, Serraglio, & Tondo, 2010). Stainless steel, glass, rubber and polypropylene surfaces can be contaminated either by spoilage or pathogenic bacteria, which under certain conditions adhere to these surfaces, initiating the cell growth and leading to the biofilm formation (Murga et al., 2001).

According to Costerton, Steward, and Greenberg (1999) biofilms are cell aggregates embedded in an organic extracellular polymeric matrix that confers resistance to involved microorganisms. Bacteria aggregated to form biofilms have greater resistance to the environmental stress than the planktonic counterparts, including the sensitivity to sanitizers (Fux, Wilson, & Stoodley, 2004; Spoering & Lewis, 2001). Bacterial aggregates detached from biofilms retain the high level of resistance to antimicrobials and may contain enough number of cells to represent a potential infectious dose. The formation of biofilms on food-contact surfaces is known as a potential risk to the consumer's health, particularly, if the cross contamination of food occurs after a bactericidal procedure (Spoering & Lewis, 2001).

Staphylococcus aureus has been frequently found in surfaces of food processing plants being responsible for outbreaks related to the consumption of fresh and processed foods worldwide (Balaban & Rasooly, 2000; Braga et al., 2005; Nostro et al., 2004). The establishment of the food poisoning caused by *S. aureus* depends on the ability of the strain to survive in/on a colonized substrate, multiply under a variety of conditions and produce several extracellular substances (Pastoriza, Cabo, Bernárdez, Sampedro, & Herrera, 2002). Although some researchers have observed the ability to adhere and form biofilm by *Staphylococcus* genera (Hussain, Becker, Von Eiff, Peters, & Hermann, 2001; Kuźman, Różalski, Walenka, Różalska, & Wysokińka, 2007), the most studies have been addressed to clinical aspects related to the

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Table 1
Physico-chemical characteristics of vegetable-based broth.

Physico-chemical parameters	Values
Proteins	0.18%
Fat	0.11%
Moisture	98.32%
Carbohydrates	1.39%
Ashes	–
pH (T: 27.5 °C)	5.69

biofilm formation by *Staphylococcus intermedius* on medical implants and materials (Herrera, Cabo, González, Pazos, & Pastoriza, 2007; Marques et al., 2007).

Currently, there is a lack of information about the capacity of *S. aureus* from food service surfaces of adhering and forming biofilm when exposed to different environmental conditions, and about the efficacy of sanitizers in removing the cells forming the biofilm. Regarding these aspects, this study was carried out with the aim of evaluating the ability of *S. aureus* isolates from food services surfaces to adhere and form biofilms on stainless steel and polypropylene surfaces when cultivated in a vegetable-based broth under different temperatures (7 and 28 °C). Still, it was observed the effect of the sanitizers peracetic acid and sodium hypochlorite in reducing the number of bacterial viable cells on a preformed biofilm.

2. Material and methods

2.1. Test isolates

S. aureus S3, *S. aureus* S28 and *S. aureus* S54 obtained from the Microorganism Collection, Laboratory of Food Microbiology, Health Sciences Center, Federal University of Paraíba (João Pessoa, Brazil) were used as test isolates. The ones were isolated from different surfaces of Food and Nutrition Services by the standard procedures (Downes & Ito, 2001). Stock cultures were kept on Nutrient Agar – NA (Difco, Brazil) slants under refrigeration (7 ± 1 °C).

Inocula used in antimicrobial assays were obtained from overnight cultures grown on NA slants at 37 °C. A loopfull of the culture was diluted in sterile saline solution (0.85 g/100 mL) to have a final concentration of approximately 8 Log of colony forming unity per mL (cfu/mL) adjusted according to the turbidity of 0.5 McFarland standard tube (Oliveira, Stamford, Gomes Neto, & Souza, 2010).

2.2. Test surfaces and experimental conditions

AISI 304 stainless steel (2 × 2 × 0.2 cm) and polypropylene coupons (2 × 2 × 0.4 cm) were used as test surfaces. The coupons were individually cleaned, sanitized and sterilized according to procedure described by Marques et al. (2007).

The adherence, detachment and biofilm formation of the test isolates on polypropylene and stainless steel surfaces and inoculated in a vegetable-based broth was assessed in two different incubation temperatures, 7 and 28 °C.

2.3. Preparation of vegetable-based broth

A mixture (1:1:1) of vegetables (carrot, lettuce and tomato) containing 300 g was mashed with 600 mL of distilled water using a domestic blender and vacuum filtered using Whatman no.1 filter paper. The material was sterilized by filtration using a Millipore 0.22 μm. The obtained broth was stored at −20 °C in aliquots of 50 mL and when required one aliquot was thawed under refrigeration (7 ± 1 °C) and used for the experimental assays.

The vegetable broth was characterized regarding its physico-chemical characteristics (moisture, protein, fat, carbohydrate, ashes and pH value) according to procedures described by IAL (2005). Physico-chemical characteristics of the vegetable-based broth used in the assays are shown in Table 1.

2.4. Adhesion to surfaces and quantification of adhered cells

An aliquot of 100 μL of the growth media was mixed to 50 μL of the bacterial inoculum, plated onto the center of each coupon and incubated under the pre-established temperatures. After 24, 48 and 72 h of incubation, coupons (two for each treatment) were withdrawn and immersed in sterile peptone water – SPW (0.1 g/100 mL) during 15 s for releasing non-adhered cells. The cells adhered to the coupons were collected by thoroughly rubbing their surfaces with two moistened swabs, which were resuspended in SPW by vigorously vortexing for 30 s. The mixture was serially diluted (10^{-1}–10^{-5}) in SPW and aliquots of 100 μL were spread plated onto sterile NA plates. The plates were incubated for 24 h at 37 °C (Herrera et al., 2007; Rode, Langsrud, Holck, & Moretto, 2007). After the incubation period, the number of viable cells was counted and the results were expressed in Log cfu/cm^2.

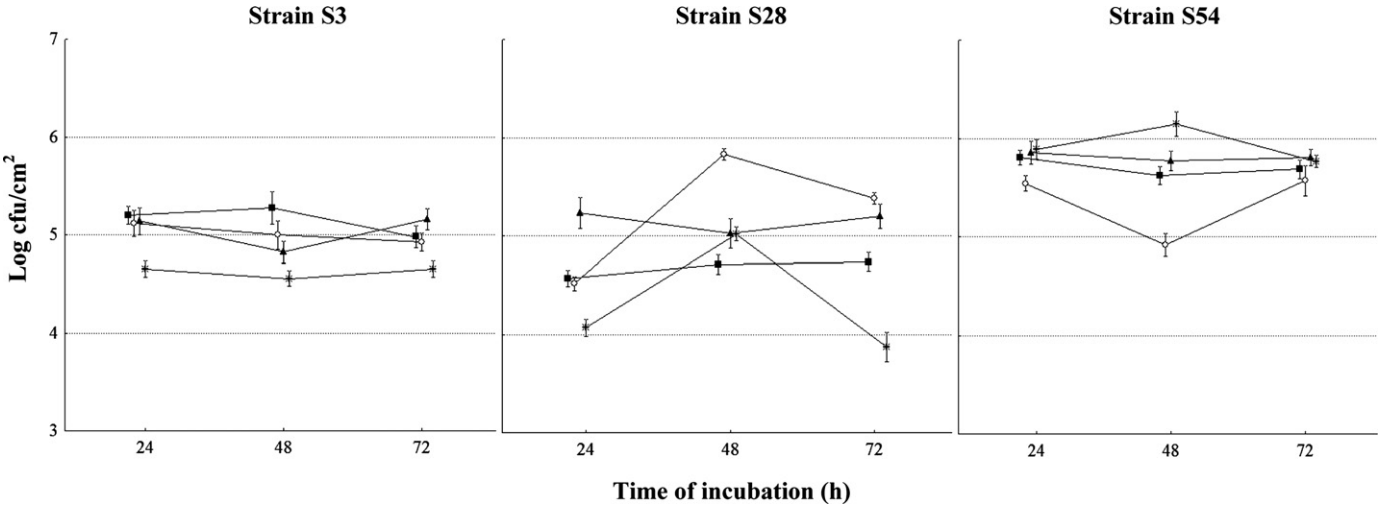

Fig. 1. Kinetics of adhesion of *S. aureus* S3, S28 and S54 to polypropylene and stainless steel surfaces in vegetable-based broth at 7 °C and 28 C over 72 h of incubation (■: polypropylene 7 °C, ○: polypropylene 28 °C, ▲: stainless steel 7 °C, ✳: stainless steel 28 °C).

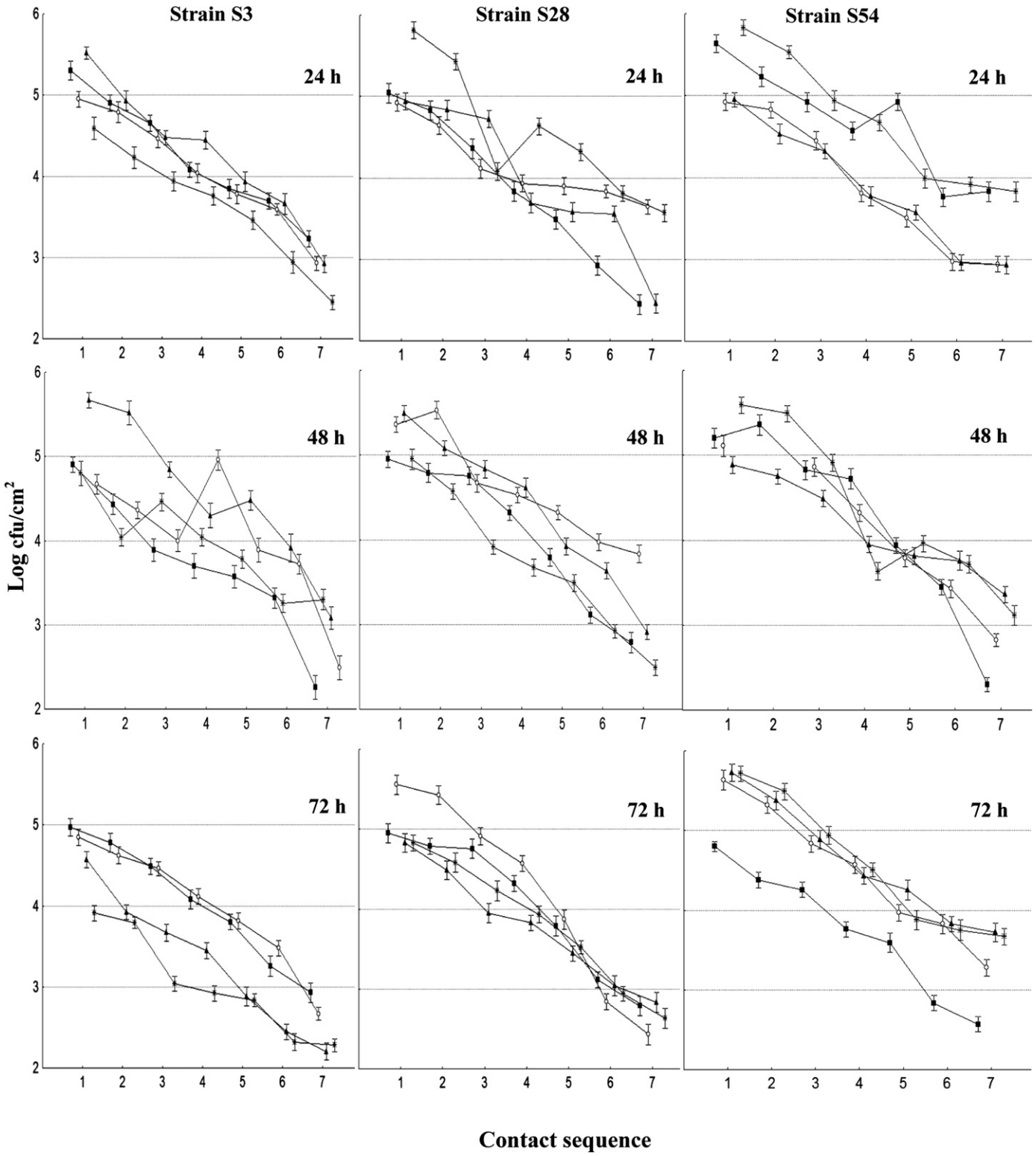

Fig. 2. Kinetics of separation of *S. aureus* S3, S28 and S54 of polypropylene and stainless steel surfaces in vegetable-based broth at 7 °C and 28 °C over 72 h of incubation (■: polypropylene 7 °C, ○: polypropylene 28 °C, ▲: stainless steel 7 °C, ✳: stainless steel 28 °C).

2.5. Detachment of adhered cells

An aliquot of 100 μL of the growth media was mixed to 50 μL of the bacterial inoculum and plated onto the center of each coupon, followed for incubation under the pre-established temperatures. After 24, 48 and 72 h of incubation, coupons (two for each treatment) were withdrawn and immersed in SPW during 15 s for releasing non-adhered cells. Each coupon was placed on a sterile NA plate, and after 2 min, removed and placed onto a second sterile NA plate. This procedure was repeated through 7 sterile NA plates.

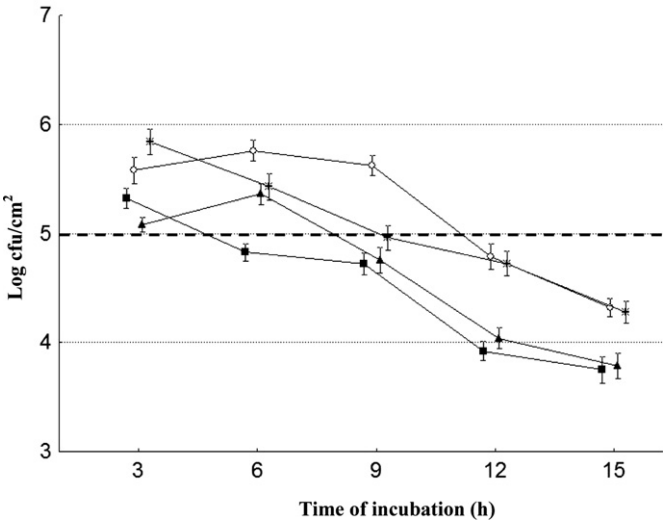

Fig. 3. Biofilm formation by *S. aureus* S3 on polypropylene and stainless steel surfaces in vegetable-based broth at 7 °C and 28 °C over 15 days of incubation (■: polypropylene 7 °C, ○: polypropylene 28 °C, ▲: stainless steel 7 °C, ✱: stainless steel 28 °C). Dotted line means the lower bacterial count (Log cfu/cm^2) needed for biofilm formation according to Wirtanen et al. (1995).

The number of detached cells on the NA plates with order number of 1, 2, 3, 4, 5, 6, and 7 was found by transferring the agar blotting from each plate to 10 mL of SPW followed by blending using a Stomacher. The mixture was serially diluted (10^{-1}–10^{-5}) in SPW and aliquots of 100 μL were spread plated onto NA plates. The plates were incubated for 24 h at 37 °C (Herrera et al., 2007). After the incubation period, the number of viable cells was counted and the results expressed in Log cfu/cm^2.

2.6. Biofilm development and quantification

The level of biofilm formation by *S. aureus* S3 on polypropylene and stainless steel surfaces incubated in vegetable broth at 7 °C and 28 °C over 15 days was assessed. For this, five stainless and five polypropylene coupons were immersed in sterile Petri dishes containing 20 mL of the growth media and 2 mL of the bacterial inoculum. The Petri dishes were sealed and incubated statically at the pre-established temperatures. After 3, 6, 9, 12 and 15 days of incubation, the coupons were withdrawn and washed with SPW to remove the non-adhered cells. Once again, the coupons were immersed in a fresh medium containing the same amount of inoculum, being the process repeated four times, completing a 15-day period.

At each incubation interval, two coupons of each treatment were submitted to bacterial count in biofilm matrix. For this, the biofilm was scraped with two moistened sterile swabs, which were resuspended in 9 mL of SPW by vortexing for 30 s. Serial dilutions were prepared in SPW and aliquots of 100 μL were spread plated onto sterile NA plates, followed for incubation at 37 °C for 24 h (Marques et al., 2007). After the incubation period, the number of viable cells was counted and the results expressed in Log cfu/cm^2.

2.7. Sanitizer application

The efficacy of the sanitizers sodium hypochlorite (250 mg/L) and peracetic acid (30 mg/L) in removing the cells of *S. aureus* S3 from the biofilm matrix grown in the vegetable-based broth at 7 and 28 °C was assessed. For this, five coupons were allowed to develop biofilm according to the experimental conditions before cited. After 15 days of incubation, the coupons were washed in SPW, immersed for 30 s in sterile Petri dishes containing 20 mL of the sanitizer solution. Afterwards, the coupons were drawn of the sanitizer solution and immersed for 3 s in a neutralizing solution (0.1 M $Na_2S_2O_3$). The remaining cells were counted after scraping by the use of two sterile moistened swabs, which were resuspended by vigorously vortexing in 9 mL of SPW. Serial dilutions were prepared in SPW and aliquots of 100 μL were spread plated onto NA plates and incubated at 37 °C for 24 h (Ammor et al., 2004). After the incubation period, the number of viable cells was counted and the results expressed in cfu/cm^2. In control assays, the solutions of the sanitizers were replaced by sterile distilled water. The efficiency of each sanitizer was calculated regarding the difference between the counts obtained for the control surfaces and for the surfaces exposed to the sanitizers.

Table 2
Effect of peracetic acid (30 mg/L) and sodium hypochlorite (250 mg/L) on the count (Log cfu/cm^2) of *S. aureus* S3 (grown in vegetable-based broth at 7 and 28 °C) adhered onto polypropylene and stainless steel surfaces.

Sanitizers	Temperature	Control	Treated	Fraction reduction
Polypropylene				
Peracetic acid	7 °C	4.9^a	2.2^b	2.7
	28 °C	5.1^a	1.8^b	3.3
Sodium hypochlorite	7 °C	4.9^a	3.4^b	1.5
	28 °C	5.1^a	3.2^b	1.9
Stainless steel				
Peracetic acid	7 °C	5.2^a	2.9^b	2.3
	28 °C	5.9^a	3.9^b	2.0
Sodium hypochlorite	7 °C	5.2^a	3.1^b	2.1
	28 °C	5.9^a	4.1^b	1.8

Values followed for the same letters in each row differ significantly ($p < 0.05$) according to the Student *t*-test.

2.8. Procedures for scanning electron microscopy

Samples submitted to biofilm formation on polypropylene and stainless steel surfaces at 28 °C according to the procedure before cited, and after exposition to the sanitizers or distilled water, were observed for remaining of viable cells in biofilm matrix using scanning electron microscopy. The coupons were pre-fixed with glutaraldehyde (2 mL/100 mL) for 2 h at 4 °C, and post fixed with osmium tetroxide (2 g/100 mL) for 30 min at 30 °C. Afterwards, the cells were washed twice with PBS, dried at a critical point in liquid CO_2 under 95 bar pressure, gold covered (Fine coat ion sputter JFC-1100, JEOL Ltd., Tokyo, Japan) and examined with a scanning electron microscope (FEI QUANTA 200F) as previously described (Kumar & Anand, 1998; Zoltai, Zottola, & McKay, 1981).

2.9. Statistical analysis

Counts were converted to decimal Logarithmic values (Log cfu/cm^2) to nearly match the assumption of a normal distribution. Counts obtained for adhesion, detachment and biofilm formation were submitted to Analysis of Variance (ANOVA) followed by Duncan test to determine significance influence of the incubation temperature and contact surface. Counts obtained for the effect of the sanitizers (before and after the application) on the biofilm matrix were compared using paired Student's *t*-test. Data were analyzed using the software Statistica 7. A probability value $p < 0.05$ was accepted as indicating significant difference.

3. Results and discussion

The number of adhered cells of *S. aureus* S3, S28 and S54 to polypropylene and stainless steel surfaces over 72 h of incubation

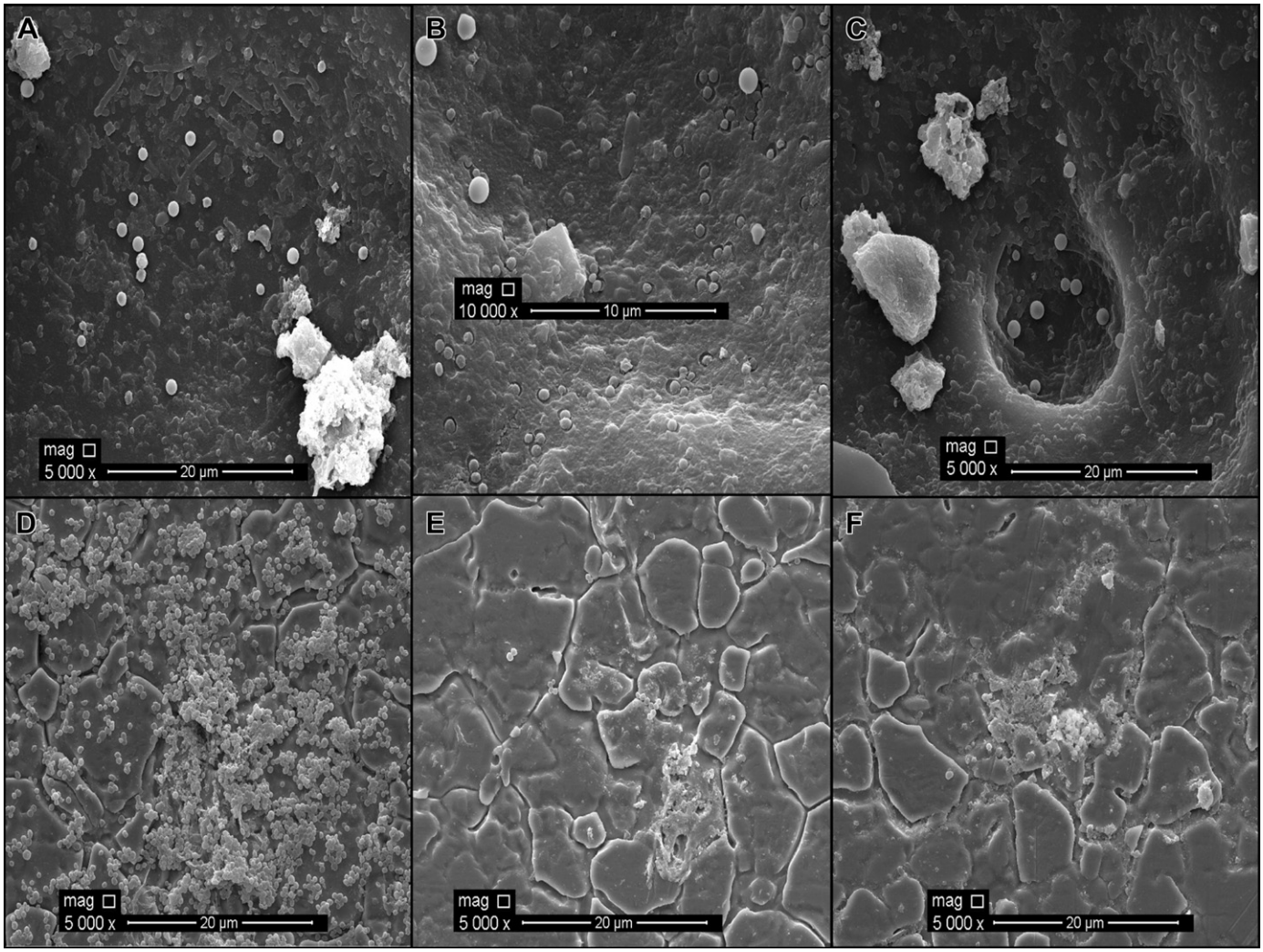

Fig. 4. Electronic microphotography of *S. aureus* S3 cells adhered on polypropylene (A, B, C) and stainless steel surfaces (D, E, F) when incubated in vegetable-based broth at 28 °C for 15 days, after the application of water (A, D – control), peracetic acid – 30 mg/L (B, E) and sodium hypochlorite – 250 mg/L (C, F).

at 7 and 28 °C is shown in Fig. 1. Regarding that the substrate and the extrinsic characteristics have been reported to interfere on the bacterial adherence, a food-based media (vegetable-based broth) and two incubation temperatures (7 and 28 °C) were tested regarding their possible influence on cell adherence and biofilm formation by isolates of *S. aureus* onto polypropylene and stainless steel surfaces. The temperature of 7 and 28 °C were chosen regarding the common temperature used to store pre-prepared vegetable products and the usual temperature found in Brazilian Food and Nutrition Services, respectively. Using liquid and/or solid food-based media as substrate for assessing the microbial growth (in planktonic or sessile form) may be useful for obtaining more realistic results than the use of laboratorial media when regarded a better feature of the nutrient availability in foods and their interaction with the surrounded environment (Herrera et al., 2007).

The highest numbers of adhered cells on polypropylene and stainless steel surfaces were found for *S. aureus* S54 in all experimental conditions. S54 showed an initial decrease in number of adhered cells on polypropylene surface at 28 °C after 48 h of incubation, followed for an increase after 72 h. *S. aureus* S3 revealed numbers of adhered cells around 5 Log cfu/cm^2 over the assessed incubation periods, with exception on stainless steel surfaces at 28 °C (counts around 4.5 Log cfu/cm^2).

The number of adhered cells of *S. aureus* S28 presented two different phases: an initial, with the number of cells on polypropylene and stainless steel surfaces ranging from 4.6 to 5.8 Log cfu/cm^2 and 4.1 to 5.0 Log cfu/cm^2, respectively, after 24 and 48 h of incubation; and a second comprising 48–72 h of incubation, when the cell counts decreased from 5.8 to 5.4 Log cfu/cm^2 and 5.0 to 3.9 Log cfu/cm^2, respectively.

Herrera et al. (2007) noted similar behavior in *S. aureus* when inoculated in phosphate buffer solution (mimicking a nutrient-lacking media) over 25 h at 25 °C. The results of the present study are in agreement with these findings which suggested that under static conditions adherent cells may be present in high numbers, but do not always increase over the incubation time. This behavior could be related with the cell division process and/or redistribution of adhered cells forming the biofilm (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003; Stoodley, Sauer, Davies, & Costerton, 2002).

There was no clear influence of the surface kind and incubation temperature on the adherence of the tested isolates. For *S. aureus* S3 highest numbers ($p < 0.05$) of adhered cells were found on polypropylene in comparison to stainless steel at both tested temperatures. For *S. aureus* S28, highest numbers ($p < 0.05$) of adhered cells were found in polypropylene at 28 °C and stainless at 7 °C.

Malheiros et al. (2010) in assays of adherence of *S. aureus* on polyethylene and stainless steel surfaces when incubated in synthetic media noted increase in counts of adhered cells at 20 °C in comparison to lower incubation temperatures (7, 10, 12 and 15 °C). Rode et al. (2007) noted highest attachment capacity in *S. aureus* on polystyrene when cultivated in synthetic media at sub-optimal temperatures (20, 25 and 30 °C). Morton, Greenway, Gaylarde, and Surman (1998) states that regardless the microbial specie or assayed surface, the adhesion process occurs at maximum intensity when bacteria are kept next to or at their optimum growth temperature.

The cell detachment (kinetic of detachment) from polypropylene and stainless steel surfaces for *S. aureus* S3, S28 and S54 at 7 and 28 °C are shown in Fig. 2. Bacterial counts revealed linear decrease in detachment rate over the contact sequence in all experimental systems. For the most systems, detached cells were around 3 Log cfu/cm^2 over the first six contacts to the agar, suggesting high persistence of cells on the surfaces over 72 h. In general, no influence ($p > 0.05$) of the surface kind and incubation temperature on the detachment of the tested strains was noted. These data regarding the detachment of *S. aureus* over a lot of contacts to a blot agar suggest a high risk source for cross contamination of foods as they passes the contaminated surfaces.

According to Heydron et al. (2000) and Cheng, Zhang, Chen, Bryers, and Jiang (2007) the maturation of a bacterial biofilm occurs between three to six days after the initial adhesion, and only after 10 days an increased population density with pronounced production and deposition of exopolysaccharide is reached (mature biofilm). Regarding these aspects, the levels of biofilm formation by *S. aureus* S3 on polypropylene and stainless surfaces over 15 days at 7 and 28 °C were assessed (Fig. 3).

The results showed a similar capacity for biofilm formation when *S. aureus* S3 was submitted to the different combinations of surface kinds and growth temperatures. The obtained counts indicated the biofilm formation on polypropylene and stainless steel surfaces at 7 and 28 °C already after 3 days of incubation. The highest numbers of cells (5.5–5.8 Log cfu/cm^2) was found after six days, with exception on polypropylene at 7 °C. For the most systems, the number of viable cells in the biofilm matrix followed a linear decrease after 6-day incubation. Highest intensity of biofilm formation on stainless steel and polypropylene was found at 28 °C.

Ronner and Wong (1993) and Wirtanen, Ahola, and Mattila-Sandholm (1995) state that a minimum of 5.0–6.0 Log cfu/cm^2 is needed for the formation of biofilm, and lower counts could indicate only an adhesion process. The literature about the biofilm formation by *S. aureus* on polypropylene and stainless steel surfaces is still scarce becoming difficult to make an extensive comparative discussion of the obtained results. Marques et al. (2007) assessing the biofilm formation by *S. aureus* on stainless steel and glass surfaces immersed in Brain Heart Infusion broth found bacterial counts of 7 and 8 Log cfu/cm^2, respectively, after 15 days of cultivation.

Counts of *S. aureus* S3 cells adhered to polypropylene and stainless surfaces after application of peracetic acid (30 mg/L) and sodium hypochlorite (250 mg/L) are shown in Table 2. When compared both sanitizers, peracetic acid was found as the most effective in reducing the viable cell count in the biofilm matrix. Log reduction caused by peracetic acid ranged from 2.0 to 3.3 Log cfu/cm^2, while for sodium hypochlorite it was from 1.5 to 2.1. However, in all experimental systems both tested sanitizers decreased ($p < 0.05$) the counts of cells adhered to the tested surfaces in comparison to the control assay.

The electron microphotography of polypropylene and stainless steel surfaces after the application of peracetic acid and sodium hypochlorite is shown in Fig. 4. It might be observed that the findings of electronic microscopy are in accordance with the results obtained in the analysis of viable cell counts on the tested surfaces when some remaining cells were found after application of peracetic acid (B, E) and sodium hypochlorite (C, F). Based on the obtained results, peracetic acid (30 mg/L) and sodium hypochlorite (250 mg/L), under the conditions used in this study, were not efficient in completely removing the cells of *S. aureus* forming a biofilm on polypropylene and stainless steel surfaces.

Even regarding the limitations of this study in assessing the adhesion, detachment and biofilm formation of a monospecies inoculum under static condition, it could be concluded that the assayed isolates of *S. aureus* presented highlighted capacity to adhere and form biofilm on stainless steel and polypropylene surfaces when immersed in a food-based broth at 7 and 28 °C. Moreover, the sanitizers peracetic acid and sodium hypochlorite were not efficient in totally removing the viable cells of *S. aureus* forming a mature biofilm. These results encourage further researches focusing on the capability of *S. aureus* to adhere, detach and form biofilm on surfaces of equipment and utensils used in food and nutrition services and the efficacy of classical and alternative sanitizers to reduce the number of cells in the biofilm matrix.

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