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Article

Adhesion and biofilm formation by *Staphylococcus aureus* from food processing plants as affected by growth medium, surface type and incubation temperature

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This study assessed the effect of different growth media [BHI broth, BHI broth plus glucose (10 g/100 mL) and BHI broth plus NaCl (5 g/100 mL)] and incubation temperatures (28 or 37 ºC) on the adherence, detachment and biofilm formation on polypropylene and stainless steel surfaces (2 x 2 cm coupons) for a prolonged period (24-72 h) by some strains of *Staphylococcus aureus* (S3, S28 and S54) from food processing plants. The efficacy of the sanitizers sodium hypochlorite (250 mg/mL) and peracetic acid (30 mg/mL) in reducing the number of viable bacterial cells in a preformed biofilm was also evaluated. *S. aureus* strains adhered in highest numbers in BHI broth, regardless of the type of surface or incubation temperature. Cell detachment from surfaces revealed high persistence over the incubation period. The number of cells needed for biofilm formation was noted in all experimental systems after 3 days. Peracetic acid and sodium hypochlorite were not efficient in completely removing the cells of *S. aureus* adhered onto polypropylene and stainless steel surfaces. From these results, the assayed strains revealed high capacities to adhere and form biofilms on polypropylene and stainless steel surfaces under the different growth conditions, and the cells in biofilm matrixes were resistant to total removal when exposed to the sanitizers sodium hypochlorite and peracetic acid.

Uniterms: *Staphylococcus aureus*/food processing. Biofilm. Surfaces. Sanitizers/efficacy.

Este estudo teve como objetivo avaliar o efeito de diferentes meios de crescimento [caldo BHI, caldo BHI adicionado de glucose (10 g/100 mL) e caldo BHI adicionado de NaCl (5 g/100 mL)] e temperaturas de incubação (28 e 37 °C) sobre a adesão, separação e formação de biofilme sobre superfícies (2 x 2 cm) de polipropileno e aço inoxidável durante longo tempo de incubação (24-72 h) por parte de cepas de *Staphylococcus aureus* (S3, S58 e S54) isoladas de plantas de processamento de alimentos. Também foi avaliada a eficácia dos sanitizantes hipoclorito de sódio (250 mg/mL) e ácido peracético (30 mg/mL) na redução do número de células bacterianas viáveis presentes em um biofilme pré-formado. As cepas de *S. aureus* aderiram em número mais elevado quando incubadas em caldo BHI em ambos os tipos de superfícies e temperaturas de incubação testadas. A separação das células das superfícies revelou alta persistência ao longo do período de incubação. Número de células necessário para a formação do biofilme foi detectado depois de três dias de incubação em todos os sistemas experimentais. O ácido peracético e o hipoclorito de sódio não foram eficientes em remover completamente a células de *S. aureus* aderidas sobre as superfícies de polipropileno e aço inoxidável. Os resultados obtidos revelaram alta capacidade das cepas ensaiadas em aderir e formar biofilme sobre superfícies de polipropileno e aço inoxidável sobre diferentes condições de crescimento e que as células na matriz do biofilme apresentaram-se resistentes à total remoção quando expostas aos sanitizantes hipoclorito de sódio e ácido peracético.

Unitermos: *Staphylococcus aureus*/ processamento de alimentos. Adesão. Superfícies. Biofilme. Sanitizantes/eficácia.

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INTRODUCTION

The relevance of contaminated surfaces in spreading pathogenic microorganisms to foods is already well established for food processing plants (Kusumaningru *et al.*, 2003; Fuster-Valls *et al.*, 2008). Some pathogenic bacteria are able to adhere to food-contact surfaces and remain viable even after cleaning and disinfection (Ammor *et al.*, 2004). One of the most common ways for bacteria to live is adhering to surfaces and forming biofilms in which they are embedded in an organic, extracellular polymeric matrix (Chae, Schraft, 2000).

Biofilm-adhered bacteria can detach during processing and contaminate food as it passes the surfaces. This cross-contamination is a high-risk source of pathogenic bacteria and may seriously affect the safety and quality of foods (Bagge-Ravn *et al.*, 2003).

Staphylococcus aureus is among the most common pathogenic bacteria isolated from surfaces in food processing plants (Pastoriza *et al.*, 2002), where it can adhere and form biofilms (Kunig, Almeida, 2001). Foodborne disease caused by *S. aureus* is typically intoxication due to the ingestion of enterotoxins preformed in food by enterotoxigenic strains (Normanno *et al.*, 2007). Most investigations regarding biofilm formation by staphylococci using *S. epidermidis* as the model microorganism have focused on clinical aspects in association with implants and medical devices (Rode *et al.*, 2007). Whereas the capacity for adhesion and biofilm formation of some food-related pathogenic bacteria has already been elucidated (Conlon *et al.*, 2002), studies focusing on the adhesion and biofilm formation of *S. aureus* are still rare. Regarding these aspects, it is important to identify the conditions under which *S. aureus* is able to survive and multiply on food processing surfaces. Early studies found that bacterial adhesion and biofilm formation depended upon the bacterial species, the nature of the surface, the growth medium and other environmental conditions (Pompermayer, Gaylarde, 2007).

The present study aimed to evaluate the capability of strains of *S. aureus* from surfaces of food processing plants to adhere and form biofilms on polypropylene and stainless steel surfaces when exposed to different growth media and incubation temperatures. The study also assessed the effects of the sanitizers peracetic acid and sodium hypochlorite in reducing the number of viable bacterial cells in a preformed biofilm.

MATERIAL AND METHODS

Test strains

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 microorganisms. These strains were isolated from different surfaces of Food Processing Plants using standard procedures. Stock cultures were kept on Nutrient Agar (NA) (Difco, Brazil) slants under refrigeration (7 ± 1 ºC).

Unless otherwise stated, inocula (10 log cfu/mL) used in assays were obtained from suspensions of the strains in the stationary phase of growth and prepared following a previously described procedure (Carson *et al.*, 2002). The suspensions obtained were serially diluted in PBS (10^{-1} - 10^{-2}) to provide a viable cell count of approximately 8.0 log cfu/mL.

Surfaces

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

Experimental conditions

The adherence, detachment and biofilm formation of the test strains were assessed under six different experimental conditions: i) in the presence of a nutrient-rich medium using Brain Heart Infusion Broth (BHI) at 28 ºC; ii) in the presence of a nutrient-rich medium (BHI) at 37 ºC; iii) in the presence of a nutrient-rich and adhesion-promoting medium, BHI supplemented with 10 g/L of glucose (BHI-Glucose) at 28 ºC; iv) in the presence of a nutrient-rich and adhesion-promoting medium (BHI-Glucose) at 37 ºC; v) in the presence of a selective medium, BHI supplemented with 5 g/L of NaCl (BHI-NaCl) at 28 ºC; and vi) in the presence of a selective medium (BHI-NaCl) at 37 ºC.

Adhesion to surfaces and quantification of adhered cells

An aliquot of 100 µL of the growth media was mixed with 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) for 15 s to release non-adhered cells. The cells adhered to the coupons were collected by thoroughly rubbing their surfaces with two moistened swabs, and the cells were resuspended in

SPW with vigorous 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 ºC (Herrera *et al.*, 2007; Rode *et al.*, 2007). After the incubation period, the number of viable cells was counted, and the results were expressed as Log cfu/cm^2.

Detachment of adhered cells

An aliquot of 100 µL of the growth media was mixed with 50 µL of the bacterial inoculum and plated onto the center of each coupon, followed by 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 for 15 s to release non-adhered cells. Each coupon was placed onto 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. 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 ºC (Herrera *et al.*, 2007). After the incubation period, the number of viable cells was counted and the results expressed in Log cfu/cm^2.

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 ºC and 28 ºC over 15 days was assessed. For this measurement, 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-adherent cells. Once again, the coupons were immersed in a fresh medium containing the same amount of inoculum, and the process was repeated four times over a 15-day period.

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

Sanitizer application

The efficacies of the sanitizers sodium hypochlorite (250 mg/L) and peracetic acid (30 mg/L) (Meira *et al.*, 2012) in removing the cells of *S. aureus* S3 from the biofilm matrix grown in the vegetable-based broth at 7 and 28 ºC were assessed. For this assessment, five coupons were allowed to develop biofilms according to the experimental conditions cited above. After 15 days of incubation, the coupons were washed in SPW and immersed for 30 s in sterile Petri dishes containing 20 mL of the sanitizer solution. Afterwards, the coupons were withdrawn from 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 with two sterile moistened swabs and 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 ºC for 24 h (Ammor *et al.*, 2004). After the incubation period, the number of viable cells was counted and the results expressed as Log cfu/cm^2. In control assays, the sanitizer solutions were replaced by sterile distilled water. The efficiency of each sanitizer was calculated as the difference between the counts obtained for the control surfaces and for the surfaces exposed to the sanitizers.

Reproducibility and statistical analysis

All analyses were carried out in triplicate, and the results are expressed as the average of the assays. 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 the Duncan test to determine the significance of the influences of the incubation temperatures and contact surfaces. Counts obtained for tests of the effects of the sanitizers (before and after the application) on the biofilm matrix were compared using the paired Student´s t-test. Data were analyzed using the software Statistica 7. A probability value $P < 0.05$ was accepted as indicating significant differences (Meira *et al.*, 2012).

RESULTS AND DISCUSSION

As substrate and extrinsic characteristics have been reported to influence bacterial adherence, different

growth media (nutrient rich-media, with or without added glucose and NaCl) and incubation temperatures (28 and 37 ºC) were tested to determine their possible influences on cell adherence and biofilm formation by isolates of *S. aureus* on polypropylene and stainless steel surfaces. The temperatures 37 ºC and 28 ºC were chosen as the ideal temperature for the growth of S. *aureus* and the common environmental temperature found in Brazilian Food and Nutrition Services, respectively.

The numbers of *S. aureus* cells adhered to polypropylene and stainless steel surfaces under different experimental conditions over 72 h of incubation are shown in Figures 1-3. The highest numbers of adhered cells (6 – 8 log cfu/cm^2) on polypropylene and stainless steel surfaces were found when the strains were cultivated in BHI. There was no indication that adherence increased in BHI-NaCl or BHI-Glucose media. It appears surprising that the presence of glucose and NaCl in the growth media caused no increase in the adhesion capacity of the assayed strains. Moretro *et al.* (2003) found that the presence of NaCl (2 g/100 mL) in tryptic soy broth (TSB) resulted in increased adhesion and biofilm formation by standard cultures of staphylococci from food and food processing environments. Herrera *et al.* (2007) also noted the same behavior for a standard culture of *S. aureus* cultured in TSB supplemented with glucose (1 g/100 mL).

S. aureus S3 and S54 cultured in BHI at both tested temperatures revealed a clear two-phase adhesion pattern regardless of the surface type: an initial phase with a progressively increasing numbers of adhered cells, with highest counts after 48 h of incubation, followed by a second phase (72 h) with a decreasing number of adhered cells. These results suggest that under static conditions, the adhered cells may be present in high numbers, but the number of adhered cells do not constantly increase over the incubation time.

S. aureus S54 presented a different pattern of adhesion in most experimental systems, with a decrease in the numbers of adhered cells over the evaluated time intervals at 28 and 37 ºC. Data for the number of adhered cells on stainless steel and polypropylene surfaces did not differ ($P > 0.05$) for strains S3 and S28 in all cultivation media at 37 ºC. For strain S54, there were greater numbers ($P < 0.05$) of adhered cells on stainless steel surfaces than on polypropylene in BHI and BHI-glucose.

Regarding the effect of the incubation temperature on the adherence capability of the tested strains, it was surprising to note that no difference ($P > 0.05$) was found between 28 ºC and 37 ºC. Some previous studies have reported a positive effect of lower temperature on the adhesion pattern of *S. aureus*. Herald and Zottola (1988) noted that *Listeria monocytogenes* and *Yersinia enterocolitica* cultivated in laboratorial media adhered to stainless surfaces in greater numbers at 21 ºC than at 30 ºC. Rode *et al.* (2007) found higher attachment capacity for *S. aureus* on polystyrene when cultivated in tryptic soy broth at sub-optimal temperatures (20, 25 and 30 ºC). Morton *et al.* (1998) reported that regardless of the species or surface assayed, the adhesion process occurs at maximum intensity when microorganisms are allowed to grow at their optimum temperatures.

Among the tested strains, *S. aureus* S3 was used for

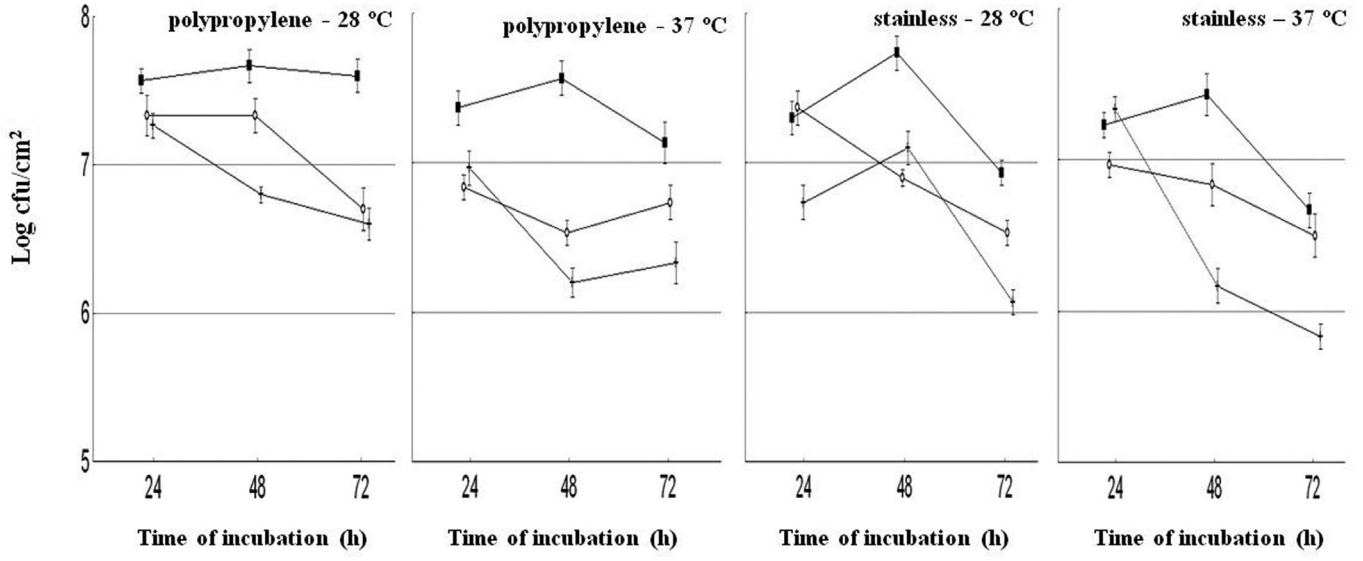

FIGURE 1 - Adhesion of *S. aureus* S3 to polypropylene and stainless steel surfaces as affected by different experimental conditions (■: BHI; ◊: BHI-Glucose; +: BHI-NaCl) over 72 h of incubation.

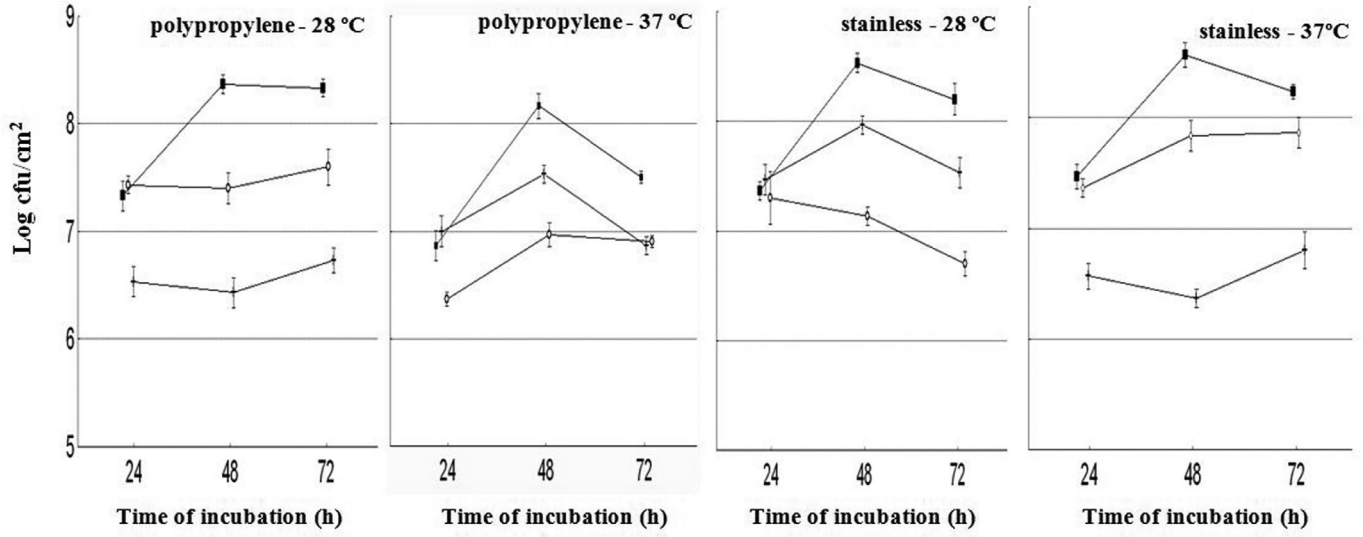

FIGURE 2 - Adhesion of *S. aureus* S28 to polypropylene and stainless steel surfaces as affected by different experimental conditions (■: BHI; ◊: BHI-Glucose; +: BHI-NaCl) over 72 h of incubation.

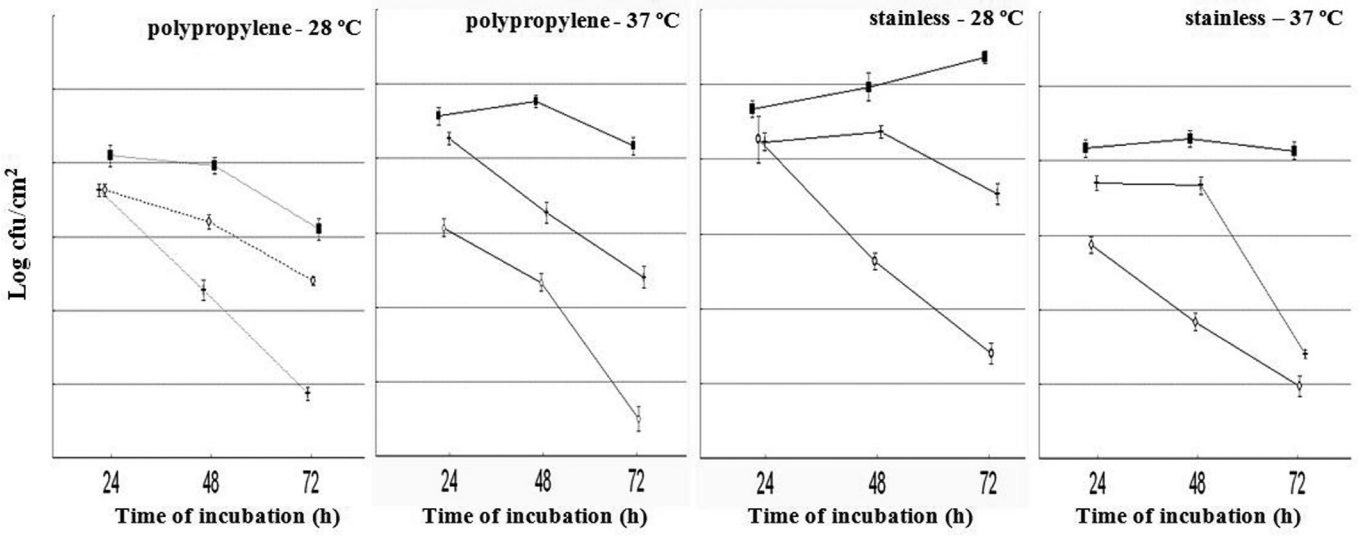

FIGURE 3 - Adhesion of *S. aureus* S54 to polypropylene and stainless steel surfaces as affected by different experimental conditions (■: BHI; ◊: BHI-Glucose; +: BHI-NaCl) over 72 h of incubation.

further assays of detachment and biofilm formation. Data for cell detachment from polypropylene and stainless steel surfaces for *S. aureus* S3 under different experimental conditions are shown in Figures 4 and 5, respectively. Bacterial counts revealed a linear decrease in the detachment rate over the contact sequence for all experimental systems. Regarding the influence of the growth media, the results obtained showed higher detachment values ($P < 0.05$) when the strain was grown in BHI compared to other media. In all experimental systems, the cell detachment was at least 3 Log cfu/cm^2 during the first 5 contacts (blots), suggesting high persistence of cells on the surfaces over 72 h. No significant influence ($P > 0.05$) of the surface type or time of incubation was noted on the detachment rate. The highest numbers of detached cells were observed when *S. aureus* S3 was incubated in BHI, which could be related to increased bacterial growth on this substrate relative to the other growth media. These data about the detachment of cells over a large number of contacts with blot agar reveal a high risk of dissemination for *S. aureus* in food processing plants.

Levels of biofilm formation by *S. aureus* S3 on polypropylene and stainless surfaces over 15 days under different experimental conditions were also evaluated. For most systems, the number of cells in the biofilm matrix followed a linear decrease over the assayed incubation

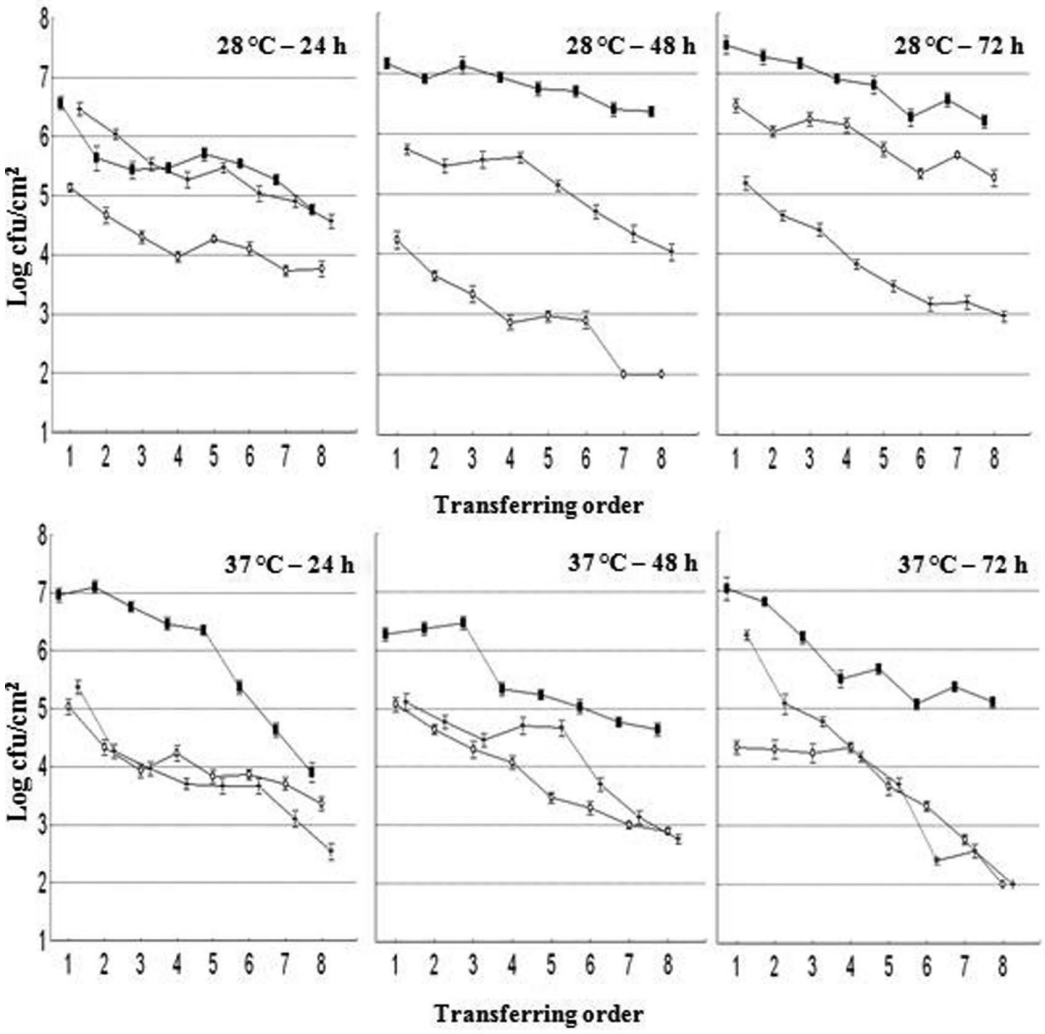

FIGURE 4 - Detachment of *S. aureus* S3 of polypropylene surfaces as affected by different experimental conditions (■: BHI; ◊: BHI-Glucose; +: BHI-NaCl).

TABLE I - Effect of peracetic acid (30 mg/L) and sodium hypochlorite (250 mg/L) on counts (Log cfu/cm²) of *S. aureus* S3 (grown in BHI broth at 28 and 37 °C) adhered to polypropylene and stainless surfaces

Sanitizer	Temperature (°C)	Control	Treated	Fraction reduced
Polypropylene				
Peracetic acid	28	7.3 (±0.3) [a]	3.8 (±0.2) [b]	3.5
	37	7.4 (± 0.3) [a]	3.3 (± 0.2) [b]	4.1
Sodium hypochlorite	28	7.1 (±0.4) [a]	2.7 (±0.3) [b]	4.4
	37	7.4 (± 0.4) [a]	2.6 (± 0.3) [b]	4.8
Stainless steel				
Peracetic acid	28	6.9 (±0.2) [a]	4.1 (±0.2) [b]	2.8
	37	7.7 (± 0.2) [a]	4.2 (± 0.4) [b]	3.6
Sodium hypochlorite	28	6.9 (±0.4) [a]	2.6 (±0.4) [b]	4.3
	37	7.7 (± 0.3) [a]	3.1 (± 0.3) [b]	4.6

Values followed by the same letters in each line differ significantly ($p<0.05$) according to the Student t test.

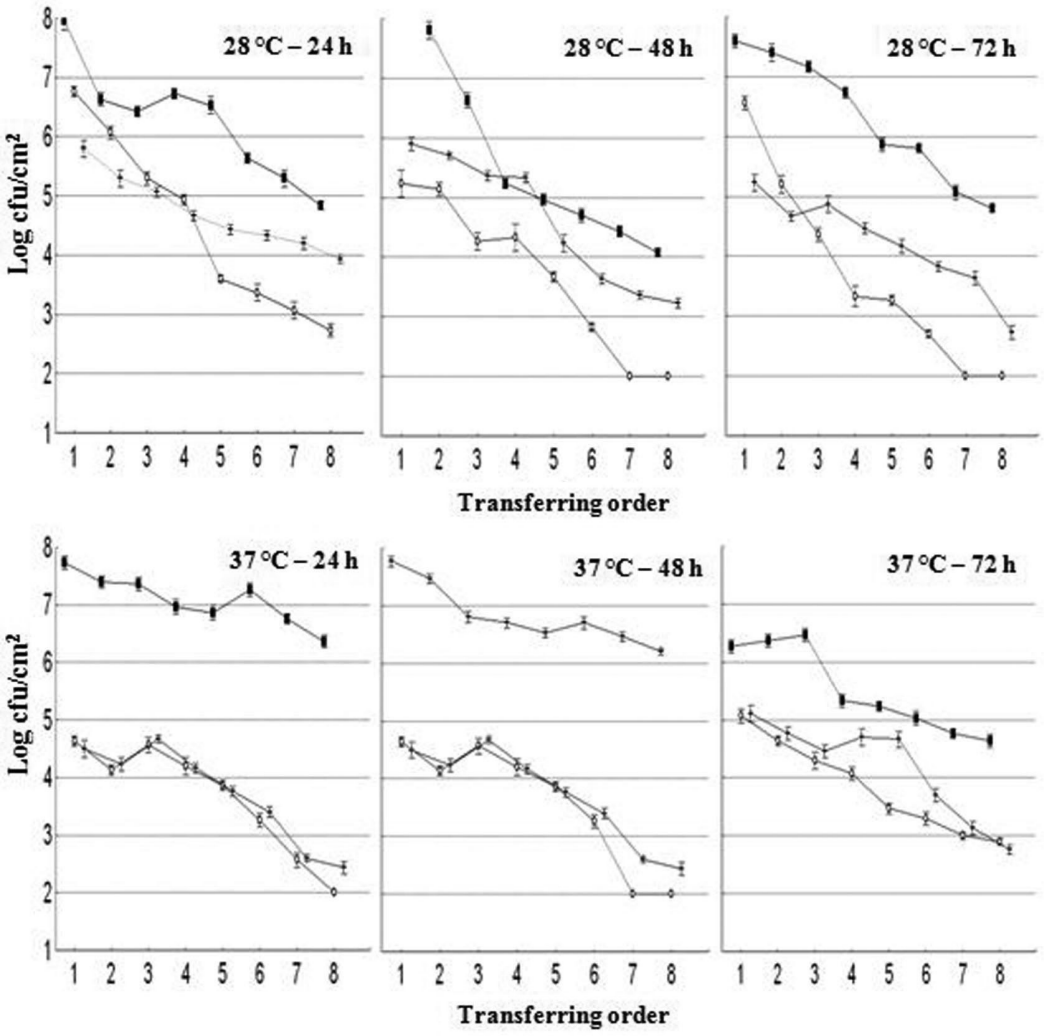

FIGURE 5 - Detachment of *S. aureus* S3 of stainless steel surfaces as affected by different experimental conditions (■: BHI; ◊: BHI-Glucose; +: BHI-NaCl).

periods (data not showed). For most experimental conditions, the maximum number of cells (approximately 6 log cfu/cm^2) was found after 6 days of incubation. The bacterial counts indicated biofilm formation on both surfaces under all experimental systems after only 3 days. Greater than 6 – 7 log cfu/cm^2 of viable cells are needed for biofilm formation, and lower counts could indicate an adhesion process (Planchon *et al*., 2006).

Counts of *S. aureus* S3 cells adhered to polypropylene and stainless surfaces before and after application of peracetic acid (30 mg/L) and sodium hypochlorite are shown in Table I. The decrease in cell counts caused by sodium hypochlorite (250 mg/L) was 2.6–3.1 Log cfu/cm^2, while for peracetic acid, the decrease was 3.3 – 4.2 Log cfu/cm^2. However, in all experimental systems, both sanitizers greatly decreased ($P < 0.05$) the counts of cells adhered to the assayed surfaces. According to the results of these experiments, the sanitizers peracetic acid and sodium hypochlorite, in the concentrations assayed, were not efficient in completely removing the cells of *S. aureus* that had formed biofilms on polypropylene and stainless steel surfaces. Residual cells adhered to the surfaces after the application of sanitizers reinforce the concept of biofilm as a substantial source of cross contamination in food processing plants.

CONCLUSIONS

The results of this study have clear implications for designing strategies to control cross-contamination in food processing environments because the strains used as test organisms were isolated directly from food-contact surfaces and the assays for adherence and biofilm formation were carried out using surfaces commonly found in food processing plants. The strains assayed here revealed significant abilities to adhere and form biofilms on the as-

sayed surfaces when exposed to different environmental conditions, suggesting that the clumping phenotype (aggregation) of the tested strains did not show any clear influence of the surrounding environment with regard to the surface type, growth media or temperature of incubation.

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