Effectiveness of cleaning and sanitizing procedures in controlling the adherence of *Pseudomonas fluorescens*, *Salmonella* Enteritidis, and *Staphylococcus aureus* to domestic kitchen surfaces

Eficiência dos procedimentos de limpeza e de sanitização no controle da adesão de Pseudomonas fluorescens, Salmonella Enteritidis e Staphylococcus aureus em superfícies usadas em cozinhas domésticas

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Abstract

The effectiveness of cleaning and sanitizing procedures in controlling *Staphylococcus aureus, Salmonella* Enteritidis, and *Pseudomonas fluorescens* adhered to granite and stainless steel was evaluated. There was no significant difference ($p \geq 0.05$) in the adherence of pure cultures of these microorganisms to stainless steel. The numbers of *P. fluorescens* and *S.* Enteritidis adhered to granite were greater ($p < 0.05$) than the numbers of *S. aureus*. Additionally, the adherence of *P. fluorescens* was similar to the adherence of *S.* Enteritidis on granite surface. In a mixed culture with *P. fluorescens*, *S aureus* adhered less ($p < 0.05$) to stainless steel surfaces (1.31 log CFU.cm^{-2}) than when in a pure culture (6.10 log CFU.cm^{-2}). These results suggest that *P. fluorescens* inhibited the adherence of *S. aureus*. However, this inhibition was not observed in the adherence process for granite. There was a significant difference ($p < 0.05$) between the number of adhered cells before and after pre-washing for *S. aureus* on stainless steel and granite surfaces, and after washing with detergent for all microorganisms and surfaces. The efficiency of the cleaning plus sanitizing procedures was not significantly different ($p \geq 0.05$) between the surfaces. However, a significant difference was observed ($p < 0.05$) between the sanitizer solutions. Sodium hypochlorite and peracetic acid were more bactericidal ($p < 0.05$) than a quaternary ammonium compound. With regard to microorganisms, *S. aureus* was the least resistant to the sanitizers. These results show the importance of good cleaning and sanitization procedures to prevent bacterial adherence and biofilm formation.
Keywords: *cleaning and sanitizing; food contact surfaces; bacterial adherence.*

Resumo

A eficiência dos procedimentos de limpeza e sanitização no controle de *Staphylococcus aureus, Salmonella* Enteritidis e *Pseudomonas fluorescens* aderidas em granito e aço inoxidável foi avaliada. Não houve diferença significativa ($p \geq 0,05$) na adesão destes microrganismos quando em cultura pura, em aço inoxidável. O número de células aderidas de *P. fluorescens* e *S.* Enteritidis foi maior ($p < 0,05$) que o número de células de *S. aureus*, em granito. Além disso, a adesão de *P. fluorescens* foi similar a de *S.* Enteritidis nesta última superfície. Em cultura mista com *P. fluorescens*, *S. aureus* aderiu menos ($p < 0,05$) em aço inoxidável (1,31 log UFC.cm^{-2}) do que quando em cultura pura (6,10 log UFC.cm^{-2}). Estes resultados sugerem que *P. fluorescens* inibiu a adesão de *S. aureus*. Entretanto, esta inibição não foi observada no processo de adesão em granito. Houve diferença significativa entre o número de células aderidas de *S. aureus* antes e após a pré-lavagem em ambas as superfícies e após o procedimento de limpeza para todos os microrganismos e todas as superfícies. A eficiência dos procedimentos de limpeza e sanitização não diferiram significativamente ($p \geq 0,05$) entre as superfícies. Entretanto, observou-se uma diferença ($p < 0,05$) entre as soluções sanitizantes utilizadas. Hipoclorito de sódio e ácido peracético apresentaram maior ação bactericida ($p < 0,05$) que o composto de amônia quaternária. Observou-se que *S. aureus* apresentou menor resistência à ação desses sanitizantes. Os resultados mostram a importância da adequada realização dos procedimentos de limpeza e sanitização para evitar a adesão bacteriana e formação de biofilme.
Palavras-chave: *limpeza e sanitização; superfícies para processamento de alimentos; adesão bacteriana.*

1 Introduction

The number of foodborne disease outbreaks due to bacteria has increased in recent years (SANTOS, 2004). Several potential causes of these outbreaks include storage temperature, inadequate thermal treatment, cross contamination, poor hygiene conditions of processing facilities, and contaminated food contact surfaces (COGAN et al., 2002; DEVERE; PURCHASE, 2007). Domestic and industrial kitchens are the most important focuses of attention for infection. In such environments, cross-contamination is the responsible factor for diseases spread by food (TEIXEIRA et al., 2007).

In Brazil, domestic kitchens, small scale food industries, and bakeries use granite to construct benches and counter top

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tables. These surfaces are able to support microbial adherence and biofilm formation for pathogenic and spoilage bacteria. Microorganisms in biofilms are more resistant to cleaning and sanitizing procedures because they are protected against chemical agents by the exopolyssacharides that they produce (CRIADO; SUÁREZ; FERREROS, 1994). The detergents applied in cleaning processes play an important role in the control of adherence, and are responsible for the removal of organic food residues such as protein, fat, and carbohydrate from surfaces (ZOTOLLA, 1997). The mechanisms proposed to explain the adherence process and biofilm formation in the first stage include conditioning of the surface by the deposition of nutrients. Subsequently, the process is consolidated and the formation of a biofilm occurs (ZOTTOLA; SASAHARA, 1994).

Inadequate cleaning and sanitizing procedures can lead to outbreaks of several pathogenic microorganisms, causing foodborne diseases. Poor cleaning or sanitizing may also lead to the contamination of food with spoilage microorganisms, also responsible for economic losses (JAY, 2005; PINTO; 2004).

This research study evaluates the efficiency of cleaning and sanitizing procedures in the control of *Staphylococcus aureus,* *Salmonella* Enteritidis*,* and *Pseudomonas fluorescens* adhered to granite and stainless steel.

2 Materials and methods

These experiments were conducted in three phases. The first phase studied the adhesion of pure cultures of *Staphylococcus aureus* ATCC 6538*, Pseudomonas fluorescens* ATCC 13525*,* and *Salmonella* Enteritidis ATCC 6539 on granite, and AISI 304 stainless steel coupons test (100 mm × 100 mm × 5 mm). The second phase assessed the efficiency of a cleaning procedure against pure-cultured cells adhered to granite and stainless steel. This stage included a pre-washing step and a washing step using a surfactant-based detergent. The third phase assessed the efficiency of the cleaning procedure plus a sanitizing procedure against the pure-cultured cells adhered to granite and stainless steel using sodium hypochlorite, peracetic acid, and a quaternary ammonium compound.

2.1 Microorganisms and culture media

The studies on adherence were conducted using suspensions of *Staphylococcus aureus* ATCC 6538*, Pseudomonas fluorescens* ATCC 13525*,* and *Salmonella* Enteritidis ATCC 6539. One mL of these cultures was kept at –80 °C in nutrient broth (Merck, São Paulo, BR) mixed with glycerol (80:20 ratio). A working culture was prepared by inoculating 100 µL of frozen culture into 10 mL of nutrient broth followed by incubation at 28 °C for 24 hours for *P. fluorescens* and 37 °C for 24 hours for *Staphylococcus aureus*, and *Salmonella* Enteritidis. The culture was sub-cultured twice before use.

2.2 Bacterial attachment

For microorganism attachments*,* three coupon test of each material were first cleaned with a neutral liquid detergent and water, rinsed with distilled water, and then sterilized at 121 °C/15 minutes. The cleaned and sanitized coupons were added to the nutrient broth-containing 1000 mL beakers previously inoculated with 1 mL of three ten-fold dilution from one of each initial suspensions containing 9 log CFU.mL^{-1}, which were determined for the plate count method in a previous test: i) pure cultures of *P. fluorescens*; ii) pure cultures of *S. aureus*; iii) pure cultures of *S.* Enteritidis; iv) mixed cultures of *P. fluorescens* plus *S. aureus*; v) mixed cultures of *S.* Enteritidis plus *S. aureus*.

The beakers were incubated statically, which better represent the conditions in domestic kitchens, at 28 °C for 12 hours. After incubation, the coupons were statically immersed in 0.1% peptone water for 1 minute to remove plancktonic cells. Then, the sessile cells on the surface were swabbed (EVANCHO et al., 2001) and appropriate dilutions of the cell suspension were plated onto nutrient agar (Merck, São Paulo, BR), containing 0.25% sodium tiossulphate to neutralize sodium hypochlorite solution, and peracetic acid, and 0.1% soy lecithin to neutralize quaternary ammonium solution. The results were expressed in log CFU.cm^{-2}.

2.3 Cleaning and sanitizing procedures

a) Cleaning procedure

The coupon tests were cleaned as follows: a pre-rinse with water at room temperature (20-25 °C) for 1 minute; a wash with neutral detergent-based biodegradable anionic surfactant at 5% by brushing with tweezer and a polystyrene sponge sterilized at 121 °C/15 minutes, followed by a water rinsing to remove mineral and organic residues from the surfaces.

b) Cleaning procedure plus sanitizing procedure

After cleaning, as in item 2.3a, the surfaces were sanitized with three different chemical agents: 60 mg.L^{-1}, pH = 3.0 of peracetic acid (Pluron, São Paulo, BR); 200 mg.L^{-1} ammonium quaternary compound, pH = 9 (Indeba); and 100 mg.L^{-1} of total available chlorine, expressed as Cl_2 prepared from sodium hypochlorite (Unilever), pH = 10. The diluted solutions were prepared from the commercial products, whose active principles were evaluated according to the methodologies proposed by APHA (1998).

2.4 Microbiological analysis

Before and after each stage of the hygiene procedure, the samples were collected by researchers wearing protective clothing and using individual equipments. Appropriate dilutions were plated onto nutrient agar to count the cells in the pure culture. Selective media were used for mixed cultures Baird Parker agar (Micromed®), Cetremide agar (Merck®), and *Salmonella Shigella* agar (Micromed®) to count *Staphylococcus aureus, Pseudomonas fluorescens,* and *Salmonella* Enteritidis, respectively.

2.5 Statistical analyses

The experiments were conducted in a random factorial scheme with three repetitions. Log 10 CFU.cm^{-2} was analyzed

by variance analysis using SAS (Statistical Analysis System), version 9.0 (2005). The average of log CFU.cm^{-2} was compared by the Duncan test. The level of significance adopted was 5%. The factors related to the first experiment were three surfaces and three microorganisms in pure culture, and seven microorganisms and three surfaces in mixed cultures. In the second experiment, the factors were two treatments (pre-washing and washing with detergent), two surfaces, and three microorganisms. In the third experiment, the factors were three microorganisms, two surfaces, and four treatments (sodium hypochlorite, peracetic acid, ammonium quaternary compound, and control).

3 Results and discussions

3.1 Bacterial adherence to surfaces

There was no significant difference ($p \geq 0.05$) in the adherence of the pure cultures of the microorganisms to stainless steel (Table 1). However, the number of *P. fluorescens* and *S.* Enteritidis adhered to granite was higher ($p < 0.05$) than the number of *S. aureus*. The adherence of *P. fluorescens* was similar to that of *S.* Enteritidis on the granite surface. According to Careli et al. (2008), *P. fluorescens* adhered to granite and marble to a greater extent after 10 hours than to stainless steel.

This similar adherence of *P. fluorescens* and *S.* Enteritidis to all surfaces (Table 1) is probably related to the fact that both microorganisms are able to express genes that induce the synthesis of cellular appendages such as flagella. This is not the case with *S. aureus*. Flagella, as well as fimbria and *pili*, which help the cells in the adherence process because they can mediate the cell's approach to surfaces by decreasing the electrostatic repulsion between the cell surfaces and food contact surfaces.

The physicochemical properties of cell surfaces are an important aspect in active bacterial adhesion. The surfaces of most bacterial cells are negatively charged, and the extent of the negative charge varies with growth environments. The net negative charge of the cell surface is adverse to bacterial adhesion due to electrostatic repulsive force. These forces keep cells a short distance away from the surface. However, the bacterial cell-surface possesses hydrophobicity due to fimbriae, flagella and lipopolysaccharide (LPS). The importance of a hydrophobic surface is to reduce the repulsive force of interaction between two surfaces. For example, thin aggregative fimbriae (Tafi) and cellulose are the two main matrix components in *Salmonella* biofilms. The co-expression of thin aggregative fimbriae and cellulose leads to the formation of a highly hydrophobic network with tightly packed cells aligned in parallel in a rigid matrix (SHI; ZHU, 2009).

All species evaluated were able to adhere to surfaces, reaching between 4.55 and 6.10 log CFU.cm^{-2} (Table 1). Similar results in the adherence of *S. aureus* to stainless steel were observed in a previous study (PARIZZI et al., 2004) that found approximately 5.0 log CFU.cm^{-2} after 12 hours of contact at 30 °C. Another study showed that the adherence of *P. fluorescens* reached 6.12 log CFU.cm^{-2} (CARELI et al., 2008).

In mixed culture with *P. fluorescens* or *S.* Enteritidis, *S. aureus* adhered significantly less ($p < 0.05$) to stainless steel surfaces (Table 2) than in pure culture (Table 1). These results suggest that *P. fluorescens* and *S.* Enteritidis were able to inhibit the adherence of *S. aureus*. However, this inhibition was not observed in the adherence process for granite. Competition between the microorganisms was observed, decreasing the number of adhered cells of both species (McELDOWNEY; FLETCHER, 1987). According to Jay (2005), *S. aureus* is a poorly competitive microorganism when compared to the antagonistic behavior of *Pseudomonas.*

A research study showed that the numbers of *S.* Enteritidis were higher than *Listeria monocytogenes* or *Yersinia enterocolitica* (CLOAK et al., 1999) in a mixed culture. This study suggests that *S.* Enteritidis is a better competitor. Another study showed that adherence of *E. coli* was higher than *S. aureus* to polypropylene. Generally, Gram-negative microorganisms adhere in higher numbers when compared to Gram-positive microorganisms (POMPERMAYER; GAYLARDE, 2000).

3.2 Evaluation of the cleaning procedures

There were significant differences ($p < 0.05$) in the log number of adhered cells before and after pre-washing of *S. aureus* adhered to stainless steel and granite surfaces as well as after washing with detergent for all microorganisms and surfaces (Table 3).

It is important to realize that *S. aureus* cells adhered more on stainless steel than on granite (Table 3), however these cells were removed more from stainless steel than from granite surfaces for all treatments. This decrease on the efficiency of cleaning and

Table 1. Number of cells of *Pseudomonas fluorescens, Salmonella* Enteritidis, and *Staphylococcus aureus* (log CFU.cm^{-2}) adhered to surfaces.

Bacteria	Stainless steel	Granite
P. fluorescens	5.94^{Aa}	5.93^{Aa}
S. Enteritidis	5.26^{Aa}	6.00^{Aa}
S. aureus	6.10^{Aa}	4.55^{Ba}

Means followed by the same capital letter in the column or by a minor letter in the same line did not differ by the Duncan test at 5% probability. Values represent the mean of three repetitions.

Table 2. Number of microorganisms cells (log CFU.cm^{-2}) in mixed cultures adhered to surfaces.

Microorganisms	Stainless steel	Granite
Mixed 1		
P. fluorescens	3.66^{Aa}	3.16^{Aa}
S. aureus	1.31^{Ba}	2.29^{Ab}
Mixed 2		
S. Enteritidis	4.22^{Aa}	4.17^{Aa}
S. aureus	3.16^{Ba}	3.93^{Aa}

Mixed 1: *Pseudomonas fluorescens* + *Staphylococcus aureus* and, Mixed 2: *Salmonella* Enteritidis + *Staphylococcus aureus.* Means followed by the same capital letter in the column or by a minor letter in the same line did not differ at 5% level of probability by the Duncan test. Values represent the mean of three repetitions.

Table 3. Number of microorganisms cells (log CFU.cm^{-2}) adhered on surfaces after pre-washing and washing with detergent.

Microorganisms	Surfaces	Initial number	After pre-washing	After washing with detergent
S. aureus	Stainless steel	6.10^{Aa}	3.72^{Bb}	0.00^{Bc}
	Granite	4.55^{Ba}	5.02^{Ab}	1.82^{Bc}
P. fluorescens	Stainless steel	5.95^{Aa}	5.82^{Aa}	4.90^{Ab}
	Granite	5.94^{Aa}	5.93^{Aa}	4.70^{Ab}
S. Enteritidis	Stainless steel	5.26^{Aa}	5.81^{Aa}	4.37^{Ab}
	Granite	6.00^{Aa}	5.22^{Aa}	3.99^{Ab}

Means followed by the same capital letter in the column or by a minor letter in the same line did not differ at 5% level of probability by the Duncan test. Values represent the mean of three repetitions.

sanitizing procedure results can be explained because granite surfaces, when examined by scanning electron microscopy, show fissures, microfissures, cracks, and crevices large enough to harbor microorganisms, particularly bacteria and food residues (CARELI et al., 2008). Moreover, the microorganism can adhere on the surface, multiply, and initiate biofilm formation. This kind of structure is very difficult to remove with standard hygiene procedures because adhered cells in biofilm are more resistant to detergent and sanitizing actions (BOWER; MCGUIRE; DAESCHEL, 1996). Consequently, the formation of biofilms can compromise the quality and safety of foods.

Krysinsky, Brown and Marchisello (1992) found that the resistance of *L. monocytogenes* was related to the type of surface to which the bacteria had attached, and they concluded that stainless steel was more easily cleaned and sanitized than polyester or polyester-polyurethane surfaces.

Brazil generally uses granite for food processing surfaces, such as benches and tables, in domestic food service kitchens. This surface is considered to be the main place responsible for foodborne outbreaks. Also, this surface is found in the processed fruits and vegetables industry as well as the meat and poultry industry. The microorganism can reach the surfaces from contaminated raw food, contaminated water, manipulators, and aerosolization that occur during food processing. The greatest aerosol sources are personnel, floor drains, ventilation systems, and water applied under pressure in the cleaning and sanitizing procedures (SALUSTIANO et al., 2002). Granite is corroded by acid, making their cleaning and sanitizing more difficult. Anticorrosive and mechanical properties and microtopography characteristics of surfaces are important parameters when choosing a proper material to construct equipment and utensils, a material that enables improved cleaning and sanitizing procedures (JULLIEN et al., 2002). AISI 304 stainless steel is mostly used in the food-processing industry and it is an ideal material for manufacturing equipment due to its physicochemical stability and high corrosion resistance (SHI; ZHU, 2009).

3.3 Evaluation of cleaning and sanitizing procedures

The efficiency of the cleaning procedures plus sanitizing procedures was not different ($p \geq 0.05$) between the surfaces. However, a significant difference ($p < 0.05$) was observed between the sanitizer solutions (Tabela 4). Sodium hypochlorite and peracetic acid were more bactericidal ($p < 0.05$) than the quaternary ammonium compound. With regard to microorganisms, *S. aureus* was the less resistant to the sanitizers (Table 4).

These results were not different from those found by Rossoni and Gaylarde (2000). These authors isolated *Escherichia coli*, *Pseudomonas fluorescens,* and *Staphylococcus aureus* from chicken carcasses and allowed them to adhere to stainless steel coupons for 1 hour before rinsing with sterile distilled water (control) and treating with the sanitizing agents at 250 or 1000 mg.L^{-1} (peracetic acid) or 100 or 200 mg.L^{-1} (hypochlorite) for 10 minutes. *P. fluorescens* showed the greatest adhesive ability, followed by *E. coli*, while *S. aureus* adhered in lower numbers. Sodium hypochlorite was more effective than peracetic acid in killing or removing the adherent cells.

Chlorination has been recommended for the elimination of psychrotrophs, such as *P. fluorescens* (BRYAN, 1980), and our results confirm this potential. Castro (1984) suggested using 50-300 mg.L^{-1} active chlorine on surfaces.

Nonetheless, another study showed that the peracetic acid was considered to be more efficient to control biofilm in stainless steel (HOLAH; THORPE, 1990; FATEMI; FRANK, 1999). Also, this sanitizing agent was more effective than hypochlorite against *L monocytogenes* and *Pseudomona*s adhered to stainless steel (FATEMI; FRANK, 1999).

There was no significant difference ($p \geq 0.05$) in the bactericidal efficiency of sanitizers against pure cultures of *S.* Enteritidis and *P. fluorescens* adhered to stainless steel or granite. Generally, Gram-negative bacteria are more resistant than Gram-positive bacteria to weak acids (RUSSEL, 1991) due to the chemical compositions of their external membranes (NIKALDO; VARRA, 1985). Peracetic acid has been suggested to act primarily on lipoproteins in the cell membrane (LEAPER, 1984) and it may be that it is equally effective against outer membrane lipoproteins, facilitating its action against Gram-negative cells.

Another reason for the resistance of Gram-negative bacteria is their tolerance towards weak acids through unknown mechanisms. However, some studies have shown that one or more proteins can be induced during bacterial exposure to acid (BRACKETT; HAO; DOYLE, 1994). Resistance systems include the induction of amino acid descarboxilases, which are able to decarboxylate amino acids under acidic conditions by consuming protons (H^+) (BEARSON; BEARSON; FOSTER, 1997)

Table 4. Number of microorganisms (log CFU.cm^{-2}) on surfaces after the cleaning and sanitizing procedures.

Treatments	Decimal reduction
Initial adherence	5.63[a]
C[1] + quaternary ammonium compound (200 mg.L^{-1}, pH = 9,0)	1.30[b]
C[1] + sodium hypochlorite (100 mg.L^{-1} of total available chlorine, pH = 10,0)	0.31[c]
C[1] + peracetic acid (60 mg.L^{-1}, pH = 3,0)	0.05[c]

C[1] – Cleaning procedure with neutral detergent based anionic surfactant. Means followed by the same capital letter in the column did not differ at 5% level of probability by the Duncan test. Values represent the mean of three repetitions.

4 Conclusions

Species of *Pseudomonas fluorescens, Salmonella* Enteritidis, and *Staphylococcus aureus* were able to adhere to AISI 304 stainless steel and granite, reaching numbers between 4.55 and 5.23 log CFU.cm^{-2}.

Chemical and mechanical actions during the cleaning procedure were more efficient against *S. aureus* adhered to stainless steel and less efficient against *P. fluorescens* and *S.* Enteritidis adhered to stainless steel and granite.

The bactericidal actions of peracetic and sodium hypochlorite solutions were more effective than the quaternary ammonium compound against adhered cells, regardless of the surface. These results show the importance of good cleaning and sanitization procedures in the prevention of bacterial adherence and biofilm formation.

Furthermore, the design of a food-contact surface can influence the degree of microbial attachment as well as the cleanability of the surface once adhesion has occurred. New materials must be investigated and tested for manufacturing equipments or novel cleaning methods may be introduced, aiming to reduce or prevent bacterial adhesion by modifying the physicochemical characteristics of surfaces. Surfaces presenting a maximum number of factors unfavorable to microbial adhesion and biofilm formation must be sought.

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