

ACTIVITY OF DISINFECTANTS AGAINST FOODBORNE PATHOGENS IN SUSPENSION AND ADHERED TO STAINLESS STEEL SURFACES

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Submitted: September 02, 2009; Returned to authors for corrections: April 04, 2010; Approved: June 07, 2012.

ABSTRACT

The purpose of this study was to investigate and compare the efficacy of various disinfectants on planktonic cells and biofilm cells of *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli*. Numbers of viable biofilm cells decreased after treatment with all tested disinfectants (iodine, biguanide, quaternary ammonium compounds, peracetic acid and sodium hypochlorite). Sodium hypochlorite was the most effective disinfectant against biofilm cells, while biguanide was the least effective. Scanning electron microscopy observations revealed that cells adhered on stainless steel surface after treatment with the disinfectants. No viable planktonic cells were observed after treatment with the same disinfectants. Based on our findings, we concluded that biofilm cells might be more resistant to disinfectants than planktonic cells.

Key words: biofilm cells, planktonic cells, disinfectants.

INTRODUCTION

Microorganisms were shown to form biofilms on the surface of materials commonly used in food processing, such as stainless steel (3, 31); thus, these surfaces become potential source of contamination that may lead to food spoilage, transmission of diseases (19, 27, 31), equipment damage and compromise the sanitation of food surfaces and environmental surfaces by spreading detached organisms to other areas of processing plants (29). Biofilm formation may be separated into the primary attachment of bacteria to surfaces, followed by proliferation of the attached bacterial cells, which leads to the accumulation of multilayered clusters of cells and extracellular polymer (glycocalyx) formation (6, 10). Biofilm bacteria can be physically and morphologically different from their

planktonic counterparts, especially in response to sanitizers and biocides (8, 17, 20, 29, 31).

It is well documented that bacteria, including foodborne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli*, can 'stick' to a variety of surfaces found in food industries (5, 14, 19, 24 28). To reduce or eliminate microorganisms on food contact surfaces, food processors have relied on techniques that have been proven over many years of use. These techniques include physical methods (e.g. hand washing, high pressure sprays) and chemical methods (e.g. hypochlorites, iodophores, quaternary ammonium compounds). Both techniques should remove and inactivate microorganisms that might be on the surface of equipment which may eventually come in contact with raw and processed food (4, 5, 14).

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Even with the use of chemical cleaning agents and acceptable clean-in-place (CIP) systems, bacteria can remain on equipment and surfaces used in the food industry. These organisms may survive for prolonged periods, depending on the amount and nature of residual soil, temperature, and relative humidity (8, 31).

The goal of this study was to compare the effects of various disinfectants on planktonic cells in suspension and on biofilm cells of *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains

The following strains of bacteria were used in this study: *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 25922. All strains were obtained from the National Institute of Quality Control in Health – Oswaldo Cruz Foundation (FIOCRUZ; Rio de Janeiro, Brazil).

Preparation of suspension

The bacterial strains were grown overnight (18 to 24h) at 37°C with shaking (150 revolutions per minute-rpm) in tryptic soy broth (DIFCO). Cells were harvested by centrifugation at 5,000 x g for 3.5 min and washed three times in phosphate-buffered saline (PBS; 0.1M, pH 7.2). Cell pellets were resuspended in PBS and adjusted by a spectrophotometer to an A660 of approximately 0.5, corresponding to $\sim 10^8$ CFU/ml (21).

Disinfectants

The disinfectants used in this study were chosen to represent those used in the food industry. The following disinfectants were used: iodine (0.20% w/v), biguanide (0.50% w/v), quaternary ammonium compounds (0.50% w/v), peracetic acid (0.50% w/v) and sodium hypochlorite (1.50% w/v). All the disinfectants used were provided by Johnson-

Diverseylever, Brazil. These agents were diluted with sterilized distilled water according to the manufacturer's instructions.

Test surface

AISI type 304 stainless steel was the surface chosen as it is used extensively throughout the food processing industry. Flat, stainless steel coupons (1 x 1cm) were used as the test surface to examine biofilm formation *in vitro*. The coupons were initially soaked overnight in acetone to remove grease. After soaking, the steel coupons were placed in a sterile tube and sonicated for 15 min in a bath sonicator. The coupons were then washed in tap water followed by three washes with distilled water, and they were autoclaved at 121°C for 15 min (23). The manipulations of coupons were assisted with a sterile surgical clamp for all assays.

Biofilm formation *in vitro*

A 20 μ l aliquot of the 10^8 CFU/mL suspension prepared as described above was placed in 50 mL polypropylene tubes (9) containing 15 mL of inoculated Mueller Hinton Broth (11) and one sterilized stainless steel coupon, and incubated at 37°C under constant agitation of 100 rpm for 5 days. The culture medium was changed every 3 days. Biofilm formation was confirmed using scanning electron microscopy (25).

Activities of the tested disinfectants against the biofilm cells

After biofilm formation, coupons were rinsed twice with 5 mL of sterile physiological saline to remove any attached bacterial cells, and separately placed in Petri dishes containing 20 mL of one of the tested disinfectants at 25 \pm 2°C for 10 min. A positive control was performed by placing a coupon in a Petri dish containing 20 mL of sterile physiological saline. The coupons were removed from the dishes and immediately transferred to 5 mL of Lethen Broth (11) for 10 min to inactivate the disinfectants and rinsed twice again with 5 mL of sterile physiological saline. They were then placed in glass tubes containing 5 mL of sterile physiological saline, sonicated

at 40kHz for 8 min (or 36kHz for 10 min) and vortexed for 10s (21). This procedure releases viable bacteria adhering to the coupons into the physiological saline. To quantify viable cells, bacteria were resuspended, serially diluted 10-fold with sterilized physiological saline and cultured in trypticase soy agar at 37°C for 24-48h (21).

The experiment was repeated three times for each strain, and the mean and standard deviation were calculated.

Activities of the tested disinfectants against the planktonic cells

A 0.05 mL aliquot of 10^8 CFU/mL suspension prepared as described above was added to glass tubes containing separately 4.95 mL of each tested disinfectant at $25^{\circ}\text{C}\pm 2^{\circ}\text{C}$ and vortexed for about 10s. A positive control was performed by adding a 0.05 mL aliquot of the suspension (10^8 CFU/mL) to a glass tube containing 4.95 mL of sterile physiological saline. From each tube, 0.5 mL was sampled after 10 min, added to 4.5 mL of Lethen Broth (11) to inactivate the disinfectants for 10 min, and vortexed again for 10s. Viable cells were counted as described above. The experiment was performed three times for each strain, and the mean and standard deviation were calculated (21).

Scanning electron microscopy observations

After treatment with the tested disinfectants, the stainless steel coupons were immersed in 2.5% glutaraldehyde in 0.1 M

phosphate buffer (pH 7.1) to fix the microorganisms, dehydrated in aqueous solutions of ethanol (15, 30, 50, 70, 95 and 100%) for 15 min, and dried in a centrifuge under vacuum, then coated with gold and examined under the scanning electron microscope JEOL-JSM [T330A](25).

Statistical analysis

Numbers of CFU/cm² and CFU/mL were transformed to log₁₀. The data were analyzed by analysis of variance (ANOVA) and the Tukey multiple comparison test using MINITAB Statistical Software (version 13.1). Statistical significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Poor sanitation of food contact surfaces, equipment, and processing environments has been a contributing factor in foodborne disease outbreaks (5, 8). In this study, we have demonstrated the efficacy of disinfectants used in food industries against foodborne pathogens in suspension and in biofilm. Table 1 shows the activities of the tested disinfectants on biofilm cells of *S. aureus*, *L. monocytogenes* and *E. coli*. The number of viable cells is presented as a logarithm. Statistically significant differences ($p < 0.05$) were found on average count of viable cells of all studied strains after treatment with the disinfectants.

Table 1. Effect of disinfectants on biofilm cells after treatment for 10 minutes

Disinfectants	Microorganisms					
	<i>S. aureus</i>		<i>L. monocytogenes</i>		<i>E. coli</i>	
	Mean ^a	SD ^b	Mean ^a	SD ^b	Mean ^a	SD ^b
Iodine	2.4 ± 1.1	(3) ^c	2.0 ± 0.0	(3)	0.8 ± 1.2	(3)
Biguanide	3.3 ± 1.2	(3)	2.9 ± 0.8	(3)	2.2 ± 0.5	(3)
Quaternary ammonium	2.8 ± 2.5	(3)	1.4 ± 0.4	(3)	1.7 ± 0.5	(3)
Peracetic acid	0.7 ± 0.7	(3)	1.1 ± 0.1	(3)	2.1 ± 0.3	(3)
Sodium hypochlorite	0.2 ± 0.3	(3)	1.0 ± 0.0	(3)	0.3 ± 0.3	(3)
Positive control ^d	5.9 ± 0.8	(3)	6.3 ± 0.6	(3)	4.7 ± 0.4	(3)

^a Mean, log CFU/cm²

^b SD, standard deviation

^c Numbers in parentheses indicate number of experiments

^d Positive control, biofilm cells not treated with disinfectants

When compared with the positive control (5.9 log CFU/cm²), the numbers of viable cells of *S. aureus* biofilm were significantly reduced after treatments with sodium hypochlorite (0.2 log CFU/cm²) and peracetic acid (0.7 log CFU/cm²). After treatment with biguanide, the numbers of viable cells of *S. aureus* biofilm (3.3 log CFU/cm²) were higher than after treatment with all other tested disinfectants, following the quaternary ammonium compounds (2.8 log CFU/cm²) and iodine (2.4 log CFU/cm²). These data show that sodium hypochlorite was the most effective against *S. aureus* biofilm cells, while the biguanide disinfectant was the least effective.

Similar results were observed for *L. monocytogenes* biofilm cells. Compared with the positive control (6.2 log CFU/cm²), the biguanide disinfectant (2.9 log CFU/cm²) was the least effective in eliminating *L. monocytogenes* biofilm cells than all tested disinfectants, following iodine (2.0 log CFU/cm²) and quaternary ammonium compounds (1.4 log CFU/cm²). The numbers of viable

cells of *L. monocytogenes* biofilm were lower after treatment with peracetic acid (1.1 log CFU/cm²) and sodium hypochlorite (1.0 log CFU/cm²), showing that these disinfectants were the most effective against *L. monocytogenes* biofilm cells.

In the case of *E. coli* biofilm cells, low numbers of viable cells were obtained after treatment with sodium hypochlorite (0.3 log CFU/cm²) and iodine (0.8 log CFU/cm²), when compared with the positive control (4.7 log CFU/cm²). These data show that sodium hypochlorite and iodine were the most effective against *E. coli* biofilm cells. The count of viable cells after treatment with biguanide (2.2 log CFU/cm²) reveals that this disinfectant was the least effective in eliminating *E. coli* biofilm cells, following peracetic acid (2.1 log CFU/cm²) and quaternary ammonium compounds (1.7 log CFU/cm²).

The activities of the tested disinfectants on planktonic cells of *L. monocytogenes*, *S. aureus* and *E. coli* are represented in Table 2. The number of viable cells is presented as a logarithm.

Table 2. Effect of disinfectants on the planktonic cells after treatment for 10 minutes

Disinfectants	Microorganisms					
	<i>S. aureus</i>		<i>L. monocytogenes</i>		<i>E. coli</i>	
	Mean ^a	SD ^b	Mean ^a	SD ^b	Mean ^a	SD ^b
Iodine	0 ± 0	(3) ^c	0 ± 0	(3)	0 ± 0	(3)
Biguanide	0 ± 0	(3)	0 ± 0	(3)	0 ± 0	(3)
Quaternary ammonium	0 ± 0	(3)	0 ± 0	(3)	0 ± 0	(3)
Peracetic acid	0 ± 0	(3)	0 ± 0	(3)	0 ± 0	(3)
Sodium hypochlorite	0 ± 0	(3)	0 ± 0	(3)	0 ± 0	(3)
Positive control ^d	5.4 ± 0.1	(3)	5.6 ± 0.1	(3)	5.6 ± 0.2	(3)

^a Mean, log CFU/cm²

^b SD, standard deviation

^c Numbers in parentheses indicate number of experiments

^d Positive control, biofilm cells not treated with disinfectants

The results show that all the studied strains exhibited a significant decrease of the survival rate of viable cells after treatment with tested disinfectants. No growth was detected after 10 min of exposure to iodine (0.20%), biguanide (0.50%), quaternary ammonium compounds (0.50%), peracetic acid (0.50%) and sodium hypochlorite (1.50%). These results, when compared with those presented in Table 1, show that biofilm

cells are more difficult to be eliminated with chemical cleaning agents than planktonic cells in suspension. Our results coincide with those of other researchers who suggested that current sanitation practices are less effective on attached microorganisms compared to free living (planktonic) microorganisms. Schwach and Zottola (26) used electron microscopy to show that microorganisms were not completely

removed from stainless steel by rinsing with up to 150 ppm sodium hypochlorite. While they did not determine the viability of the remaining cells, their findings did not rule out the possibility that viable cells remained. Marques *et al.* (18) evaluated the efficiency of sodium dichloroisocyanurate, hydrogen peroxide and peracetic acid in inactivating *Staphylococcus aureus* cells adhered on stainless steel and glass surfaces. This researchers comproved that peracetic acid was the most efficient in removing adhered cells. Frank and Koffi (12) showed that a biofilm composed completely of *L. monocytogenes* on glass survived more than 10-times longer than free-living cells when exposed to anionic acid sanitizers. Similarly, Andrade *et al.* (2) demonstrated that *Enterococcus faecium* cells adhering to stainless steel were more resistant to chemical sanitizers than non-adherent cells. Trachoo and Frank (30) determined the survival of *Campylobacter jejuni* in mixed-culture biofilms grown on polyvinyl chloride (PVC) plastic coupons after treatment with chemical sanitizers. They showed that chlorine was the most effective sanitizer since it completely inactivated *C. jejuni* in the biofilms after treatment at 50 ppm for 45s while quaternary ammonia, peracetic acid and a peracetic acid/peroctanoic acid mixture at 50 and 200 ppm for 45 s not completely inactivated *C. jejuni* in the biofilms.

It may be difficult to compare results from these different studies because the conditions for attachment and biofilm development vary greatly and these differences can be significant (5).

Once the microorganisms have attached, they must be capable of withstanding normal disinfection processes. Biofilm bacteria display a resistance to biocides that may be considered stunning (15).

According to Characklis and Marshall (7), incomplete removal of the biofilm will allow it to quickly return to its equilibrium state, causing a rebound in total place counts following sanitization. Surviving organisms rapidly create more extracellular polymers as a protective response to irritation by chemical cleaning agents.

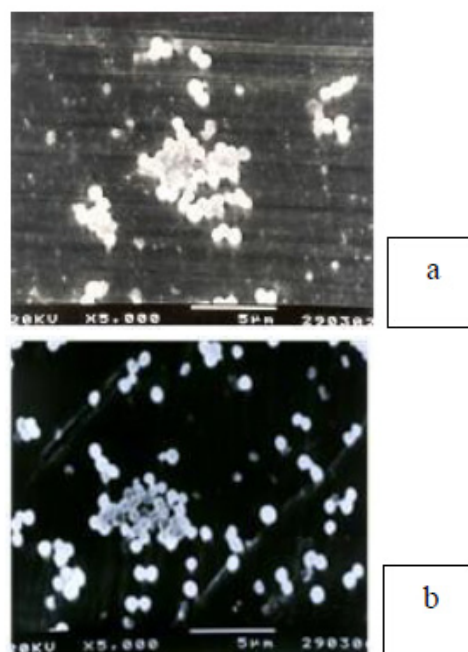
It has become clear that biofilm-grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents (17). Studies have indicated that slow growth and/or induction of an *rpo S* – mediated

stress response could contribute to biocide resistance (13, 16). Adams and Mc Lean (1) reported that deletion of *rpo S* greatly reduces the ability of *E. coli* to grow in biofilm yet has little effect on the growth of planktonic bacteria. The physical and/or chemical structure of exopolysaccharides or other aspects of biofilm architecture could also confer resistance by exclusion of biocides from the bacterial community. Finally, biofilm-grown bacteria might develop a biofilm-specific biocide-resistant phenotype (5, 17).

The microbiological evaluations observed using scanning electron microscope showed bacterial adherence and/or biofilm formation on stainless steel surface before and after treatment with studied disinfectants to strains: *S. aureus* (Figs 1a-f), *E. coli* (Figs 2a-f) and *L. monocytogenes* (Figs 3 a-f).

Results of this study indicate that biofilm cells are more resistant to chemical cleaning agents when compared with planktonic cells in suspension. Sodium hypochlorite seems to be the best chemical agent to eliminate biofilm cells formed on stainless steel surfaces, while biguanide seems to be the worst.

Additional studies should be performed to further elucidate how and why bacteria growing in complex surface-attached communities can protect themselves from the action of antimicrobial agents.



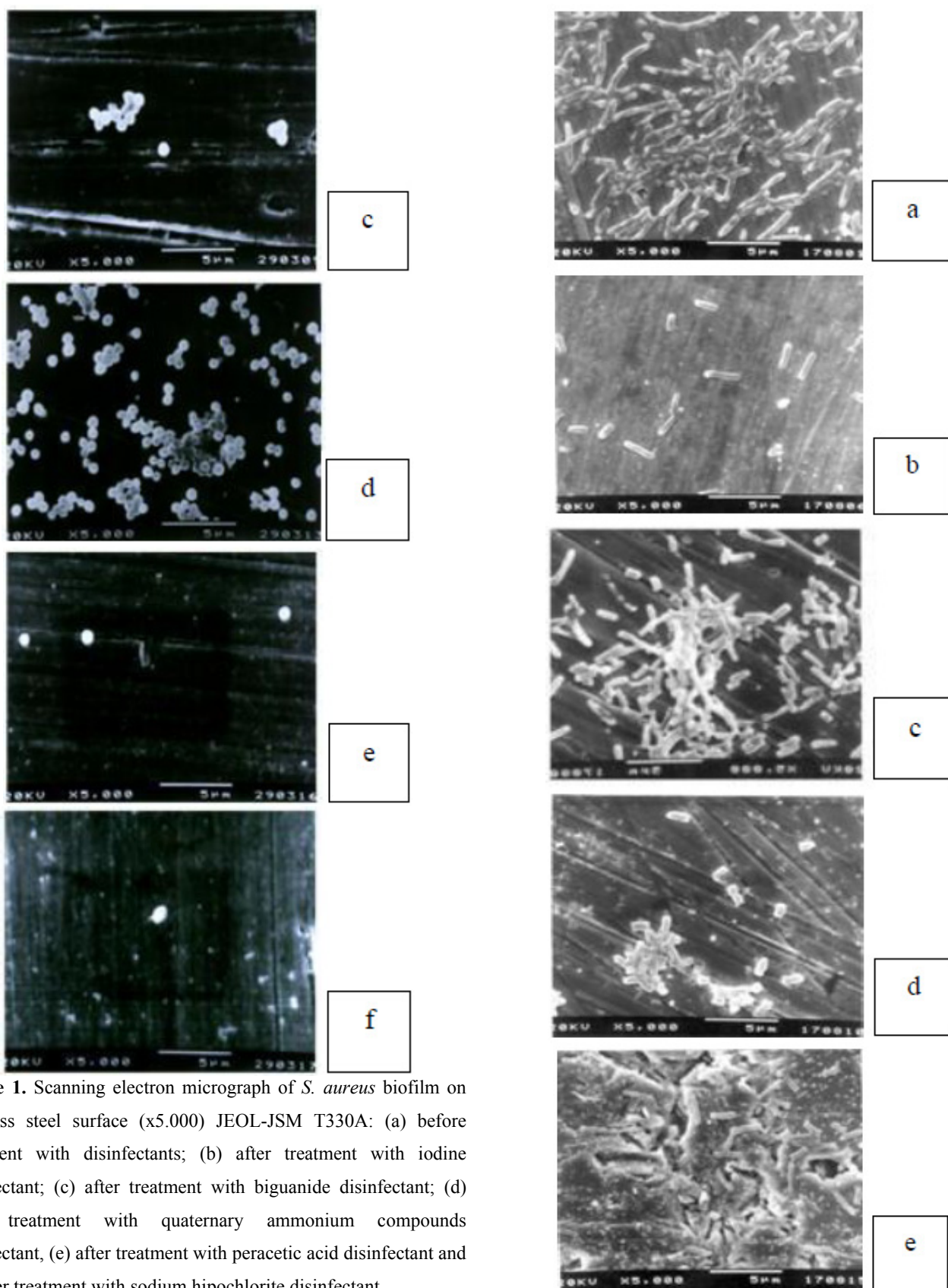


Figure 1. Scanning electron micrograph of *S. aureus* biofilm on stainless steel surface (x5.000) JEOL-JSM T330A: (a) before treatment with disinfectants; (b) after treatment with iodine disinfectant; (c) after treatment with biguanide disinfectant; (d) after treatment with quaternary ammonium compounds disinfectant, (e) after treatment with peracetic acid disinfectant and (f) after treatment with sodium hypochlorite disinfectant.

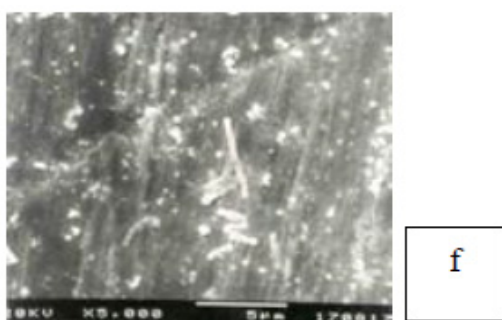


Figure 2. Scanning electron micrograph of *E. coli* biofilm on stainless steel surface (x5.000) JEOL-JSM T330A: (a) before treatment with 294 disinfectants; (b) after treatment with iodine disinfectant; (c) after treatment with biguanide disinfectant; (d) after treatment with quaternary ammonium compounds disinfectant, (e) after treatment with peracetic acid disinfectant and (f) after treatment with sodium hypochlorite disinfectant.

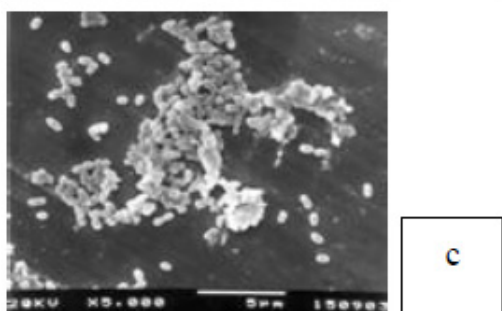
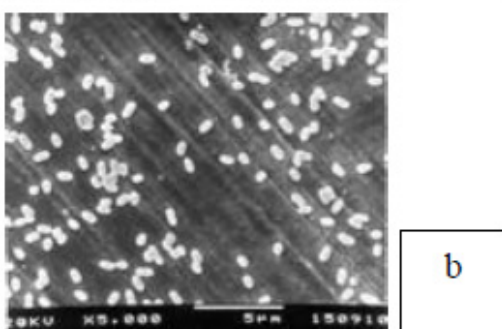
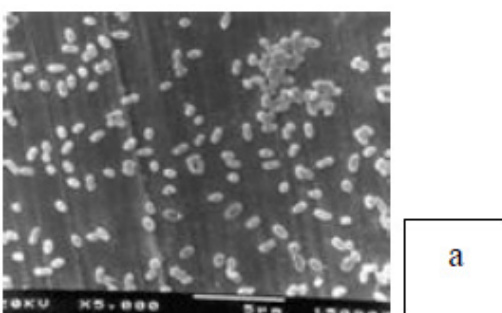
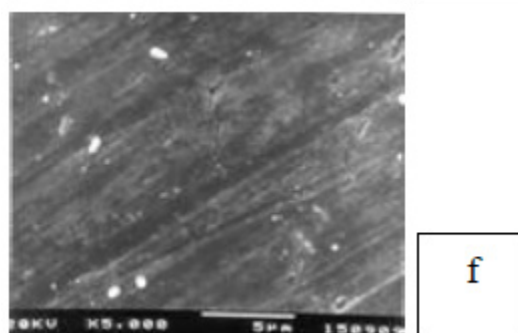
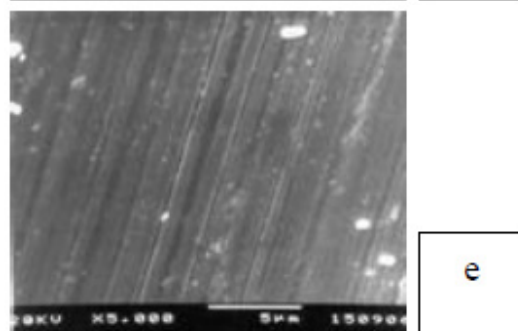
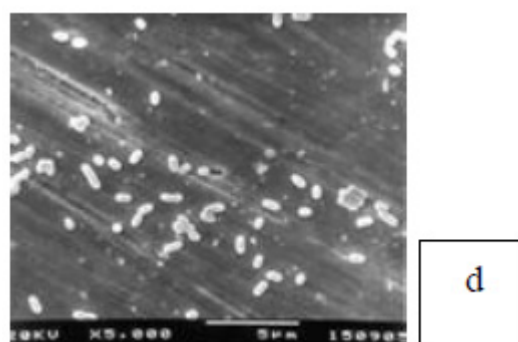


Figure 3. Scanning electron micrograph of *L. monocytogenes* biofilm on stainless steel surface (x5.000) JEOL-JSM T330A: (a) before treatment with disinfectants; (b) after treatment with iodine disinfectant; (c) after treatment with biguanide disinfectant; (d) after treatment with quaternary ammonium compounds disinfectant, (e) after treatment with peracetic acid disinfectant and (f) after treatment with sodium hypochlorite disinfectant.

ACKNOWLEDGEMENTS

This work was supported by The Diagnostic Centre at the Community Assistance Nucleus – NAC – UNESP - Sao Paulo State University, School of Pharmaceutical Sciences, Araraquara, Sao Paulo, Brazil

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