

Evaluation of biofilm formation by *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* on coatings for stainless steel composed of polypropylene with zeolite and polypropylene with glass and silver

Avaliação da formação de biofilme por *Staphylococcus aureus*, *Staphylococcus epidermidis* e *Escherichia coli* em revestimentos para aço inox compostos por polipropileno com zeolita e polipropileno com vidro e prata

Evaluación de la formación de biopelículas por *Staphylococcus aureus*, *Staphylococcus epidermidis* y *Escherichia coli* en revestimientos de acero inoxidable compuestos de polipropileno con zeolita y polipropileno con vidrio y plata

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Abstract

The presence of microbial biofilm on milking equipment caused by inadequate cleaning is a constant source of contamination for milked animals and the milk that is sent to the industry. Biofilm also compromises the health of animals and the quality of milk produced. The aim of this study was to evaluate biofilm formation on four different coatings for stainless steel, composed of virgin polypropylene, polypropylene with 3% zeolite, polypropylene with 6% zeolite and polypropylene with glass and silver. The formation of biofilm on the four coatings tested was assessed by counting colony-forming units of cells in biofilm, extracting the matrix by sonication, and quantifying extracellular proteins and polysaccharides, as confirmed by analysis of images generated by confocal laser scanning microscopy. Five samples of biofilm-producing *Staphylococcus aureus*, one of *Staphylococcus epidermidis* and five of *Escherichia coli* were selected, the biofilm production of which had been evaluated in a previous study to assess adhesion to the different coatings. *Escherichia coli* was the bacterium with the greatest biofilm production on all the tested coatings, and greater adhesion of microbial biofilm occurred on polypropylene with 6% zeolite when compared with polypropylene with silver and glass. The material composed of silver, glass and polypropylene was expected to offer less adherence to microbial biofilms, but in this study, no difference was observed between the coatings with virgin polypropylene, with 3% zeolite, and with glass and silver.

Keywords: Bovine mastitis; Dairy cattle; Stainless steel.

Resumo

A presença de biofilme microbiano aderido em equipamentos de ordenha, formado por limpezas inadequadas, constituem uma constante fonte de contaminação para os animais ordenhados e para o leite que vai para a indústria, comprometendo tanto a saúde do animal quanto a qualidade do leite produzido. O objetivo desse estudo foi avaliar a formação de

biofilme em quatro revestimentos para aço inox diferentes, compostos por polipropileno virgem, polipropileno com zeolita a 3%, polipropileno com zeolita a 6% e polipropileno com vidro e prata. Para a determinação da formação de biofilme nos quatro revestimentos testados, foi realizada a contagem de unidades formadoras de colônias das células em biofilme, extração da matriz por sonicação, quantificação de proteínas extracelulares e polissacarídeos, confirmados por análise de imagens geradas pela microscopia confocal a laser. Foram selecionadas cinco amostras de *Staphylococcus aureus*, uma de *Staphylococcus epidermidis* e cinco de *Escherichia coli* produtoras de biofilme cuja produção de biofilme foi avaliada em estudo anterior realizado para avaliar a adesão nos diferentes revestimentos. A *Escherichia coli* foi a bactéria com maior produção de biofilme em todos os revestimentos testados e o polipropileno com zeolita a 6% apresentou uma maior adesão de biofilme microbiano quando comparado ao polipropileno com prata e vidro. Esperava-se que o material composto por prata, vidro e polipropileno tivesse menor aderência de biofilmes microbianos, mas nesse estudo observou-se que não houve diferença entre os revestimentos de polipropileno virgem, com zeolita a 3% e com vidro e prata.

Palavras-chave: Inox; Mastite bovina; Rebanho leiteiro.

Resumen

La presencia de biofilm microbiano adherido a los equipos de ordeño, formado por una limpieza inadecuada, es una fuente constante de contaminación para los animales ordeñados y para la leche que va a la industria, comprometiendo tanto la salud del animal como la calidad de la leche producida. El objetivo de este estudio fue evaluar la formación de biopelículas en cuatro recubrimientos diferentes de acero inoxidable, compuestos de polipropileno virgen, polipropileno con 3% de zeolita, polipropileno con 6% de zeolita y polipropileno con vidrio y plata. Para la determinación de la formación de biofilm en los cuatro recubrimientos ensayados, se contaron las unidades formadoras de colonias de las células en biofilm, extracción de matriz por sonicación, cuantificación de proteínas extracelulares y polisacáridos, confirmada por análisis de imágenes generadas por microscopía confocal láser. Se seleccionaron cinco muestras de *Staphylococcus aureus*, una de *Staphylococcus epidermidis* y cinco de *Escherichia coli* productoras de biofilms, cuya producción de biofilm fue evaluada en un estudio previo realizado para evaluar la adherencia a diferentes recubrimientos. *Escherichia coli* fue la bacteria con mayor producción de biopelícula en todos los recubrimientos probados y el polipropileno con 6% de zeolita mostró una mayor adhesión de biopelícula microbiana en comparación con el polipropileno con plata y vidrio. Se

esperaba que el material compuesto por plata, vidrio y polipropileno tuviera menor adherencia a las biopelículas microbianas, pero en este estudio se observó que no hubo diferencia entre los recubrimientos de polipropileno virgen, con 3% de zeolita y con vidrio y plata.

Palabras clave: Acero inoxidable; Mastitis bovina; Ganado lechero.

1. Introduction

Biofilms are formed by bacteria that bind themselves to aggregated surfaces in a hydrated polymeric matrix of their own synthesis. The formation of these sessile communities and their inherent resistance to antimicrobial agents means that they are the source of many persistent and chronic bacterial infections (Costerton et al., 1999).

These structures naturally occur in a range of different types of biotic and abiotic environments. However, the presence of biofilm in environments that come into contact with food, such as 304 stainless steel, creates a point of contamination by causing a constant release of pathogenic microorganisms such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*, thus compromising the microbiological quality of raw materials, pre-finished and finished products (Boari, Alves, Tebaldi, Savian & Piccoli, 2009; Ciccio et al., 2015; Fuster-Valls, Hemandez-Herrero, Marín-de-Mateo & Rodríguez-Jerez, 2008). Therefore, it is essential to define strategies that prevent the adhesion of biofilms and thereby reduce the risk of contamination of products.

A number of studies have been conducted on the action of metals that inhibit the formation of microbial biofilm, mainly in dentistry, for periodontal diseases and prostheses (Tamanai-Shacoori, Chahandad, Rébillard, Cillard & Bonnaure-Mallet, 2014), and in the form of additives for consumer products, such as socks, shirts, water filters, antiperspirants, combs and paints (Nagy et al., 2011).

In a study to isolate *Listeria monocytogenes* biofilm, the presence of biofilm was observed on milking equipment and in the milking environment (Latorre et al., 2010). Therefore, research into a coating that inhibits the formation of biofilm, to be added to milking utensils and equipment, can reduce risks to human and animal health.

The use of silver nanoparticles as a coating has been evaluated due to its antimicrobial effect and ability to destroy biofilm (Khameneh, Zarei & Bazzaz, 2014). However, because of the high cost, other types of products are also being studied, such as zeolite, a crystalline, porous, hydrated, aluminosilicate mineral found in volcanic sedimentary rock. It possesses physical-chemical properties that include ion exchange, molecular sieving, catalysis and

adsorption, and a strong affinity for ionic heavy metals (Eriksson, 2008; Wang & Peng, 2010). Raw zeolite is considered an affordable material and it provides bactericidal activity (Ebrahimi, Zandi & Gharibi, 2014).

The creation of a material that prevents biofilm formation is an interesting option for rural producers and entrepreneurs in the milking equipment sector. Moreover, a material containing substances that inhibit microbial adhesion to the stainless steel equipment used in the daily routine can bring numerous benefits, such as prevent the spread of diseases and reduce corrosion of equipment and qualitative damage to the raw material. Consequently, this material will eliminate contact between the mammary glands of cows and biofilm-producing bacteria, which cause difficulties and resistance in the treatment of bovine mastitis. Currently, many reports of biofilm formation on equipment used in rural production, but these articles do not propose possibilities for solving this problem (Lee et al. 2014; Martin et al. 2016). In the fields of medicine and dentistry, more options are available for preventing the formation of biofilms (Cos, Toté, Horemans & Maes, 2010; Tran & Webster, 2013).

This study aimed to evaluate the formation of microbial biofilms of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* on four kinds of stainless steel coating materials, composed of virgin polypropylene, polypropylene with 3% zeolite, polypropylene with 6% zeolite and polypropylene with glass and silver.

2. Materials and methods

All methodology used in this work is in accordance with the methodologies used for the study of biofilms. The respective authors are mentioned in each methodology.

2.1. Strain Selection

The experimental protocol was approved by the Ethics Committee for the Use of Animals (CEAU) at the Federal University of Uberlândia, under protocol 111/14. Five strains of *Staphylococcus aureus*, one strain of *Staphylococcus epidermidis* and five strains of *Escherichia coli* were identified after being isolated from the milk of cows with bovine mastitis from a farm in the municipality of Uberlândia, Minas Gerais, Brazil, according to the methodology described by Koneman (2001). Initially, Chagas et al. (2017) the following tests were performed to determine if strains produced biofilm: the Congo red agar test (Freeman, Falkiner & Keane, 1989) and the microplate test with crystal violet dye

(Cucarella et al., 2004), both of which are recommended in the literature for this assessment. The tests were carried out at the Infectious-Contagious Diseases Laboratory of the Federal University of Uberlândia, Minas Gerais.

2.2. Preparation of material

The following types of coating for 304 stainless steel were tested: virgin polypropylene, polypropylene with 3% zeolite, polypropylene with 6% zeolite and polypropylene with glass and silver. These samples were prepared by cutting fragments of each material into 1cm x 1cm squares, which were then sterilized in an autoclave for 15 minutes at 121°C.

2.3. Evaluation of Biofilm Formation on Coatings

The selected bacteria were cultured individually for one night in Tryptic Soy Broth (TSB) at 37°C, with agitation. Then, the cells in suspension were inoculated onto sterile 6-well flat-bottomed polystyrene plates, along with the tested material, diluted 1:200 in TSB with 0.25% glucose and incubated for 24 hours at 37°C with agitation and medium renewal after 12 hours (Gomes, 2010).

The microbial biofilm formed on the materials was washed three times with 200µL of saline solution (NaCl 0.85%). Then, 200µL of saline solution was added to the material in a sterile petri dish for the biofilm scraping procedure, during which 200µL tips were used to scrape each side of the material a total of 40 times. The scraped solution was placed in an Eppendorf-type microtube for sonication (Ultrasonic Processor) for 20 seconds at 22% amplitude. After sonication, the strains were homogenized in a vortex (Gomes, 2010).

Next, a range of dilutions (10^{-1} to 10^{-5}) were prepared for counting the colonies in petri dishes containing a trypticase soy agar (TSA) medium using the method of dripping the scraped sample (three separate 10µL drops were added to the TSA and the plate was tilted so the drops would run across the medium). The dishes were incubated in an oven at 37°C for 48 hours. Soon after, the colonies were counted in each plate and calculations were made for the dilutions, expressed in Colony Forming Units (UFC/ml) (Gomes, 2010).

Five strains of *S. aureus*, one of *S. epidermidis* and five of *E. coli* were used to test each material. The test was performed in triplicate, three times with each bacterium, resulting in 15 repetitions for each treatment and each 10^{-3} , 10^{-4} and 10^{-5} dilution for *S. aureus*

and *E. coli* and 3 repetitions for *S. epidermidis* (Gomes, 2010). For the evaluation of each coating with the result for all bacteria, 33 repetitions were performed for each treatment and each 10^{-3} , 10^{-4} and 10^{-5} dilution.

2.4. Extraction of Extracellular Substances from the Matrix and Quantification of Extracellular Proteins and Polysaccharides

The biofilm-producing strains that strongly adhered to the tested materials (above 1.0 on the microplate test after reading the absorbance at 490nm) were selected, resulting in two strains of *Staphylococcus aureus*, one of *Staphylococcus epidermidis* and two of *Escherichia coli*. After taking readings on the spectrophotometer, all strains were incubated with the tested materials at a concentration of 0.5, with TSB and 0.25% glucose on sterile 6-well flat-bottomed polystyrene plates at 37°C for 24 hours with agitation and medium renewal after 12 hours. Then, the material was scraped with phosphate-buffered saline (PBS) to perform the tests described below (Gomes, 2010).

2.4.1. Extraction of the Matrix by Sonication

The biofilm matrix was extracted by scraping the material in the PBS. Afterward, the biofilm suspension was sonicated for 30s at 30W/Hz on ice, homogenized in a vortex for 2 minutes and centrifuged at 3000 g for 10 minutes at 4°C. The supernatant was filtered through a 0.2 µm membrane and the membrane was dried at 60°C for about 20 minutes. The dry weight was determined by the weight difference (subtracting the weight of the filter) (Azeredo, Lazarova & Oliveira, 1999).

2.4.2. Quantification of Proteins from the Matrix (BCA Kit)

For this analysis, 25µL of the scraped sample was added to a 96-well microplate. Soon after, 200µL of the mixed reagents from the BCA kit (Sigma) was added, followed by homogenization for 30 seconds and incubation for 30 minutes at room temperature. Readings were taken at 562nm using the ELISA reader (Thermo Scientific, Multiskan GO) and a phosphate buffer as background (Azeredo et al., 1999).

2.4.3. Quantification of Polysaccharides - Phenol Sulfuric Acid Method

Polysaccharide content was determined following the method described by Dubois, Gilles, Hamilton, Rebers and Smith (1956). Consequently, 0.5 ml of the scraped sample was added to a test tube, followed by 0.5 ml of phenol (50g/L) and, immediately afterward, 2.5 ml of sulfuric acid (95-97% - Isofar). The solution was homogenized in a vortex and left to react for 15 minutes at room temperature. Measurements were performed taken at 490nm using the ELISA reader (Thermo Scientific, Multiskan GO) with a phosphate buffer as background.

2.5. Confocal Laser Scanning Microscopy (CLSM)

Microscopy was performed on one strain of *Staphylococcus aureus*, one of *Staphylococcus epidermidis* and one of *Escherichia coli*, incubated with the four tested materials in a sterile 24-well flat-bottomed polystyrene plate at 1:200 in TSB with 0.25% of glucose, at 37°C for 24 hours with agitation and medium renewal after 12 hours (Gomes, 2010).

At the Federal University of Uberlândia's (UFU) Confocal Microscopy Laboratory, the materials were washed with sterile saline solution, stained with a LIVE/DEAD Kit (Live/Dead, BacLight L7012) and assessed using the confocal laser scanning microscope (Zeiss 510 Meta). The lasers used for the analysis were Argonio and HeNe, with excitations of 488 nm and 561 nm, respectively. The emission filters used were 500-545 nm and 580-680 nm. The ideal parameters for the marking of living (viable) and dead (non-viable) cell constituents in the kit are Syto 9 and propidium iodide, respectively. Subsequently, the image data were processed using the specific software Fuji (Kunze et al., 2010).

2.6. Statistical analysis

The chi-square test was performed to check the relationship between the treatment factors (coatings) and the number of colonies in the TSA. The Kruskal-Wallis non-parametric test was used to compare the treatments and dilutions, with a significance level of 5%.

The procedures for statistical analysis are described in Triola (1999) and Ayres, Ayres Junior, Ayres and Santos (2007) with use of the Action tool (2015), based on the R Program (R Development Core Team, 2015).

3. Results and Discussion

The capacity of *S. aureus*, *S. epidermidis* and *E. coli* to form on the tested materials was evaluated using the counting test for colony-forming units per ml (CFU/ml). It was found that bacterial growth ranged from 1000 to 100,000 CFU/ml and that this variation occurred according to the type of material and bacteria, as shown in Table 1. According to the chi-square test, a statistically significant difference (p-value <0.05) was observed when comparing the coatings and the dilutions with the number of colonies of *S. aureus*, *S. epidermidis* and *E. coli*. A statistically significant difference between all bacteria was verified by the non-parametric Kruskal-Wallis test. However, following the hypothesis of testing by coating, the comparison for *S. aureus* and *S. epidermidis* showed no significant difference. Furthermore, no statistically significant difference was observed with *E. coli*. All of the other comparisons between the bacteria and materials tested showed a significant difference (p-value <0.05) (Table 1). As such, it is possible to infer that microbial biofilm formed on all of the materials, indicating there is no way to select the best material by bacteria although *E. coli* showed greater adhesion to all of the tested coatings (Table 1).

Table 1. Count of the colony forming units of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* in the dilutions of 10^3 , 10^4 and 10^5 , of the biofilms adhered to the coatings composed of virgin polypropylene, polypropylene and 3% zeolite, polypropylene and 6% zeolite and polypropylene, glass and silver.

Coating material	Microorganism								
	<i>Staphylococcus aureus</i>			<i>Staphylococcus epidermidis</i>			<i>Escherichia coli</i>		
	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}
Virgin polypropylene	5	2	1	1	0	0	5	5	0
Polypropylene with zeolite 3%	5	2	0	1	0	0	5	5	0
Polypropylene with zeolite 6%	5	3	0	1	1	0	5	5	0
Polypropylene with glass and silver	5	2	1	1	0	0	5	5	0

Source: The authors.

Table 2 shows the overall results for the coatings tested, incorporating all of the bacteria results from the counting test for colony-forming units of the cells in

biofilm. The chi-square test and the Kruskal-Wallis test showed greater microbial biofilm formation on polypropylene with 6% zeolite than polypropylene with silver and glass (p-value <0.05). However, no statistically significant difference was observed between polypropylene with glass and silver, polypropylene with 3% zeolite and virgin polypropylene at this dilution, although biofilm formed on all of the tested materials.

Table 2. Counting of colony forming units in the 10^3 , 10^4 and 10^5 dilutions, of the biofilms adhered to the coatings composed of virgin polypropylene, polypropylene and zeolite 3%, polypropylene and zeolite 6% and polypropylene, glass and silver.

Coating material	Microorganism		
	10^{-3}	10^{-4}	10^{-5}
Virgin polypropylene	11	7	1
Polypropylene with zeolite 3%	11	7	0
Polypropylene with zeolite 6%	11	9	0
Polypropylene with glass and silver	11	7	1
Total of strains tested	44	30	2

Source: The authors.

Cowan, Abshireb, Houk and Evans (2003) tested the adhesion of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 7644 to a stainless steel coating material composed of 2.5% zeolite and silver and 14% zinc. These authors observed that zeolite concentrations of 1.5 or 3.1 mg/mL and silver concentrations of 39 or 78 $\mu\text{g/mL}$ were ideal for bactericidal activity. The mixture of these particles reduced the formation of biofilms on the coating by 99% and after 6 hours of TSB incubation and after 24 hours no adhesion was observed.

In this study, the silver and zeolite particles were not able to inhibit bacterial cell adhesion fully. Cowan et al. (2003) observed that the bactericidal effect of the two particles in association is greater than when separate. Thus, in order to reduce the costs of producing the material, an attempt was made to use zeolite and silver separately and consequently inhibit the formation of biofilm on polypropylene.

Neves, Agnelli, Kurachi and Souza (2014) tested the efficacy of silver nanoparticles in resins at dosages of 0.3% and 0.6% to prevent biofilm formation by *Streptococcus mutans* and *Lactobacillus acidophilus* after dental restoration, and observed an excellent bactericidal effect at both dosages. They found a smaller number of colonies with both materials than on

the control on days one, four and seven of incubation. In the present study, *S. aureus*, *S. epidermidis* and *E. coli* had lower adhesion to the surface with biofilm formation. However, no statistical difference was observed between the particle coatings and the control, virgin polypropylene.

Due to the major environmental impact caused by the toxicity of metals and the serious implications for the treatment of wastewater caused by nitrifying bacteria, Nagy et al. (2011) created a membrane containing zeolite to stabilize the inserted silver particles. They used *E. coli* bacteria to verify the presence of adhesion and biofilm formation. The progressive release of silver was evaluated for destroying bacterial cells, and after 30 minutes elimination was lower than after 180 minutes of exposure, when the strains were almost completely eradicated. The present study evaluated the exposure of bacteria to the coating after 24 hours, but these bacteria were still viable after that period. Thus, zeolite and silver did not efficiently inhibit adhesion and biofilm formation.

When analyzing the reports of previous authors and the raw results of this research, the silver coating was expected to have less biofilm formation. After statistical analysis, however, no difference was observed between the virgin polypropylene, polypropylene with 3% zeolite and polypropylene with glass and silver coatings.

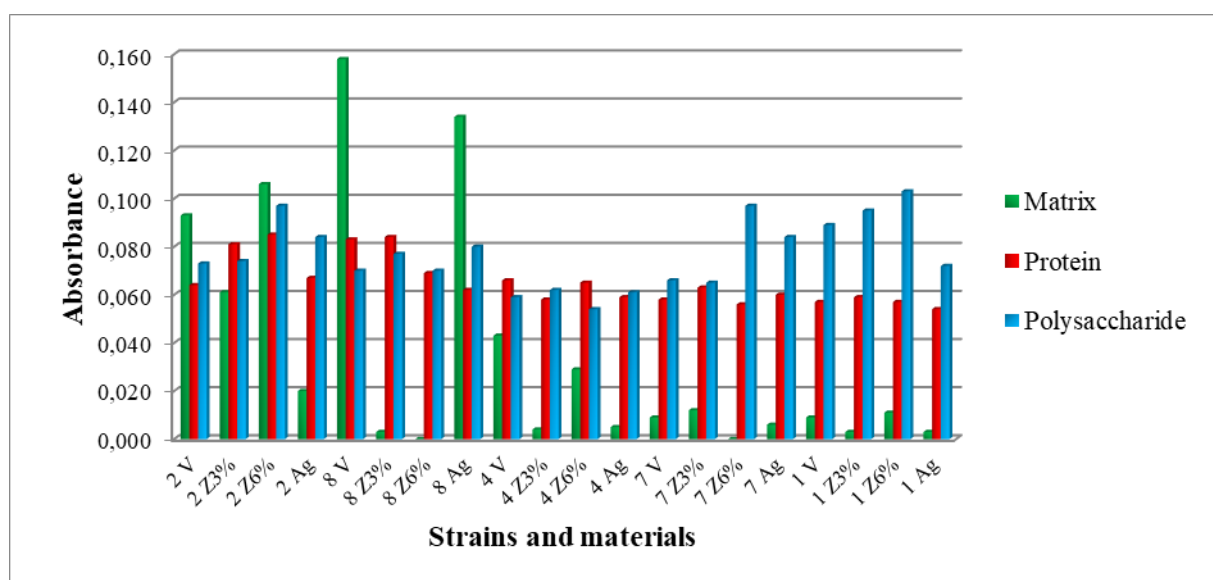
The greater efficiency of these materials for destroying gram-negative bacteria when compared to gram-positive bacteria is due to the reduced quantity of peptidoglycans in the cell walls of gram-negative species (Nagy et al., 2011; Saengmee-Anupharb et al., 2013), which influences the entry of silver and zeolite to act as a bactericide. That is why all the strains of *E. coli* tested demonstrated greater adherence than the other bacteria tested.

The samples used in this study were collected from the milk of cows with mastitis and from milking equipment. The contact of this raw material with stainless steel increases hydrophobicity and electron receptor properties due to the amount of fat in the product (Hamadi et al., 2014). Because this component is an important factor in the formation of microbial biofilms, the equipment used during milking and for cow udders should be correctly cleaned and disinfected at every milking.

Several physical-chemical parameters of the surface can influence the formation of microbial biofilms, including surface tension, hydrophobicity, electrostatic interaction, surface roughness, cytotoxicity and different dosages of nanoparticles in the materials (Lavery, Gorman, & Gilmore, 2013; Neves et al., 2014; Sousa, Teixeira & Oliveira, et al., 2009). These factors may also determine the effectiveness of a material that inhibits the formation of biofilms.

Figure 1 summarizes the results of the extraction of substances from the matrix and quantitative analysis of proteins and polysaccharides with the different materials tested. The increase in protein and polysaccharides in the samples when exposed to silver and zeolite is due to the probable lysis of the bacterial cells with release of intracellular content and greater interaction of the particles with the bacterial matrix (Monteiro et al., 2013).

Figure 1. Extraction of extracellular substances from the matrix and quantification of extracellular proteins and polysaccharides from 2 samples (2 and 8) of *Staphylococcus aureus*, 2 samples (4 and 7) of *Escherichia coli*, and 1 sample (1) of *Staphylococcus epidermidis*, grown for 24 hours in the TSB with coating materials with virgin polypropylene (V), with polypropylene and zeolite 3% (Z3%), with polypropylene and zeolite 6% (Z6%) and with polypropylene, glass and silver (Ag).



Source: The authors.

Estimating the amount of protein and polysaccharide is an indirect way of estimating the deposit of biomass since these parameters are the main components of the biofilm (Lazarova & Manem, 1995). As shown in Figure 1, the matrix quantity diminishes when the biofilm is reduced by the presence of zeolite and silver, compared to the control (virgin polysaccharide). In sample 8 of *S. aureus*, 4 and 7 of *E. coli* and 1 of *S. epidermidis*, zeolite reduced the quantity of matrix, while in sample 2 of *S. aureus*, 4 and 7 of *E. coli* and 1 from *S. epidermidis*, silver reduced the quantity of matrix.

Similar to the data in this study, Oliveira (2013) found a greater amount of polysaccharide compared to protein in aerobic and anaerobic bacteria and fungi adhered to

carbon steel coupons in contact with freshwater, assessed after 30, 60 and 90 days of incubation.

Leite (2013) also assessed the amount of protein and polysaccharide in two strains of *S. epidermidis* after one treatment with N-acetylcysteine (NAC) and another with NAC and rifampicin. A significant increase was observed in the protein and polysaccharide after exposure to the agents, mainly the polysaccharides. However, when the bacteria came into contact with the combined agents, protein increased even further. Similar results were obtained in the present study, in which bacteria, when exposed to zeolite or silver, increased the number of proteins and especially polysaccharides from *S. epidermidis* when compared to virgin polypropylene.

Based on the images recorded by the confocal laser scanning microscope, the results of the previous tests confirm the effectiveness of the polypropylene material with silver and glass (Figure 2). These results were similar to those obtained by Morita et al. (2014), who observed the bactericidal and antibacterial activity of silver on orthodontic wires with biofilms of *Streptococcus sobrinus*. This activity probably occurs through the following mechanisms: interference with biofilm quorum sensing, inhibition of DNA replication and mitosis, effects on cell membrane permeability and control of glucose oxidation, as reported by other researchers (Giulio, et al. 2013; Gurunathan, Han, Kwon & Kim, 2014; Morones et al., 2005).

With the microbial cell viability kit, the presence of dead bacterial cells was observed on the three materials (polypropylene with 3% zeolite, polypropylene with 6% zeolite and polypropylene with silver and glass), due to the bactericidal effects of zeolite and silver. In addition, the toxin released by dead cells can influence the survival of living cells.

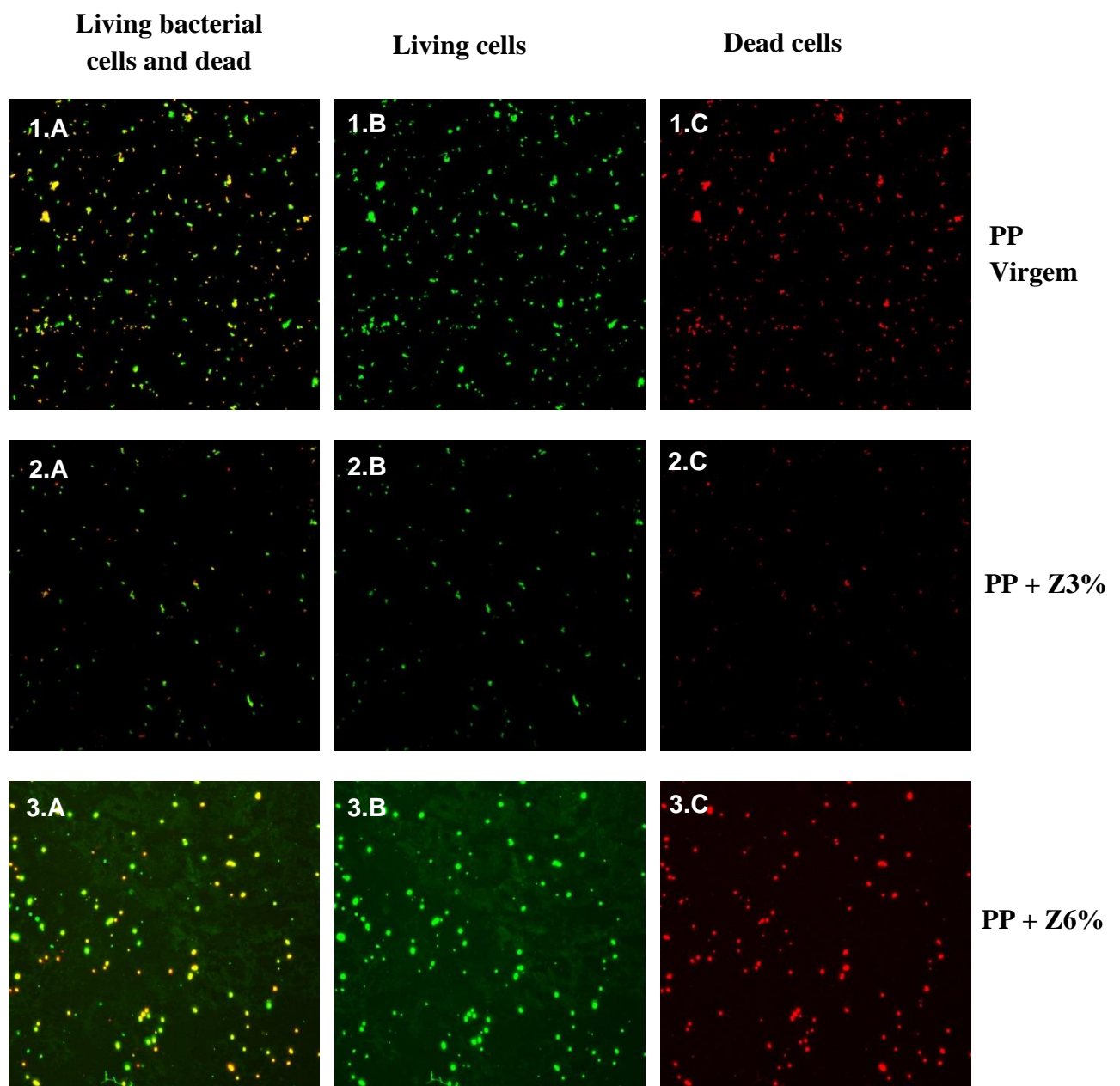
In the photos with the presence of live and dead bacteria - green and red respectively (A) - yellow cells appear because of the overlap of the two types of cells and because the 40x magnification makes it impossible to see them separately due to the several layers of bacteria. Confocal laser scanning microscopy is a good technique for visualizing biofilm and the effectiveness of materials but is not effective for quantification.

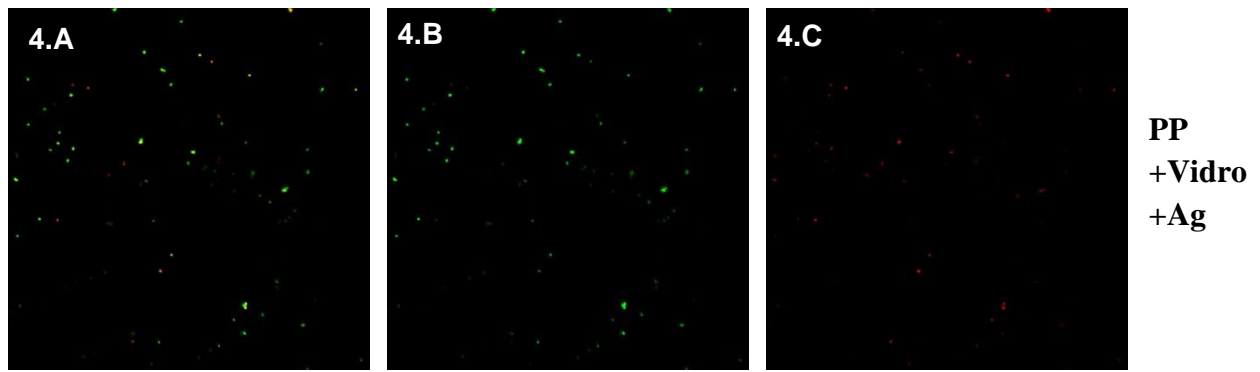
The presence of gram-negative biofilm-producing bacteria, such as *Escherichia coli*, is of concern to rural producers because, as seen in this study, such bacteria are highly capable of adhering to surfaces and thus becoming a source of continuous infection to animals. The presence of gram-positive biofilm-producing bacteria, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, is also of concern, as they are considered infectious and contaminating. Furthermore, after adhering to the animal, the likelihood of contaminating

other animals and fomites is enormous and, since chemical agents and antibiotics are unable to penetrate the biofilm, eliminating the bacteria from the herd can be difficult.

The use of particles such as silver and zeolite with polypropylene as a coating for stainless steel was not very effective since no statistical difference was observed to prove a good capability.

Figure 2. Confocal laser microscopy (Zeiss 710) of *Staphylococcus epidermidis* (sample 1) in virgin material (1), with 3% zeolite (2), with 6% zeolite (3) and with polypropylene, glass and silver (4) , images in stacks, all bacteria (A), live bacteria (B) and dead bacteria (C) at 40x magnification.





Source: The authors.

In figures 1A, 1B and 1C it is observed that in virgin polypropylene a high density of adherent cells was observed, both viable and dead. In figures 2A, 2B and 2C shows a decrease in adhered cell density when 3% zeolite was added to polypropylene. In figures 3A, 3B and 3C a higher percentage of zeolite acted negatively and did not prevent bacterial adhesion. There is an adhesion and cell density very close to virgin polypropylene. In figures 4A, 4B and 4C a better result was observed with less bacterial adhesion when added to silver and glass in polypropylene with a significant reduction in bacterial density and adhesion.

4. Final Considerations

It was concluded that all of the microorganisms tested formed biofilm on all of the coatings tested, particularly polypropylene with 6% zeolite when compared to polypropylene with silver and glass. The material composed of silver, glass and polypropylene was expected to offer less adherence to microbial biofilms, but no difference was observed between this material and the virgin polypropylene coating, with 3% zeolite and with glass and silver. Confocal microscopy revealed that microbial adhesion occurs in relation to the different compositions of stainless steel surfaces. The best result obtained was on the surface composed of polypropylene, glass and silver, with low bacterial adhesion, thus revealing the possibility of increasing the concentration of silver on this surface in future projects due to its anti-adhesive properties.

It is suggested that future research can evaluate these interactions between bacteria and the components of surfaces, so that surfaces, with anti-bacterial adhesion and biofilm formation can be increasingly used.

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Suppliers

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Material supplied by the company Jacto (Pompéia, SP).

References

Action. (2013). *Estatcamp - Consultoria estatística e qualidade*, São Carlos, SP, Brasil. Retrieved from <http://www.portalaction.com.br/>

Ayres, M., Ayres J. M., Ayres, D. L., & Santos, A. A. S. (2007). *BioEstat 5.0: aplicações estatísticas nas áreas das ciências biológicas e médicas*. Belém: Sociedade Civil Mamirauá; Brasília: Conselho Nacional de Desenvolvimento Científico e Tecnológico.

Azeredo, J. A., Lazarova, V., & Oliveira, R. (1999). Methods to extract the exopolymeric matrix from biofilms: a comparative study. *Water Science Technology*, 39(7), 243-250. doi:10.1016/S0273-1223(99)00174-2

Boari, C. A., Alves, M. P., Tebaldi, V. M. R., Savian, T. V., & Piccoli, R. H. (2009). Formação de biofilme em aço inoxidável por *Aeromonas hydrophila* e *Staphylococcus aureus* usando leite e diferentes condições de cultivo. *Ciência e Tecnologia de Alimentos*, 29(4), 886-895. doi: 10.1590/S0101-20612009000400029

Chagas, L. G. S., Melo, P. C., Brasão, S. C., Silvestre, G. B. R., Guimarães, E. C., & Lima, A. M. C. (2017). Evaluation of biofilm formation by bacterial strains isolated from milking equipment and milk samples from cows with mastitis. *Semina: Ciências Agrárias*, 38(4), 1887-1896. doi: 10.5433/1679-0359.2017v38n4p1887

Ciccio, P. D., Vergara, A., Festino, A. R., Paludi, D., Zanardi, E., Ghidini, S., & Ianieri, A. (2015). Biofilm formation by *Staphylococcus aureus* on food contact surfaces: Relationship with temperature and cell surface hydrophobicity. *Food Control*, 50(1), 930-936. doi: 10.1016/j.foodcont.2014.10.048

Cos, P., Toté K., Horemans T., & Maes, L. (2010). Biofilms: an extra hurdle for effective antimicrobial therapy. *Current Pharmaceutical Design*, 16(20), 2279-2295. doi: 10.2174/138161210791792868

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections, *Science*, 284(5418), 1318-1322. doi: 10.1126/science.284.5418.1318

Cowan, M. M., Abshireb, K. Z., Houk, S. L., & Evans, S. M. (2003). Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel. *Journal of Industrial Microbiology and Biotechnology*, 30(2), 102-106. doi: 10.1007/s10295-002-0022-0

Cucarella, C., Tormo, M. A., Ubeda, C., Trotonda, M. P., Monzón, M., Peris, C., Amorena, B., Lasa, I., & Penadés, J. R. (2004). Role of biofilm associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*. *Infection and Immunity*, 72(4), 2177-2185. doi: 10.1128/IAI.72.4.2177-2185

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28(3), 350-356. doi: 10.1021/ac60111a017

Ebrahimi, R., Zandi, S., & Gharibi, F. (2014). Removal of nickel and total chromium using *Escherichia coli* biofilm supported on clinoptilolite. *Journal of Advances in Environmental Health Research*, 2(2), 126-133. doi: 10.22102/jaehr.2014.40153

Eriksson, H. (2008). Controlled release of preservatives using dealuminated zeolite Y. *Journal of Biochemical and Biophysical Methods*, 70(6), 1139-1144. doi: 10.1016/j.jbbm.2007.05.010

Freeman, D. J., Falkiner, F. R., & Keane, C. T. (1989). New method for detecting slime production by coagulase negative staphylococci. *Journal Clinical Pathology*, 42(8), 872-874. doi:10.1136/jcp.42.8.872

Fuster-Valls, N., Hemández-Herrero, M., Marín-de-Mateo, M., & Rodríguez-Jerez, J. J. (2008). Effect of different environmental conditions on the bacteria survival on stainless steel surfaces. *Food Control*, 19(3), 308-314. doi: 10.1016/j.foodcont.2007.04.013

Giulio, M. D., Bartololomeo, S. D., Campli, E. D., Sancilio, S., Marsich, E., Travan, A., Cataldi, A., & Cellini, L. (2013). The effect of a silver nanoparticle polysaccharide system on Streptococcal and saliva-derived biofilms. *Internation Journal Molecular Sciences*, 14(7), 13615–13625. doi: 10.3390/ijms140713615

Gomes, F. I. A. (2010). *New therapeutic strategies against Staphylococcus epidermidis biofilms*. Tese de Doutorado, Universidade do Minho, Braga, Portugal. Retrieved from <http://repositorium.sdum.uminho.pt/handle/1822/11720>

Gurunathan, S., Han, J. W., Kwon, D. N., & Kim, J. H. (2014). Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. *Nanoscale Research Letters*, 9(375), 1-17. doi: 10.1186/1556-276X-9-373

Hamadi, F., Asseme, F., Elabed, S., Bensouda, S., Mabrouki, M., & Latrache, H. (2014). Adhesion of *Staphylococcus aureus* on stainless steel treated with three types of milk. *Food Control*, 38(1), 104-108. doi: 10.1016/j.foodcont.2013.10.006

Khameneh, B., Zarei, H., & Bazzaz, B. S. F. (2014). The effect of silver nanoparticles on *Staphylococcus epidermidis* biofilm biomass and cell viability. *Nanomedicine Journal*, 1(5), 302-307. doi: 10.7508/nmj.2015.05.003

Koneman, E. W. (2001). *Diagnóstico Microbiológico – Texto e Atlas Colorido*. (5 ed.). Rio de Janeiro: Médica e Científica Ltda.

Kunze, B., Reck, M., Dotsch, A., Lemme, A., Schummer, D., Irschik, H., Steimmetz, H., & Wagner-Dobler. (2010). Damage of *Streptococcus mutans* biofilms by carolacton, a

secondary metabolite from the myxobacterium *Sorangium cellulosum*. *BMC Microbiology*, 10(199), 2-13. doi: 10.1186/1471-2180-10-199

Latorre, A. A., Van Kessel, J. S., Kams, J. S., Zurakiwski, M. J., Pradhan, A. K., Boor, K. J., Jayarao, B. M., Houser, B. A., Daugherty, C. S., & Schukken, Y. H. (2010). Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes*. *Journal of Dairy Science*, 93(6), 2792–2802. doi: 10.3168/jds.2009-2717

Laverty, G., Gorman, S. P., & Gilmore, B. F. (2013). Biomolecular mechanisms of staphylococcal biofilm formation. *Future Microbiology*, 8, 509-524. doi: 10.2217/fmb.13.7

Lazarova, V., & Mamem, J. (1995). Biofilm characterization and activity analysis in water and wastewater treatment. *Water Research*, 29(10), 2227–2245. doi: 10.1016/0043-1354(95)00054-O

Lee, S. H. I., Mangolin, B. L. C., Gonçalves, J. L., Neeff, D. V., Silva, M. P., Cruz, A. G., & Oliveira, C. A. F. (2014). Biofilm-producing ability of *Staphylococcus aureus* isolates from Brazilian dairy farms. *Journal of Dairy Science*, 97(1), 1812–1816. doi: 10.3168/jds.2013-7387

Leite, B. A. (2013). *Estudo da susceptibilidade e resposta dos biofilmes de estafilococos aos agentes antimicrobianos*. Tese de Doutorado, Universidade Federal de São Carlos, São Carlos, SP, Brasil. Retrieved from <https://repositorio.ufscar.br/handle/ufscar/263>

Martin, J. G. P., Oliveira e Silva, G., Fonseca, C. R., Morales, C. B., Belmonte, C. P., Miquelutti, D. L. & Porto, E. (2016). Efficiency of a cleaning protocol for the removal of enterotoxigenic *Staphylococcus aureus* strains in dairy plants. *International Journal of Food Microbiology*, 238(1), 295-301. doi: 10.1016/j.ijfoodmicro.2016.09.018

Monteiro, D. R., Silva, S., Negri, M., Gorup, L. F., Camargo, E. R., Oliveira, R., Barbosa, D.B., & Henriques, M. (2013). Silver colloidal nanoparticles: effect on matrix composition and structure of *Candida albicans* and *Candida glabrata* biofilms. *Journal of Applied Microbiology*, 114(4), 1175–1183. doi: 10.1111/jam.12102

Morita, Y., Imai, S., Hanyuda, A., Martin, K., Hanada, N., & Nakamura, Y. (2014). Effect of silver ion coating of fixed orthodontic retainers on the growth of oral pathogenic bacteria. *Dental Materials Journal*, 33(2), 268–274. doi: 10.4012/dmj.2013-216

Morones, J.R., Elechiguerra, J. L., Camacho, A., Holt, K., Kouri, J. B., Ramirez, J.T., & Yacaman, M. J. (2005). The bactericidal effect of silver nanoparticles. *Nanotechnology*, 16(10), 2346–2353. doi: 10.1088/0957-4484/16/10/059

Nagy, A., Harrison, A., Sabbani, S., Munson, Jr. R. S., Dutta, P.K., & Waldman, W. J. (2011). Silver nanoparticles embedded in zeolite membranes: release of silver ions and mechanism of antibacterial action. *International Journal of Nanomedicine*, 6(1), 1833–1852. doi: 10.2147/IJN.S24019

Neves, P. B. A., Agnelli, J. A. M., Kurachi, C., & Souza, C. W. O. (2014). Addition of silver nanoparticles to composite resin: effect on physical and bactericidal properties in vitro. *Brazilian Dental Journal*. 25(2), 141-145. doi: 10.1590/0103-6440201302398

Oliveira, E. S. D. (2013). *Biocorrosão em cupons de aço carbono expostos à água doce*. Dissertação de Mestrado, Universidade Federal do Pernambuco, Recife, Brasil. Retrieved from <https://repositorio.ufpe.br/handle/123456789/13342>

R Development Core Team. R. (2015). A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria. Retrieved from <http://www.r-project.org>

Saengmme-Anupharb, S., Srikririn, T., Thaweboon, B., Thaweboon, S., Amornsakchai, T., Dechkumakor, S., & Suddhasthira, T. (2013). Antimicrobial effects of silver zeolite, silver zirconium phosphate silicate and silver zirconium phosphate against oral microorganisms. *Asian Pacific Journal of Tropical Biomedicine*. 3(1), 47-52. doi: 10.1016/S2221-1691(13)60022-2

Sousa, C., Teixeira, P., & Oliveira, R. (2009). Influence of surface properties on the adhesion of *Staphylococcus epidermidis* to acrylic and silicone. *International Journal of Biomaterials*, 9(1), 1-9. doi: 10.1155/2009/718017

Tran, P. A., & Webster, T. J. (2013). Understanding the wetting properties of nanostructured selenium coatings: the role of nanostructured surface roughness and air-pocket formation. *International Journal of Nanomedicine*, 8(1), 2001-2009. doi: 10.2147/IJN.S42970

Triola, M. F. (2014). *Introdução à Estatística*. (7^a ed.). Rio de Janeiro: LTC.

Tamanai-Shacoori, Z., Chahandad, F., Rébillard, A., Cillard, J., & Bonnaure-Mallet, M. (2014). Silver-zeolite combined to polyphenol-rich extracts of *Ascophyllum nodosum*: potential active role in prevention of periodontal diseases. *Plos One*, 9(10), 1-8. doi: 10.1371/journal.pone.0105475

Wang, S., & Peng, Y. (2010). Natural zeolites as effective adsorbents in water and wastewater treatment. *Chemical Engineering Journal*, Laussane, 156(1), 11-24. doi:10.1016/j.cej.2009.10.029

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