

Affinity of *Staphylococcus aureus* for prostheses colonization compared to other bacteria. An *in vitro* study

Afinidade de Staphylococcus aureus para colonização de próteses em comparação com outras bactérias. Um estudo in vitro

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Abstract

Staphylococcus aureus biofilms have been recognized as a leading cause of multiple infections, including implant-associated infections and chronic wounds. We evaluated the colonization capacity of two distinct textured prostheses by different bacterial strains. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Proteus mirabilis* and *Enterococcus faecalis* were evaluated. Initially, the hydrophobicity and biofilm formation capacity were determined. Subsequently, 20 fragments of vascular prosthesis and 20 silicone prostheses were embedded in

suspensions with the microorganisms and incubated. The prostheses were then sown in culture medium and incubated for 48 hours. Petri dishes were photographed and analyzed by fractal dimension. The Kruskal-Wallis test and the Dunn test were applied for the analysis of biofilm formation. To compare the mean intensity for the type of bacteria and the type of prosthesis, a general linear model was applied. *Staphylococcus aureus* was the bacterium with the highest colonization density in both prostheses ($p = 0.0001$). *E. coli* showed strong adherence in the biofilm formation capacity test ($p = 0.0001$), however, it did not colonize either prosthesis. We demonstrated that *Staphylococcus aureus* has a greater affinity for vascular and silicone prostheses than other bacteria.

Keywords: Biofilms; Prostheses and implants; Polytetrafluoroethylene; Vascular surgical procedures; Breast implant.

Resumo

Biofilmes de *Staphylococcus aureus* foram reconhecidos como uma das principais causas de infecções múltiplas, incluindo infecções associadas a implantes e feridas crônicas. Avaliamos a capacidade de colonização de duas próteses texturizadas distintas por diferentes cepas bacterianas. Foram avaliados *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Proteus mirabilis* e *Enterococcus faecalis*. Inicialmente, foram determinadas a hidrofobicidade e a capacidade de formação de biofilme. Posteriormente, 20 fragmentos de próteses vasculares e 20 próteses de silicone foram incluídos em suspensões com os microrganismos e incubados. As próteses foram então semeadas em meio de cultura e incubadas por 48 horas. As placas de Petri foram fotografadas e analisadas pela dimensão fractal. O teste de Kruskal-Wallis e o teste de Dunn foram aplicados para a análise da formação de biofilme. Para comparar a intensidade média para o tipo de bactéria e o tipo de prótese, foi aplicado um modelo linear geral. *Staphylococcus aureus* foi a bactéria com maior densidade de colonização em ambas as próteses ($p = 0,0001$). *Escherichia coli* apresentou forte aderência no teste de capacidade de formação de biofilme ($p = 0,0001$), porém não colonizou nenhuma das próteses. Demonstramos que o *Staphylococcus aureus* tem maior afinidade por próteses vasculares e de silicone do que outras bactérias.

Palavras-chave: Biofilmes; Próteses e implantes; Politetrafluoroetileno; Procedimentos cirúrgicos vasculares; Implantes mamários.

Resumen

Se ha reconocido que las biopelículas de *Staphylococcus aureus* son una de las principales causas de infecciones múltiples, incluidas las infecciones asociadas a implantes y las heridas crónicas. Evaluamos la capacidad de colonización de dos prótesis texturizadas distintas por diferentes cepas bacterianas. Se evaluaron *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Proteus mirabilis* y *Enterococcus faecalis*. Inicialmente se determinó la hidrofobicidad y la capacidad de formación de biopelículas. Posteriormente, se embebieron 20 fragmentos de prótesis vasculares y 20 prótesis de silicona en suspensiones con los microorganismos y se incubaron. A continuación, las prótesis se sembraron en medio de cultivo y se incubaron durante 48 horas. Las placas de Petri se fotografiaron y analizaron por dimensión fractal. Se aplicaron la prueba de Kruskal-Wallis y la prueba de Dunn para el análisis de la formación de biopelículas. Para comparar la intensidad media para el tipo de bacteria y el tipo de prótesis, se aplicó un modelo lineal general. *Staphylococcus aureus* fue la bacteria con mayor densidad de colonización en ambas prótesis ($p = 0,0001$). *Escherichia coli* mostró una fuerte adherencia en la prueba de capacidad de formación de biopelículas ($p = 0,0001$), sin embargo, no colonizó ninguna de las prótesis. Demostramos que *Staphylococcus aureus* tiene una mayor afinidad por las prótesis vasculares y de silicona que otras bacterias.

Palabras clave: Biopelículas; Prótesis e implantes; Politetrafluoroetileno; Procedimientos quirúrgicos vasculares; Implante mamario.

1. Introduction

According to the National Institutes of Health (NIH), over 80% of all microbial infections are related to biofilm formation. These types of infections are difficult to diagnose and treat. Biofilms are also able to colonize medical devices such as catheters and implants (Rabin et al., 2014).

In studies carried out with patients with different types of wounds associated or not with surgery, the most common bacterial species detected were *Staphylococcus aureus* (in most cases), followed by *Proteus mirabilis*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella ssp* and *Corynebacterium spp* (Bessa et al., 2015; Mengesha et al., 2014).

The most common microorganisms found on the skin are *S. aureus* and *Staphylococcus epidermidis* (Igari et al, 2014). Contamination of vascular or silicone prostheses often occurs in the preoperative period, when the prosthesis contact the

skin during surgery, so these are the bacteria that most contaminate these two types of prostheses (Ghiselli et al., 2001; Alp et al., 2014).

S. aureus is a major cause of nosocomial and community-acquired infections, and infections caused by this bacterium represent a high cost to the healthcare system (Lister & Horswill, 2014). Interest in infections caused by the *S. aureus* biofilm has been associated with the rapid increase in the use of implants and medical prostheses and the concomitant increase in infections related to these devices (Bhattacharya et al., 2015).

The surface of an implant is rich in proteins such as fibronectin, which is present at the surgical wound site. These proteins are recognized by microbial surface components that recognize matrix adhesion molecules, providing a niche for bacteria to form biofilms. The adhesion of bacteria can be influenced by the material that prosthesis is made and by its texture (Bhattacharya et al., 2015).

The hypothesis of this study is that the prevalence of *S. aureus* as a causal agent of biofilms or infections in prostheses and implants is due to its greater ability to adhere to medical devices when compared to other bacteria. So, we evaluate the *in vitro* colonization capacity of different bacterial strains on two prostheses with different textures (silicone prostheses and vascular prostheses that are two medical devices widely used in clinical practice) to assess whether *S. aureus* has greater prosthesis colonization capacity when compared to other bacteria that commonly colonize prostheses and wounds and whether the texture of the prostheses can influence this colonization.

2. Methods

This is a prospective, quantitative, and experimental study (Pereira et al., 2018).

2.1 Bacterial strains and culture condition

The bacterial strains (Microbiologics, Inc., St. Cloud, Minnesota, USA) used were *Escherichia coli* ATCC® 25922™, *Proteus mirabilis* ATCC® 25933™, *Staphylococcus aureus* subspecies *aureus* ATCC® 25923™, *Staphylococcus epidermidis* ATCC® 12228™ and *Enterococcus faecalis* ATCC® 29212™.

About 50µL of bacterial frozen stocks samples (10^8 CFU/mL) were inoculated in BHI (brain heart infusion) and incubated at 37°C for 24 hours.

2.2 Determination of cellular hydrophobicity

The hydrophobicity of the bacterial cell surface was assessed by measuring the microbial adhesion to solvents (MATS) (Locatelli et al., 2004). Bacterial cultures were adjusted to an optical density (OD) 600 nm of 1.0 in 0.9% saline. A 0.4 mL volume of xylene was added to 3.6 mL of bacterial suspension. The mixture was vortexed for 5 minutes, and then the tubes were conditioned at room temperature for 20 minutes. The aqueous phase was removed, and the OD 600 nm was measured by a spectrophotometer (Digital Visible Spectrophotometer - Q898DRM, QUIMIS Scientific Apparatus, Diadema, São Paulo, Brazil). In this method, a greater interaction of microorganisms with a nonpolar liquid (p-xylene) represents a greater cellular hydrophobicity. Bacteria were considered hydrophobic when OD was less than 0.8 and hydrophilic when OD was equal to or greater than 0.8 (Locatelli et al., 2004).

2.3 Biofilm formation analysis

For biofilm formation analysis, cultures were adjusted to the turbidity corresponding to McFarland scale tube 0.5 (1.5×10^8 colony-forming units/mL). Then, 20 µL aliquots of the cell suspension from each isolate were added to 200 µL of BHI

broth present in the wells of 96-well polystyrene microplates (CRALPLAST, CRAL Laboratory Articles Ltd., Cotia, Brazil) and incubated at 37°C for 24 hours. The plates were then washed and the adhered cells were stained with 200 µl of 0.1% (w / v) violet crystal for 5 minutes (Ziuzina et al., 2015). The optical density (OD) of the solution was read at a wavelength of 600 nm (Microplate reader MR-96A, Shenzhen Mindray Bio-Medical Electronics Co., Ltd, Shenzhen, China). The mean OD of the negative control (OD_c), BHI without inoculum, was used as the cutoff point. Isolates were classified as follows: OD_c ≤ OD nonadherent; OD_c < OD ≤ 2x OD_c weakly adherent; 2x OD_c < OD ≤ 4x OD_c moderately adherent; 4x OD_c < OD strongly adherent (Tiba et al., 2009). The analysis was performed in quadruplicate for each bacterium.

2.4 Prostheses colonization test

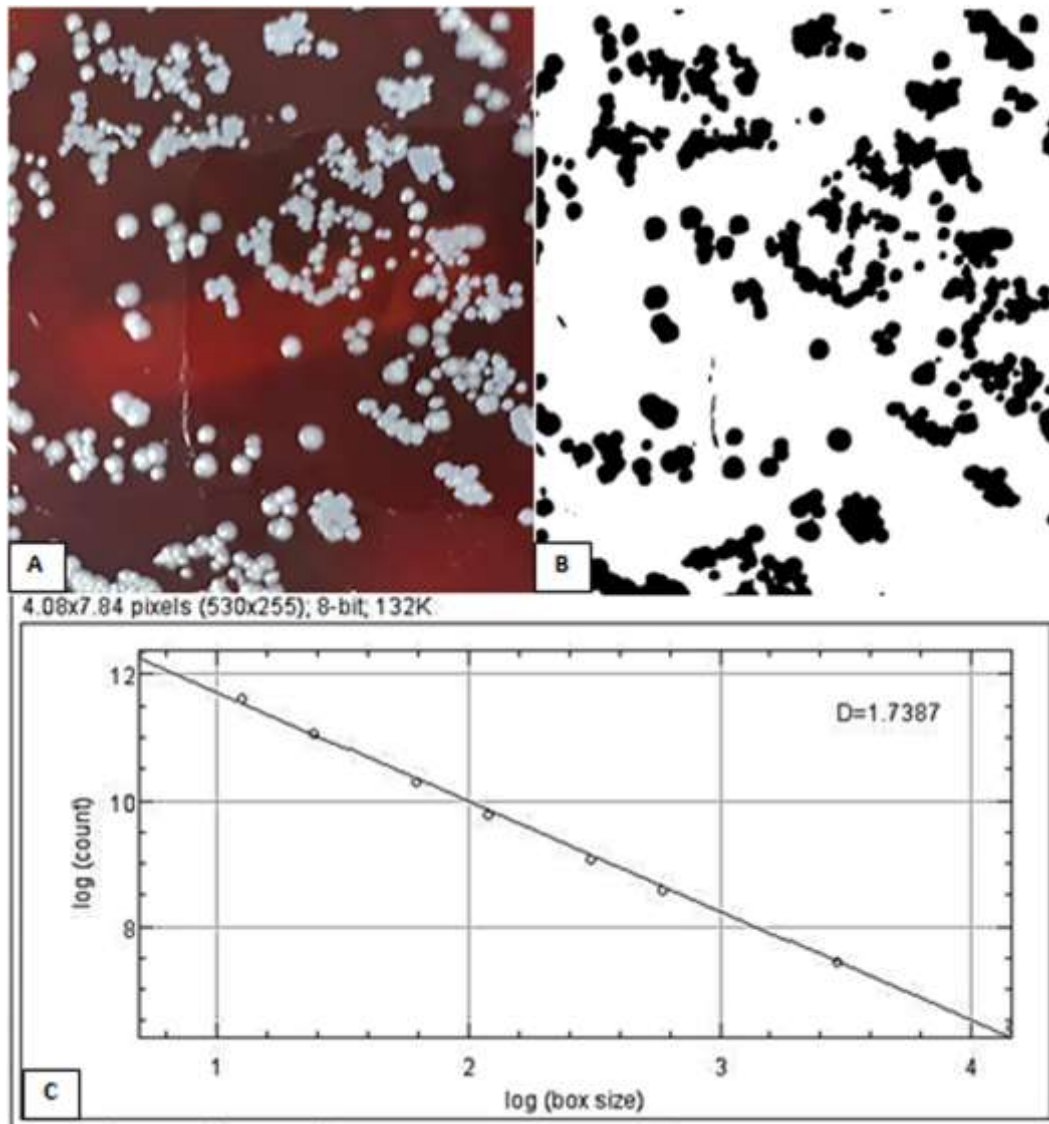
To calculate the minimum sample size, the “pwr” package in R was used (Champely, 2018). The test power was 80%, significance level was 5%, number of groups to be compared was 10 (2 prostheses x 5 bacteria), and effect size (Cohen's d) was 0.50. So, it was concluded that at least 4 of each prosthesis per group were necessary.

Twenty silicone prostheses measuring 2 cm diameter (Model Forma Malhas Compressivas e Produtos Hospitalares Ltda. - EPP, São Caetano do Sul, Brazil) and 20 fragments of the Exxcel Soft expanded polytetrafluoroethylene (ePTFE) synthetic vascular prosthesis (Maquet Cardiovascular LLC, Wayne, NJ, USA) measuring 1 cm each were used. Silicone and ePTFE prosthesis were soaked in suspensions in 1 ml of TSB (Tryptic Soy Broth) with the microorganisms, which were adjusted to the turbidity corresponding to McFarland scale tube 0.5. The prostheses were incubated in sterile flasks in an oven at 37°C for one week. One milliliter of TBS was added to each flask every 2 days during the incubation. The analysis was performed in quadruplicate for each bacterium and prosthesis.

After incubation, the prostheses were seeded by rolling in Petri dishes (15x150 mm) containing 40 mL of agar (Electrolyte Deficient Cystine Lactose Agar – CLED – for evaluation of *P. mirabilis* and blood agar for evaluation of other bacteria) and were incubated at 37°C for 48 hours.

After incubation, the plates were photographed with a digital camera (Cyber Shot W830 20.1MP, Sony, Japan) and analyzed with ImageJ[®] software (<http://rsbweb.nih.gov/ij/>). Fractal dimension analysis was performed by the box-counting method (Figure 1) (Stolze et al., 2019; Nai et al., 2021).

Figure 1. A - Original image of Petri dishes inoculated with ePTFE prostheses contaminated with *S. aureus*. B - Binarized image. C - Box-counting of fractal dimension analysis.



Source: Authors.

2.5 Statistical analysis

The homogeneity of the variances was verified by the Levene test and the normality by the Kolmogorov-Smirnov test.

The analysis of biofilm formation did not present a normal distribution or homogeneity of variances, so the Kruskal-Wallis test was applied and the Dunn test to make the comparison between the groups.

To compare the mean intensity for the type of bacteria and the type of prosthesis, a general linear model was applied. As the intensity did not present a normal distribution or homogeneity of variances, the Kruskal-Wallis test was also applied to compare the positions of each group, regardless of the type of prosthesis.

Differences were considered statistically significant when $p < 0.05$. The tests were performed with SPSS v. 23.0.

3. Results

3.1 Determination of cellular hydrophobicity

S. aureus, *E. coli* and *E. faecalis* were found to be hydrophobic, and *S. epidermidis* and *P. mirabilis* were found to be hydrophilic (Table 1).

Table 1. Result of the determination of cellular hydrophobicity by optical density (OD) of each bacterium evaluated in the study.

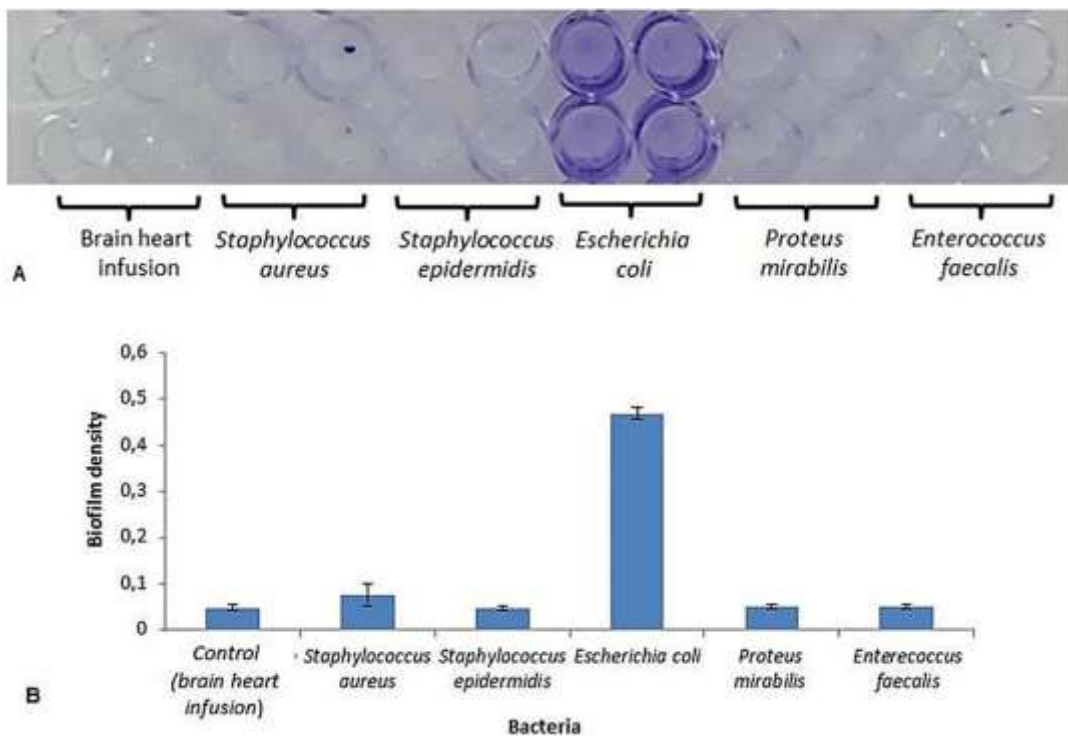
| Bacteria | OD |
|-----------------------|-------|
| <i>S. aureus</i> | 0.410 |
| <i>E. coli</i> | 0.489 |
| <i>E. faecalis</i> | 0.442 |
| <i>S. epidermidis</i> | 0.861 |
| <i>P. mirabilis</i> | 0.853 |

Fonte: Autores (2021).

3.2 Biofilm formation analysis

There was a significant difference in the analysis of biofilm formation between the bacterial groups ($p = 0.0001$) (Figure 2).

Figure 2. A - Microtiter plate showing biofilm production. B - Density of biofilm formation according to the bacteria studied (mean and standard deviation).



Fonte: Autores (2021).

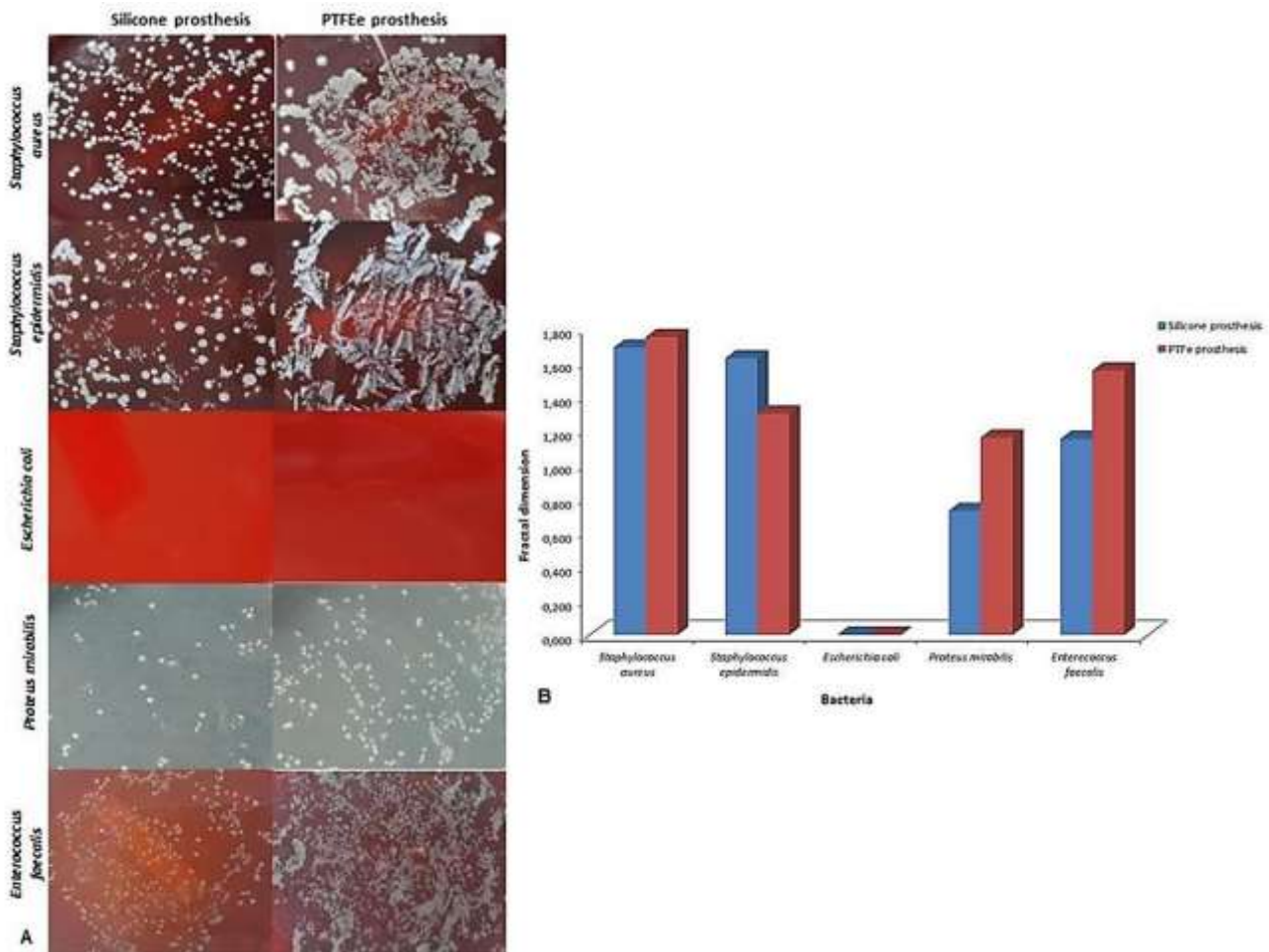
Bacteria were considered non-adherent when OD was less than 0.049, weak adherent when OD was between 0.049 and 0.098, moderately adherent when OD was between 0.098 and 0.196 and strong adherent when OD was greater than 0.196.

S. epidermidis (OD= 0.005228129) was considered “nonadherent”, *S. aureus* (OD= 0.07575), *E. faecalis* (OD= 0.005560276) and *P. mirabilis* (OD= 0.004041452) were considered “weakly adherent”, and *E. coli* (OD= 0.015986974) was considered “strongly adherent”.

3.3 Prostheses colonization test

There was a difference in intensity only for the types of bacteria ($p = 0.0001$), but there was no difference for the type of prosthesis ($p = 0.496$) or for the interaction prosthesis x bacteria ($p = 0.586$) (Figure 3).

Figure 3. A - Image of Petri dishes inoculated with silicone prostheses and ePTFE prostheses colonized by the five bacteria studied. B - Fractal dimension of Petri dishes inoculated with silicone prostheses and ePTFE prostheses colonized by the five bacteria studied.



Fonte: Autores (2021).

4. Discussion

In this study, *S. aureus* was the bacterium that colonized both types of prostheses evaluated the most, although in the biofilm formation test, it was “weakly adherent”. In contrast, *E. coli* was “strongly adherent” to the biofilm formation test but

did not colonize either type of prosthesis evaluated. This corroborates our hypothesis that *S. aureus* is the most frequent cause of infection because it has a higher affinity for the materials from which medical devices are made.

Silicone breast implant infection occurs in 7 to 24% of implant breast reconstructions and the most common pathogens are *S. epidermidis* and *S. aureus* (Arad et al., 2013), and we corroborate these data.

ePTFE is chemically inert, hydrophobic and mechanically durable (Campbell *et al.* 1976). *S. aureus* and coagulase-negative *Staphylococcus* are responsible for 70 to 90% of vascular prosthesis infections (Turtiainen et al., 2014; Van De Vyver et al., 2017). Apparently, the microporous structure and synthetic composition of ePTFE grafts provide niches for bacterial accumulation and interfere with the ability of leukocytes to fight infections (Nadzam et al., 2000). In this study, the bacteria that most colonized the ePTFE prostheses were *S. aureus* followed by *E. faecalis*. These two bacteria have a hydrophobic character, which may justify having colonized more ePTFE prostheses. However, *E. coli* also presented a hydrophobic character but did not colonize these prostheses.

Interstitial porosity, surface tension and electronegativity of medical devices have been shown to attract and maintain bacterial adherence (Chang et al., 2003). This may explain the differences we found between the biofilm formation test, where *E. coli* was “strongly adherent” and *S. epidermidis* was “nonadherent”, and the prosthesis colonization test, where *E. coli* did not colonize both prostheses, but *S. epidermidis* colonized the two types of prostheses in greater concentration. The microplates that were used for the biofilm formation test were made with polystyrene, a material very distinct from silicone or ePTFE, which may have led to this difference in results.

Although *E. coli* is a biofilm-forming bacterium (Henriques et al., 2013; Ziuzina et al., 2015), in this study, *E. coli* did not colonize silicone or ePTFE prostheses. Biofilm formation capacity seems to be associated with specific genes expressed in different strains of *E. coli* (Tiba et al., 2009). Additionally, most studies have evaluated the ability of biofilm formation by *E. coli* in tissues (Tiba et al., 2009; Henriques et al., 2013; Ziuzina et al., 2015). Some studies have evaluated catheters associated with urinary tract infection and *E. coli* is the bacterium that colonizes these devices the most (Maharjan et al., 2018). These data may justify the fact that we did not observe colonization by *E. coli* in the prostheses; the difference may be due to the bacterial strain used in our study, which would make a difference in its ability to form biofilm, or because *E. coli* causes nosocomial infection by colonizing more tissue and only certain medical devices. *In vivo* studies will be needed to clarify this point.

5. Conclusion

With the data from this study, we demonstrated that *S. aureus* showed a higher affinity for colonization of silicone and ePTFE prostheses than the other bacteria tested and that *E. coli*, although showed strong adherence in the biofilm formation capacity test, did not colonize either prosthesis in the *in vitro* evaluation. This difference observed for *E. coli* between the biofilm formation capacity test and the adhesion in the evaluated prostheses shows that the bacterial strain and the material that prosthesis is made can influence the bacterial adhesion to them.

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