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# Dynamic of Biofilm Formation on Surgical Clamps by *Staphylococcus aureus* and *Acinetobacter baumannii* Strains

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors SGS and ACO designed the study. Authors JPAH and CDV performed the statistical analysis. Authors MJVT, CDV and LMF wrote the protocol and wrote the first draft of the manuscript. Authors MJVT, JKTS and JFGF managed the experiments and the analyses of the results. Author MJVT and SGS managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Background:** Understanding biofilm formation and the interaction between bacterial species on surgical instrument surfaces is of great importance. Due to the scarcity of studies on this subject, the present study proposes to investigate this dynamic process.
**Methods:** *Staphylococcus aureus* and *Acinetobacter baumannii* reference strains, associated

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or not, were cultured on Cushing tissue dissecting forceps. In an attempt to compare and investigate biofilm in different anatomic parts of this surgical instrument, quantitative microbial culture and electronic microscopy were performed at different times.

**Results and Discussion:** The quantitative culture and electronic microscopy analysis of single-species biofilm showed that *A. baumannii* cells were more adherent and prevalent on the instruments' surface at all examined times, whereas mixed-species biofilm results showed that *S. aureus* cells prevail after the sixth hour and represent the majority of the aggregated cells at 12 and 24 h.

**Conclusions:** Our results indicated a possible antagonistic interaction between the two tested species. The findings also showed that the biofilm formation occurred after the first analyzed time, reinforcing the need to follow the existing guidelines to process medical devices.

*Keywords: Biofilm formation; Staphylococcus aureus; Acinetobacter baumannii; medical devices.*

## 1. BACKGROUND

Medical devices contaminated with dirt and microorganisms could be a result of failures in professional handling and cleaning leading to high rates of surgical site infections (SSIs) [1,2]. SSIs are defined by the Centers for Disease Control and Prevention [3] (CDC) as those infections that happen in the same part of the body as where the surgical procedure was performed. Sometimes they could be a superficial infection involving the skin only and in other events could be more serious involving tissues under the skin, organs, or implanted materials. According to the World Health Organization [4] and the CDC [3], hospital-acquired infections (HAIs) are responsible for significant morbidity and mortality inside healthcare institutions. The onset of these infections is directly associated with biofilm formation. SSIs are included among the leading types of HAIs, being responsible for almost 31% of hospital infections, increasing costs and the length of hospital stay. Despite the extremely high importance of SSIs, HAI studies focused on this subject are scarce and almost all published papers are related to outbreaks [3,5].

Biofilms are complex microbial aggregates embedded into a matrix of exopolysaccharide (EPS). This structure can adhere to biotic and/or abiotic surfaces whose composition can be mono- or polymicrobial [6,7]. Medical devices represent a propitious surface for microbial adhesion and biofilm formation corresponding to nearly 65% of HAIs.

Pathogens from the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp*) are the main causes of SSIs associated with medical devices and represent a serious public healthcare problem. Among this group, *S. aureus* and *A. baumannii* are two of the most important pathogens. Both secrete a wide range of virulence factors, such as those responsible for biofilm formation. This structure allows microbial survival in hospital settings and also inside the host [8].

Biofilm-associated diseases are difficult to diagnose and treat and are therefore a challenge for healthcare workers. Biofilm enables resistance to environmental conditions, resistance to immune system responses including phagocytosis and also difficult antimicrobial and chemical agent action [6,9]. These characteristics are related to the EPS matrix and the differences in gene expression, which results in microorganisms with different physiological profiles. Detachment and dispersal of cells from biofilms could occur. The presence of impurities inside the EPS matrix could exacerbate the infectious process and inflammatory reactions [1]. Due to the scarcity of studies on biofilm formation on surgical instrument surfaces, the present study proposes to investigate this dynamic process using *S. aureus* and *A. baumannii*, associated or not, on surgical forceps surfaces. This knowledge could contribute to a better understanding of the biofilm formation process and the mechanisms of cell adhesion on medical devices.

## 2. MATERIALS AND METHODS

### 2.1 Biofilm Formation

This *in vitro* study was conducted in the Laboratory of Oral Microbiology and Anaerobes of the Federal University of Minas Gerais, Belo Horizonte city, Brazil. The biofilm formation

process and its dynamics was performed according to published methods [10,11,12] with some modifications. Cushing tissue dissecting forceps without teeth (Edlo®) were chosen to perform all tests due to their intimate contact with body tissues and fluids during surgical procedures. To investigate biofilm formation, *Staphylococcus aureus* ATCC 25923 and *Acinetobacter baumannii* ATCC 19606 strains were distributed into three study groups. The first one was composed of dissecting forceps colonized by *S. aureus*, the second one with *A. baumannii* and the third one with the two microbial strains associated. Each experimental group was tested in triplicate.

## 2.2 Analysis of Biofilm Formation over the Entire Forceps

Sterilized dissecting forceps were placed in a sterile container with 700 mL of Tryptic Soy broth (TSB) (BD®) and an inoculum of 1.5 x $10^5$ colony-forming units (CFUs)/mL of *S. aureus* and/or *A. baumannii* strains was added. To allow biofilm formation, experimental containers were incubated at 37 °C with shaking at 60 rpm (Ovan®) for 2, 4, 6, 8, 12 and 24 hours. At these times, the forceps were removed from the container and rinsed six times with sterile phosphate-buffered saline (PBS). After washing, all instruments were transferred to a sterile plastic bag with 100 mL of PBS and submitted to five minutes of vortexing (Vortex-Genie 2, Daigger®). After mixing, the instruments were sonicated at a frequency of 40 KHz (model DES500, Unique®) for five minutes to remove the formed biofilm. One aliquot of 100 µL of the mixed solution was spread, in duplicate, onto Tryptic Soy Agar (TSA) (Difco®) to quantify the total bacterial cells, and also onto Mannitol Salt Agar (BD®) and MacConkey Agar (BD®) selective medium to evaluate the gram-positive and gram-negative strains, respectively. After 24 hours of incubation at 37℃, the number of colonies on each plate was counted and the results were expressed as colony-forming units (CFUs)/mL.

## 2.3 Analysis of Biofilm Formation on Forceps Fragments

In an attempt to compare and investigate biofilm formation on different anatomic parts of the dissecting forceps, quantitative culture and electronic microscopy were performed. The dissecting forceps were fragmented into three different areas according to their design and potential possibility of biofilm accumulation. Portions of 1 cm of the tip, the flat part of the body and the part with indentations were selected. The biofilm formation analyses of fragments were similar to that performed on the entire forceps, considering the three different study groups. Each experimental group was also tested in triplicate. Sterilized fragments were placed in 5 mL of TSB inoculated with 1.5 x $10^5$ CFUs/mL of bacterial strains. The containers with the fragments were incubated at 37°C under shaking at 60 rpm for 2, 4, 6, 8, 12 and 24 hours. The fragments were removed from the culture medium, rinsed six times with sterile PBS and transferred to a sterile plastic bag with 10 mL of PBS. The fragment was mixed for five minutes and then sonicated at a frequency of 40 KHz for five minutes. Aliquots of 100 µL were recovered and spread in duplicate onto TSA, Mannitol Salt Agar and MacConkey Agar (BD®). After 24 hours of incubation at 37℃, the number of colonies on each plate was counted and the results were expressed as CFUs/mL.

## 2.4 Scanning Electron Microscopy

To evaluate biofilm composition a method for high-resolution imaging of surfaces was applied – QUANTA FEI 200 (ESEM™). Experiments and analyses involving electron microscopy were performed in the Center of Microscopy at the Federal University of Minas Gerais, Belo Horizonte city, Brazil (http://www.microscopia.ufmg.br). The biofilm was immediately fixed by immersion in 3 mL of glutaraldehyde 2.5% and 0.2 M phosphate buffer. The fragments remained in this solution for 24 hours at 4℃. After this time, three washes with 0.2 M phosphate buffer and distilled water 1:1 were performed. After rinsing, the fragment was stored in 0.2 M phosphate buffer and sent to the Center of Microscopy. There, the fragments were submitted to drying and metallization, by depositing a 0.5 nm layer of gold.

## 2.5 Statistical Analysis

Statistical analyses were performed using linear regression with STATA statistics software, version 14.0 (STATA Corp., TX, USA).

## 3. RESULTS

### 3.1 Biofilm Formation on Dissecting Forceps

It was observed that *S. aureus* cells were able to adhere at 2 h, tripled at 4 h and increased significantly from 6 h to 12 h. After 12 h, the biofilm composition remained stable until 24 h. At 2 h, the number of *A. baumannii* cells was more abundant and continued to increase slowly until 12 h, reaching a peak at 24 h. These results suggest that biofilm formation was better expressed by *Acinetobacter baumannii* than in *Staphylococcus aureus* strains when they were analyzed individually.

The study of mixed-strain biofilm showed that *A. baumannii* reached higher cell numbers than *S. aureus* in 2 and 4 hours. From 6 h, *S. aureus* reached higher numbers than *A. baumannii*. At 12 h a peak for both species was observed, with a decrease at 24 h (Table 1). There were statistical significant differences when we observed all species during the experimental time ($p<0.05$).

### 3.2 Analysis of Biofilm Formation on Forceps Fragments

Analysis of *S. aureus* biofilm showed that rough fragments exhibited more complex and better-structured cell formation than the smooth body and tip parts for the first 12 hours (Table 2). At 24 h a different pattern of biofilm formation was detected. In the smooth part associated with *S. aureus* cells, more cells were observed than in the rough and in the tip parts. Microbial adhesion of *A. baumannii* cells was higher in the smooth fragment at all evaluated times. The part with indentations followed by the tip represented the second and third most adherent cell regions, respectively.

Quantitative analysis of mixed-strain biofilm did not demonstrate any pattern of cell adhesion. It was observed that during the first six hours *S. aureus* cell counts were higher than those of *A. baumannii*. From the eighth hour onward, the volume of cells dropped for the two bacteria, except for the fragment with indentations where it was observed that *S. aureus* cells almost doubled. At the twelfth hour an increasing number of cells for the two species could be seen and at the twenty-fourth another fall was observed, except for *A. baumannii* on the indentation part of the forceps. When comparing microbial cell adhesion on the different fragments, it was found that *S. aureus* biofilm grows better on indentations followed by the tip and smooth part. In contrast, *A. baumannii* biofilm was more abundant on the indentations, followed by the flat portion and on the tip. There were statistically significant differences between *A. baumannii* single-biofilm cells that were 0.9 log10 CFU/mL higher than *S. aureus* ($p<0.001$) and of *A. baumannii* mixed biofilm cells that were 0.48 log CFU/mL lower than *S. aureus* ($p=0.005$). Statistically significant differences were also observed among species during the experimental time ($p<0.05$).

### 3.3 Scanning Electron Microscopy (SEM)

The results of bacterial adhesion and biofilm formation from 2 to 24 hours are shown in Figs. 1 to 3. SEM images exhibited bacterial aggregation after the first evaluated time (Figs. 1 and 3, letters A and F; Fig. 2, letters A, E and I). An increase in biofilm formation was observed from the sixth hour (Figs. 1 and 3, letters B, G and L; Fig. 2 letters B, F and J) to the twenty-fourth hour (Figs. 1 and 3, letters D, I and N; Fig. 2, letters D, H and L). The pictures showed that *A. baumannii* was more adherent and prevalent on the instrument surface at all analyzed times. Cell aggregates were detected from the twelfth hour (Fig. 1M) and appeared larger and more frequent at 24 h (Fig. 1N). From the second hour small numbers of *S. aureus* cell adhesion were detected. Bacterial aggregates of *A. baumannii* cells were found later, at 24 h (Fig. 2D).

Initial images of mixed-species biofilms exhibited cells of the two species on different areas of the forceps surfaces with no interaction and in lower numbers than in single-species microbial biofilm (Fig. 3, letters B, C, G, K and L). Pictures of mixed-species biofilm showed that *S. aureus* cells prevail after the sixth hour (Fig. 3G) and represent the majority of the aggregated cells at 12 and 24 h (Fig. 3, letters D, I and N). These results pointed to a possible antagonistic interaction.

## 4. DISCUSSION

As previously mentioned, biofilm on indwelling medical devices compromises routine clinical diagnosis and treatment, raising morbidity and mortality inside healthcare institutions [9]. The present study investigated the process of biofilm

formation on tissue dissecting forceps (full and fragmented body) and demonstrated that it was possible to recover a large number of cells during the first eight hours. This finding

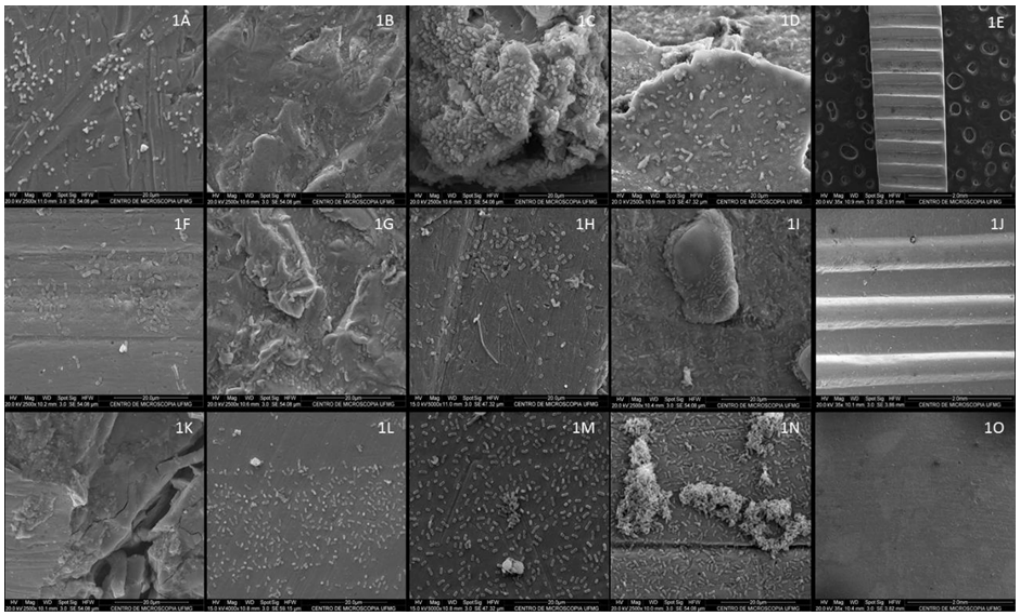


**Fig. 1. Scanning electron microscopy images of *Acinetobacter baumannii* single- strain biofilm on surgical forceps**

*1A to 1D - Tip; 1F to 1I: Rough part; 1K to 1N: Smooth part. A, F and K: 2 h; B, G and L: 6 h; C, H and M: 12 h; D, I and N: 24 h. E: image representing the tip; J: image representing the Rough part; O: image representing the smooth part. 20.0 KV, 5,000X (1H and 1M); 20.0 KV, 4,000X (1L); 20.0 KV, 2,500X (1A to 1G, 1I to 1K, 1N and 1O)*

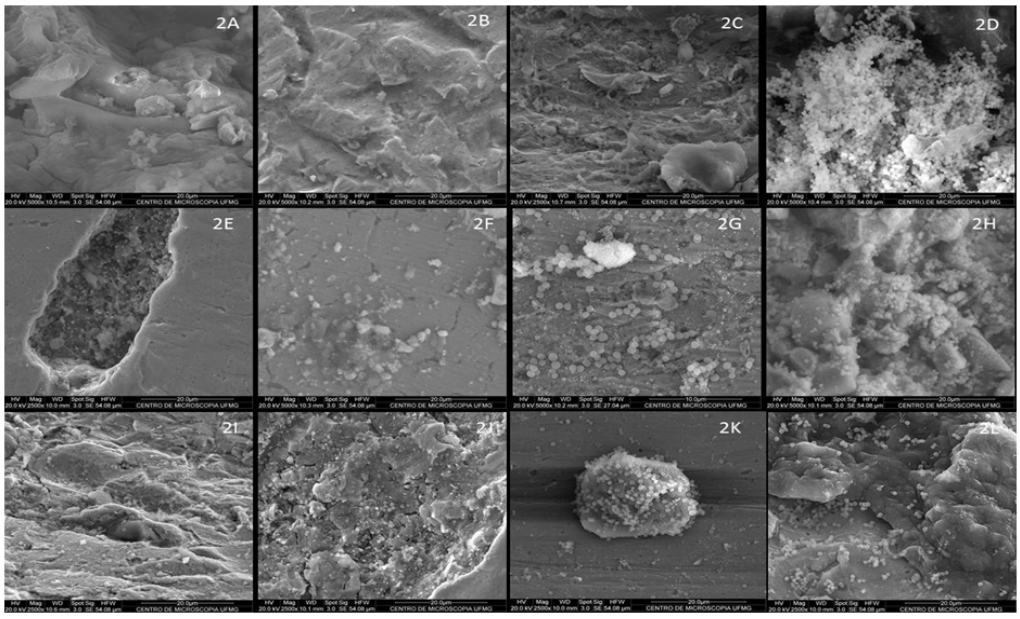


**Fig. 2. Scanning electron microscopy images of *Staphylococcus aureus* single-strain biofilm on surgical forceps**

*2A to 2D - Tip; 2E to 2H: Rough part; 2I to 2L: Smooth part. A, E and I: 2 h; B, F and J: 6 h; C, G and K: 12 h; D, H and L: 24 h. 20.0 KV, 5,000X (2A, 2B, 2D, 2F to 2H); 20.0 KV, 2,500X (2C, 2E, 2I to 2L)*

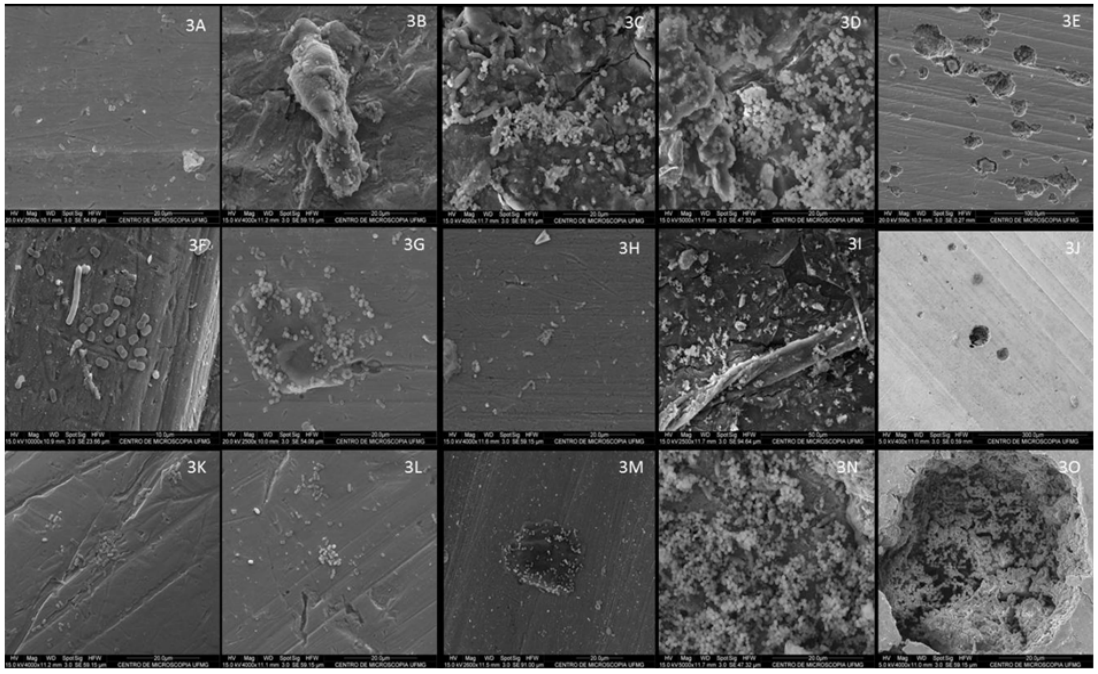


**Fig. 3. Scanning electron microscopy images of *Staphylococcus aureus* and *Acinetobacter baumannii* mixed-strains biofilm on surgical forceps**

*3A to 3D - Tip; 3F to 3I: Rough part; 3K to 3N: Smooth part. A, F and K: 2 h; B, G and L: 6 h; C, H and M: 12 h; D, I and N: 24 h. 3E, 3J and 3O: pores on the stainless steel. 15.0 KV, 10,000X (3F); 15.0 KV, 5,000X (3D and 3N); 15KV, 4,000X (3B, 3C, 3H, 3K and 3L); 5 KV, 4,000X (3O); 20KV, 2,500X (3A and 3G); 15KV, 2,500X (3I and 3M); 20KV, 500X (3E); 5KV, 400X (3J)*

**Table 1. Evaluation of biofilm formation by *Staphylococcus aureus* and/or *Acinetobacter baumannii* strains on surgical forceps: results of quantitative culture[1]**

| Biofilm | Reference strains | Time[2] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2[b] | 4[c] | 6[d] | 8[e] | 12[f] | 24[g] |
| Single-strain[3] | *Staphylococcus aureus*[4],a | 2.26 | 2.83 | 3.96 | 4.36 | 4.63 | 4.68 |
| | *Acinetobacter baumannii*[5],a | 3.47 | 4.18 | 4.33 | 4.44 | 4.47 | 4.64 |
| Mixed-strains[3] | *Staphylococcus aureus*[4],a | 2.80 | 3.72 | 4.11 | 4.13 | 4.24 | 3.99 |
| | *Acinetobacter baumannii*[5],a | 3.49 | 3.80 | 3.41 | 3.42 | 3.82 | 3.76 |

*[1]-Tested in triplicates; [2]- Expressed in hours; [3]- Medium values expressed in log10 CFUs/mL; [4] - Staphylococcus aureus ATCC 25923; [5]- Acinetobacter baumannii ATCC 19606; [a] - same letter in the same column: there is no statistically significant difference between isolated strains, considering the log10CFU. [b] to [g] – different letters in the same line: there is statistically significant difference between the different times in all species ($p<0.05$)*

suggests that bacterial adhesion seems to occur soon after the cell comes into close contact with the surgical instrument surface. Fragmented part analysis exhibited higher numbers of log 10 CFUs/mL when compared with the full body. This could be due to the efficacy of the sonication process on small fragments.

The selected parts of the instrument are considered critical during cleaning procedures once their roughness can difficult the organic and inorganic matter removal. The proteins secreted and the EPS matrix itself make the methods of cleaning and sterilizing difficult and also reduce the action of chemical agents [2]. In

the present study, it was observed that *S. aureus* cells were more adherent to indented parts and *A. baumannii* to smooth ones. Other studies [13,14] showed that serrations, indentations and teeth have a larger number of microbial cells than flat parts. It has been proved that bacterial adhesion depends on the surface design but it is also related to the bacteria-instrument surface interaction. The synthesis of virulence factors is responsible for this interaction and the present study demonstrated that the two evaluated species adhere differently to the forceps surface. It is important to highlight that the small size of the tip could influence the small number of viable recovered cells.

Our results demonstrated that *Acinetobacter baumannii* cells were more adherent to surfaces and more capable to grow during biofilm formation than those of *Staphylococcus aureus.* According to Eijkelkamp et al. [15], *A. baumannii* has the ability to adhere to medical devices and expresses various virulence factors such as *pili*, outer membrane protein (OmpA) and biofilm-associated protein (Bap) that contribute to its adherence to abiotic surfaces. Virulence factors of *S. aureus* are related to biofilm formation and adherence inside a host. Arciola et al. [16] stated that the adhesion of these bacteria depends on interactions between bacterial proteins and those proteins secreted by the host.

The analysis of mixed-species biofilm on fragmented and on entire forceps demonstrated that *S. aureus* achieved higher numbers of CFUs/mL than *A. baumannii*. This suggests that the former is the best competitor in the proposed experimental situation. The differences in log10 CFUs/mL in favor of *S. aureus* during the experimental period could be the consequence of the competition between the two bacteria. The results suggest that an antagonistic interaction could exist between them. Studies showed that bacterial interactions in mixed biofilms may be of synergistic type, helping the aggregation of cells and increasing resistance against antimicrobial agents, and of antagonistic interaction. This second type happens when there is competition for the same nutritional substrate or antimicrobial substance production. Knowledge of the stages in biofilm formation could contribute to an understanding of these structures in different environments, to improving cleaning guidelines and also to studying new antimicrobial agents and biocides [17]. Given the importance of understanding the dynamics of biofilm formation on surgical instruments and the impact of this knowledge in preventing HAIs, new studies in this field are needed.

**Table 2. Evaluation of biofilm formation in fragments by *Staphylococcus aureus* and/or *Acinetobacter baumannii* strains on surgical forceps*: Results of quantitative culture***

| | Variable | Coefficient | p-value | Conf. interval (95%) | |
|---|---|---|---|---|---|
| | A | Baseline | - | - | - |
| Area | B | + 0.148 | 0.309 | - 0.141 | + 0.438 |
| | C | + 0.105 | 0.407 | - 0.184 | + 0.394 |
| | a | Baseline | - | - | - |
| | b | + 0.904 | < 0.001 | 0.570 | 1.238 |
| Specie | c | - 0.246 | 0.146 | - 0.580 | 0.882 |
| | d | - 0.483 | 0.005 | - 0.818 | - 0.149 |
| | 0 | Baseline | - | - | - |
| | 1 | 0.874 | <0.001 | 0.465 | 1.284 |
| Time | 2 | 1.498 | <0.001 | 1.088 | 1.907 |
| | 3 | 1.233 | <0.001 | 0.813 | 1.632 |
| | 4 | 2.024 | <0.001 | 1.614 | 2.434 |
| | 5 | 2.163 | <0.001 | 1.753 | 2.573 |
| Medium value** | | 2.342 | <0.001 | 1.950 | 2.734 |

** - Tested in triplicates. A- Tip; B: Rough part; C: Smooth part. a - Staphylococcus aureus ATCC 25923 in single-strain biofilm; b - Acinetobacter baumannii ATCC 19606 in single-strain biofilm; c - Staphylococcus aureus ATCC 25923 in mixed-strains biofilm; d - Acinetobacter baumannii ATCC 19606 in mixed-strains biofilm. 0- 2 h; 1-4 h; 2- 6 h; 3- 8 h; 4- 12 h; 5- 24h. Adj. R-squared: 0.7577. ** - Medium values expressed in log10 of log10 CFU/mL*

SEM images confirmed the results of quantitative culture, indicating that *A. baumannii* formed more robust biofilms. From the second hour of the study cell adhesion was observed as well as the nonexistence of a pattern over the surface. Bacterial attachment to a surface is the first step in biofilm formation and depends on environmental conditions. Physical and chemical interactions are responsible for the transition from a planktonic to a surface-bounded condition. During primary and reversible adhesion, *pili*, fimbriae and flagella are involved. Secondary and irreversible adhesions are mediated by specific adhesins and the EPS matrix [17]. Electron microscopy images showed *A. baumannii* cell aggregation from the twelfth hour, indicating that this bacterium was more able of biofilm formation than *S. aureus* [18].

Another amazing finding from the present study is related to the stainless steel quality. The images demonstrated that the instruments' surface contained pores and scratches promoting bioburden accumulation (Fig. 3, letters E, J and O). The association of proteins, salts and dirt on medical devices could prevent the action of sterilant agents from reaching microorganisms and also benefit cell adhesion and biofilm formation [19]. It is important to highlight that these imperfections could not be monitored by the recommendation proposed in international guidelines due to their microscopic size.

## 5. CONCLUSION

*Staphylococcus aureus* ATCC 25923 and *Acinetobacter baumannii* ATCC 19606 strains had the ability to adhere and to aggregate on surgical instruments under the proposed experimental conditions. From the beginning of the investigation it was possible to recover bacterial cells by quantitative culture and also to visualize their adhesion on surfaces. Using SEM images, it was possible to confirm that biofilm formation was a dynamic process considering the bacterial species and time used in the present study. The obtained images also demonstrated that there was an antagonistic interaction between the tested bacteria. Single-species and mixed-species microbial biofilm behaved differently. It is important to highlight that mixed-species biofilm could represent the routine of clinical and surgical procedures where there is always more than one bacterial species involved. The antagonistic interaction observed herein reinforces the need for, and importance of, better understanding the dynamics of biofilm formation.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## ACKNOWLEDGEMENTS

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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