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Bactericidal activity and biofilm inhibition of F18 bioactive glass against *Staphylococcus aureus*

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ABSTRACT

Antimicrobial treatment failure has been increasing at alarming rates. In this context, the bactericidal properties of biocompatible antimicrobial agents have been widely studied. F18 is a recently developed bioactive glass that presents a much wider working range when compared to other bioactive glasses, a feature that allows it to be used for coating metallic implants, sintering scaffolds or manufacturing fibers for wound healing applications. The aim of this study was to investigate the *in vitro* bactericidal and anti-biofilm activity of F18 glass as a powder and as a coating on steel samples, and to explore the effects of its dissolution products at concentrations from 3 mg/mL to 50 mg/mL against the *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms. Furthermore, we intend to verify whether changes in the medium pH could influence the bactericidal activity of F18. The results indicated that F18 presented bactericidal activity in preformed *S. aureus* and MRSA biofilms, reducing more than 6 logs of the viable cells that remained in contact with 50 mg/mL for 24 h. Moreover, an anti-biofilm activity was observed after 12 h of direct contact, with a drop of more than 6 logs of the viable bacterial population. Neutralization of the F18 solution pH decreased its bactericidal efficacy. These results indicate that the F18 glass could be considered as an alternative material for controlling and treating infections by *S. aureus*.

1. Introduction

Bioactive glasses are widely known to promote a chemical bonding between the implanted material and the host tissue [1]. These materials stimulate various biological responses when in contact with the physiological fluids, and have advantages that exceed bone regeneration, such as bactericidal properties [2,3].

In fact, preventing and treating bacterial infections have become increasingly necessary. In recent years, hospitalization rates due to bacterial infections have grown exponentially, and are frequently associated with complications in conventional treatments, resulting in long periods of antibiotic therapy, which ultimately lengthens the cost and delay the patient"s recovery [4].

One of the most common microorganisms found in hospital infection cases is *Staphylococcus aureus*, and most infections are due to biofilm formation [5]. When a biofilm is formed, bacteria achieve an arsenal of properties that allow them to survive in unfavorable environments, increasing their resistance to antimicrobial agents, such as methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which facilitates the spread of infections and increase their complexity [5–7].

Consequently, various studies are being conducted to evaluate the antimicrobial activity of new biomaterials that seem to be promising substitutes for conventional treatments.

Special attention has been paid to the potential bactericidal properties of bioactive glasses. Different compositions of this unusual material have already been tested against a wide spectrum of microorganisms, including *S. aureus*. However, most studies have been developed on planktonic life forms, which makes studying these material properties against bacterial biofilms extremely relevant [8–13]. Besides that, in the biofilm form, *S. aureus* presents enormous resistance to antimicrobial agents, 1000 times greater than in the planktonic form, which can lead to a decrease in the activity of antimicrobial agents, such as bioactive glasses [14].

Recently, a bioactive glass denominated F18 was developed at LaMaV-DEMa/UFSCar. Previous studies have demonstrated the *in vitro* and *in vivo* biocompatibility of this glass and its ability to stimulate the

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formation of new bone and soft tissues [15]. Furthermore, it was also observed that F18 showed a high bactericidal effect *in vitro* against *E. coli*, *S. aureus*, *S. epidermidis*, and *P. aeruginosa*, all in planktonic form [16].

Despite this, F18 has not been studied regarding its antibiofilm activity. Thus, the objective of this study was to investigate the antibiofilm and bactericidal activity of bioactive glass F18 in powder form on *Staphylococcus aureus* biofilms, evaluating its capability of inhibiting biofilm formation and also its efficacy in reducing preformed biofilms.

2. Material and methods

2.1. Bioactive glass and sample preparation

The bioactive glass F18 belongs to the SiO_2-Na_2O-K_2O-MgO-CaO-P_2O_5 system. Its fabrication process is described in more details elsewhere [15,17]. Briefly, powder samples were prepared by melting the chemical reagents in a platinum crucible, the glass blocks formed were then ground in an agate mortar. The desired particle size with an average diameter of 50 μm was obtained using nylon sieves. For the coating of the test samples, stainless steel 304 disks with 13 mm of diameter and 1 mm of thickness, the technique described in [18,19] was used. The glass particles covered 60% of the steel surface (n = 12). All samples were sterilized, either by dry heat (powder sample at 170 °C for 2 h) or by ultraviolet light (coated samples for 20 min each surface).

2.2. Antimicrobial tests

2.2.1. Inoculum preparation

For inoculum preparation, the bacterial strain *Staphylococcus aureus* (ATCC 25923) and methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591) were used. The bacteria strains were transferred from their stock culture (−20 °C) to the medium and incubated for 24 h at 36 °C ± 1 °C.

Inocula were standardized by measuring spectrophotometer absorption at 600 nm, reaching an absorbance value of approximately 10^8 Colony Forming Units per milliliter (CFU/mL). Standardized suspensions were diluted to obtain a concentration of 10^6 CFU/mL required for the initial inoculum [20,21].

Biofilm formation and adhesion were verified by the Cristal Violet assay, following the methodology described by Xu et al. [22].

2.2.2. Kinetic study

The selected methodology was based on JIS Z 2801:2010 and ISO 22196:11 standards [20,21]. The bacterial inoculum (33 μL of *S. aureus* in PBS) was added to the coated test sample, based on the studies described by Souza et al. [16], and incubated at 36 ± 1 °C in approximately 85% humidity. To perform the kinetic studies, 1 mL of the Müller Hinton Broth medium was added to each specimen after preestablished experimental times (4 to 12 h). After this procedure, all samples were incubated at 36 ± 1 °C for complete 24 h with Müller Hinton Broth medium followed by quantification of viable cells. Tests were performed in triplicate.

2.2.3. Reduction of bacterial biofilms

To evaluate the reduction of bacterial biofilms of *S. aureus* and MRSA, 1 mL of the standardized bacterial inoculum as described in Section 2.2.1, was placed on the surface of the sample, and incubated at 36 ± 1 °C for 24 h. After the incubation, the samples were washed with PBS and exposed to concentrations of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, and 3.12 mg/mL of the F18 glass. The samples were incubated at 36 ± 1 °C for 24 h, followed by quantification of viable cells.

2.2.4. Influence of pH on the bactericidal action

To evaluate the effect of the change of the medium pH on the bactericidal action of the F18 glass, an extract was produced using 25 mg/mL of the biomaterial powder in PBS solution for 24 h at 36 ± 1 °C. After that, all the particles were removed by filtration. Then, three different solutions were prepared: 1. An extract of F18 as obtained, 2. an extract of F18 at neutral pH, obtained by adding 2 M HCl to the extract 1, and 3. an F18 extract diluted with distilled water. The extracts were inoculated separately in 24-hour preformed *S. aureus* biofilms and incubated at 36 ± 1 °C for 24 h followed by viable cells count. In addition, the supernatant from SEM tested samples was filtered on a cellulose acetate filter membrane with 0.2 μm pores.

2.2.5. Quantification of viable cells

In every performed test, the bacterial biofilms were disaggregated and serial dilutions of 10 to 10,000 times were obtained. Spreading was performed according to the *Spread Plate* and *Pour-Plate* methods. All plates were incubated at 36 ± 1 °C for 24 h followed by the viable CFU count.

2.2.6. Scanning electron microscopy

All samples, including the cellulose acetate filter membrane, were washed with PBS and fixed in 2.5% glutaraldehyde solution, followed by gradual dehydration with ethyl alcohol, for SEM analysis, a Philips model XL30 FEG microscope equipped with EDS accessory (energy dispersion X-ray spectroscopy) was used.

2.2.7. Statistical analysis

The data were analyzed by ANOVA with a 95% confidence level, followed by a Tukey Test using *OriginPro 8* software.

3. Results

3.1. Kinetic study

The kinect test results indicated that keeping the bacterial sample in direct contact with F18 for 6 h was sufficient to effectively reduce 5.36 logs of the *S. aureus* population. For all experimental times, the direct contact of the inoculum with F18 significantly decreased the CFU when compared to the respective control groups (Fig. 1). After 6 h, no significant difference was observed between the treated groups, and after 12 h the samples showed no visible bacterial growth, but since the experiment detection limit is 200 CFU/mL, this was the assigned value.

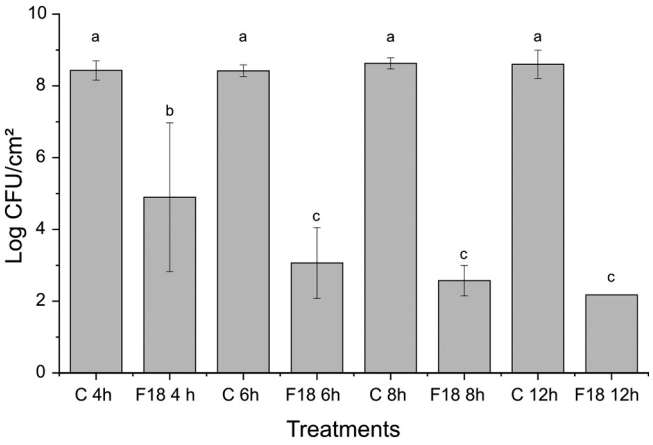

Fig. 1. Graphical representation of log CFU/cm^2 of *S. aureus* biofilm after performing the kinetic study. Test samples coated 60% with F18 bioactive glass compared to the control (C), uncoated. Bars represent standard deviation. Different letters indicate a significant difference from the Tukey Test ($p < 0.05$).

3.2. Biofilm reduction test

Fig. 2 depicts the bactericidal effect of F18 different concentrations on preformed *S. aureus* biofilms in both forms: powder and dissolution extracts. Results showed that F18 treated groups were significantly different from the control.

The 50 mg/mL concentration reached the technique detection limit of 1 CFU/mL, and no apparent microbial growth was observed.

No significant statistical difference was seen between the treatments regarding the two types of the studied application forms: the dissolution products and the direct application of F18 powder. This may suggest that both application forms would result in a representative reduction in the number of viable cells in the *S. aureus* biofilm.

From the 12.5 mg/mL to higher concentrations, a minimal reduction of 4 logs in the number of viable bacterial cells was observed. SEM analysis depicted this decrease in the quantity of cells on the samples' surface, from the smallest to the highest concentration tested (Fig. 3). From the SEM images, it can be observed that bacterial cells that were in contact with F18 appear to be damaged, showing an irregular pattern in their membranes (Fig. 3B, C, and E).

Similarly, however more intense, results were found when performing the reduction test on preformed methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms. Fig. 4 depicts the statistical analysis indicating that all F18 treated groups were significantly different from the control.

Comparing the different methodologies performed on preformed MRSA biofilms, we observed that both procedures resulted in a representative reduction of the CFU values. Concentrations higher than 12.5 mg/mL did not show a significant statistical difference from each other, and the technique detection limit of 1 CFU/mL was reached by applying 50 mg/mL of F18 in power and dissolution extract forms.

3.3. Influence of pH on the bactericidal action of F18

Regarding the pH effect on the CFU count, the analysis showed that 25 mg/mL of F18 led to the solution pH increase, reaching 11.4 ± 0.1 after 24 h. After the sample neutralization with HCl, the pH value was reduced to 7.5 ± 0.1.

Fig. 5 shows the results after applying the F18 extract, F18 extract diluted with distilled water, the neutralized samples, and the control group. As can be seen, there was no representative effect of CFU reduction on the neutralized sample surface after 24 h, and according to the statistical analysis, the obtained average values were not significantly different between this group and the control.

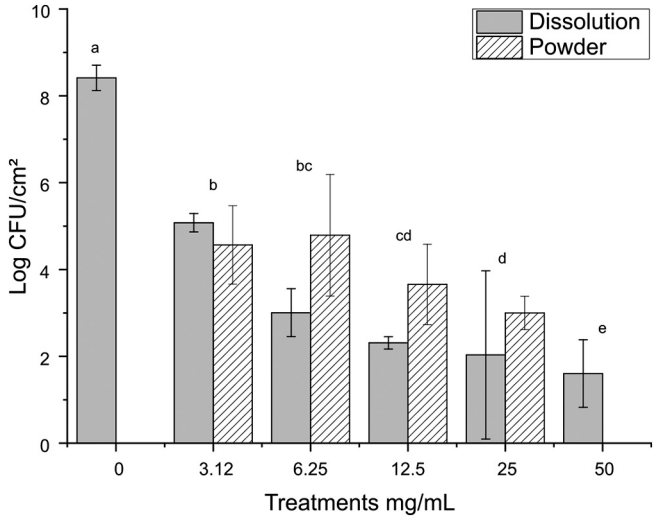

Fig. 2. Graphical representation of the log of CFU/cm^2 of *S. aureus* biofilm after performing the reduction test with different F18 concentrations. Are compared the both forms of application, powder and dissolution products with control group (0 mg/mL). Bars represent standard deviation. Different letters show that there is a significant difference from the Tukey Test ($p < 0.05$).

SEM images validate the presented quantitative data, depicting a decrease in the number of cells after exposure to 25 mg/mL dissolution products of F18 at alkaline pH (Fig. 6B and C) when compared to the control (Fig. 6A). In contrast, the surface of the specimens maintained in neutral F18 solution (Fig. 6D and E) presented no significant difference in bacterial development.

Fig. 6E presents the apparent formation of a silica gel-rich layer covering the surface of *S. aureus* cell clusters after maintaining the preformed bacterial biofilm in contact with the neutralized solution of F18 for 24 h.

The SEM images of the membrane filtered supernatants show the disaggregated cells that were retained in the filter for the control group and also for both F18 treatment methodologies (alkaline pH and neutral pH). It can be observed that the cells that were in contact with the F18 solution of alkaline pH, indicated by the white arrow, have been damaged, changing their morphology (Fig. 6G, H and I).

This data shown by Figs. 3 and 6 are still under investigation, however, compared to the control samples, the cells appear to be much more injured, giving an indication that this phenomenon is not only linked to the specimen preparation for SEM analysis.

Along with the SEM images, an energy-dispersive X-ray spectroscopy (EDS) analysis was also conducted, showing the presence of 43.8% silicon (mass percentage) indicating the possible formation of silica gel layer.

4. Discussion

In a previous study, a minimal reduction value of 6.0 logs on planktonic *S. aureus* after 24 h of contact with F18 was reported [16]. In the present study, the same reduction value was observed with only 8 h of direct contact of F18 and *S. aureus* biofilm. In addition, Souza et al. [16] showed that 50 mg/mL of F18 could make the whole planktonic population of *S. aureus* microorganisms unfeasible, which corroborates with the data obtained herein.

The antimicrobial effect of some bioactive glass compositions has been explored over the past few years. The bactericidal activity of Bioglass® 45S5 powder was verified when particles with an average diameter of 50 μm and a concentration of 50 mg/mL were used. The authors reported a 98% (~2.0 logs) elimination of planktonic cells of *S. aureus* [9]. In another study, a reduction of 6 logs in the amount of viable bacterial cells of *S. aureus* biofilms was observed, however the experiments were conducted using a 500 mg/mL concentration of S53P4 particles with 45 μm in diameter. Therefore, despite the small differences in the applied methodology, a concentration 10 times higher of this biomaterial was necessary to achieve the same efficacy bactericidal activity of F18 glass [12].

In the present study, the use of 50 mg/mL of F18 powder (50 μm in diameter) resulted in a reduction of more than 8 logs of the bacterial population of the *S. aureus* biofilm.

Zhang et al. showed that a concentration of 100 mg/mL of S53P4 reduced the viable bacterial cells of sixteen different species, among them, *S. epidermidis* tested in the planktonic form [2]. This study confirmed a dose-dependent relationship on the effectiveness of the bioactive glasses and their bactericidal action, which could also be observed in the present study. Despite the similarity on the trend behavior, the results obtained with F18 herein showed greater efficiency in reducing viable microorganisms in bacterial biofilms and in much lower concentrations.

Another study achieved good results of bacterial activity with bioactive glass 77S nanoparticles against *E. faecalis* biofilms [23]. The concentration used was 50 mg/mL, one of the concentrations used in

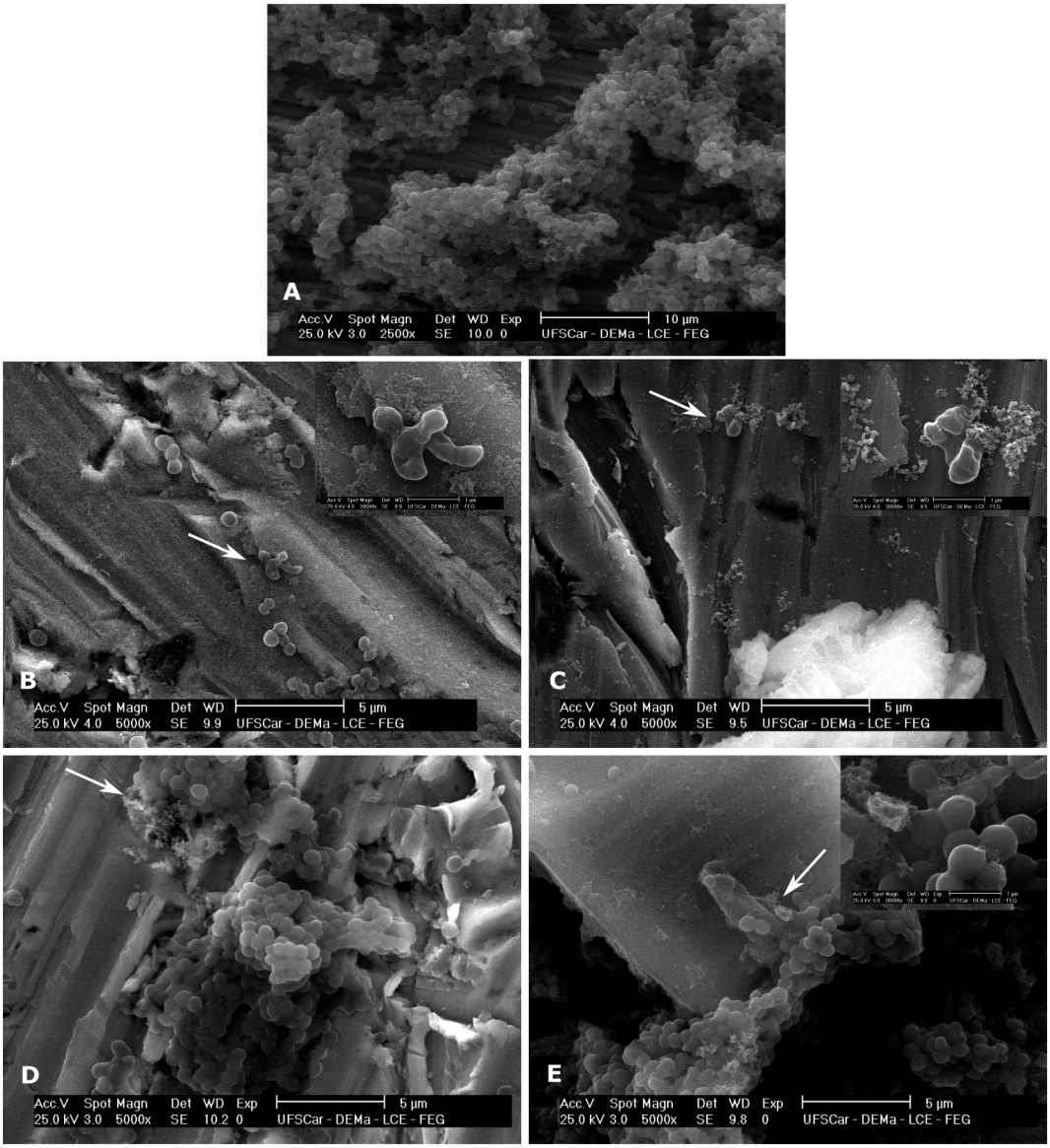

Fig. 3. SEM 24-hour biofilm images of *S. aureus* formed on test sample surface after reduction test. Control, not coated with biomaterial (A) test sample after application of dissolution products and direct application of powder, 50 mg/mL F18 (B) and (C) and 3 mg/mL (D) and (E). In the upper right corner of the images (B), (C) and (E) 30,000× enlargement of the bacterial cell. Arrow indicates bacterial cells of *S. aureus*.

the present study for F18, but they observed that it took one week to kill all the viable cells, sevenfold the time required for the F18 powder and fibers [24].

Even taking into account the different experimental procedures used in previous studies, the F18 presented a faster and more effective bactericidal effect when compared to other bioactive glasses.

In a study conducted by Drago et al., the bactericidal effect of bioactive glass S53P4 against MRSA biofilms was evaluated using particles with an average diameter of 45 µm and a concentration of 400 mg/mL. The authors reported an 80% reduction in the total biomass obtained by the violet crystal technique [25].

The results found herein indicated the achievement of approximately 99.9999% of MRSA biofilm reduction when a concentration of 50 mg/mL (20 times lower) of F18 powder was applied (a reduction of more than 6.77 logs).

The mechanism of action of bioactive glasses in the elimination of microorganisms is still being studied. Common points that appear in some studies are the increase of the pH of the aqueous medium and the increase in osmotic pressure, besides the use of needle-shaped glass particles, which can damage the cell wall and induce the death of the bacteria [2,8–12,16,23].

Malpartida et al. also reported another mechanism that could be involved in the bactericidal activity of bioactive glasses. Bacteria cells can be attached to the glass particles that could result in a high calcium release in the proximity of the plasma membrane, which could distort the electrochemical potential gradient, leading to cell death [26].

It is known that the direct application of F18 powder adds the variable of “particle shape” in the biomaterial's bactericidal mode of action when compared to the application of its dissolution products. However, based on the obtained results it was observed that there were no significant differences between both F18 application forms (powder and extract) in the reduction of bacterial cells. Thus, under the tested conditions, this variable did not appear to interfere representatively in the bioactive glass antibacterial activity. Similar results were observed by Allan et al., in which the direct contact between the 45S5 particle and the bacterial cell was not necessary for the efficacy of the biomaterial's bactericidal activity [27].

However, some evidence shows that increasing pH is a critical factor

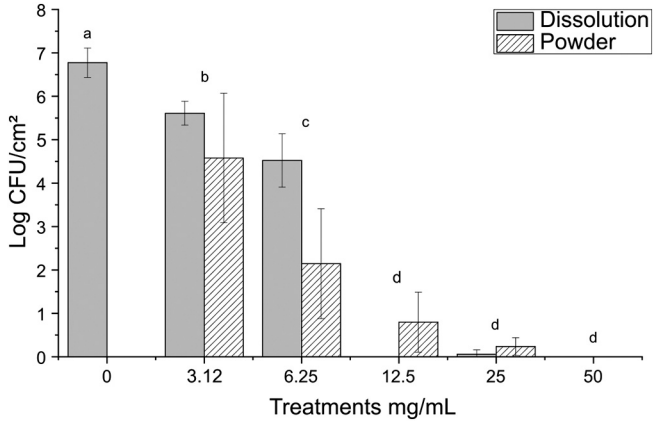

Fig. 4. Graphical representation of the CFU log/cm^2 of methicillin-resistant *S. aureus* biofilm (MRSA) after performing the reduction test with different F18 concentrations. Compare treatments where dissolution products and powder were applied with control (0 mg/mL in PBS). Bars represent standard deviation. Different letters show that there is a significant difference from the Tukey Test ($p < 0.05$).

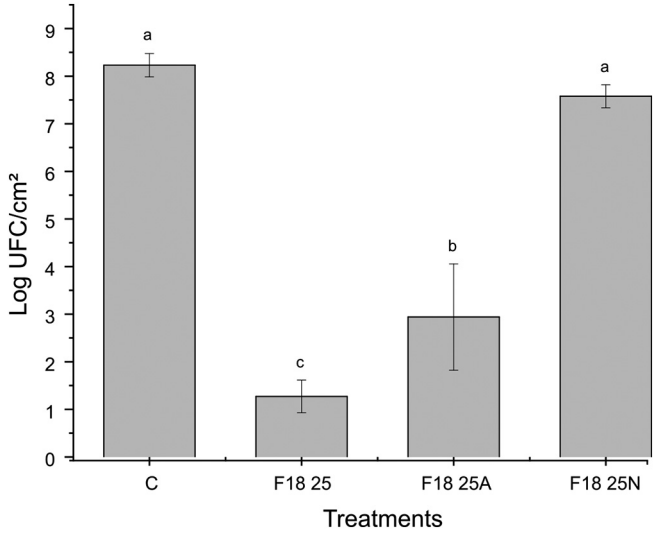

Fig. 5. Graphical representation of the log of CFU/cm^2 of *S. aureus* biofilm after verifying the influence of pH on F18 action. Treatment with 25 mg/ml F18 at alkaline pH (F18 25), diluted with distilled water (F18 25A), at neutralized pH (F18 25N) and control (C), are compared. Bars represent standard deviation. Different letters indicate a significant difference from the Tukey Test ($p < 0.05$).

for the effect of bioactive glass bactericidal activity. According to Begum et al., alkaline environments may alter the pH of the bacterial cytoplasmic membrane, which partly explains growth inhibition and toxic effects on the cells after their exposure to this kind of biomaterial [11]. It is well known that alkaline environments are often not tolerated by microorganisms and that the optimum pH value for *Staphylococcus aureus* cell growth is between 7.0 and 7.5, however they are capable of growing between 4.5 and 9.3 [28].

A reduction greater than 90% in the viability of bacterial cells of *S. sanguis* in planktonic form was observed when in contact with 45S5 supernatant at pH 9.8, which was also observed by adding NaOH in the NB (Nutrient Broth) medium culture [27].

Hu and colleagues noted in their study that the water solution containing 45S5 bioactive glass changed the pH value from 7.0 to 10.3, as did Zhang and colleagues with the S53P4, which reached a value of local pH of 11.0 in SBF solution (Simulated Body Fluid) [2,9]. Both studies indicated a relationship between the increase of pH value and the effectiveness of the bactericidal activity of these bioactive glasses.

On the other hand, Begum et al., indicated that 45S5 particles with less than 63 μm at 10 mg/mL did not show bactericidal activity against planktonic microorganisms of *S. aureus* and *E. coli* when the pH of the NB medium was neutralized to 7.3 [11]. The same trend was observed by Allan et al. [27]. The authors reported that by neutralizing the pH of NB medium to 7.2, the reduction efficiency of a 45S5 solution (with grain size ranges of 90–710 μm and 1000 mg/mL concentration) decreased by more than 60% when compared to the alkaline pH solution (9.8). These data corroborate to the present study, where a decrease of 6.31 logs in the reduction value of viable bacterial cells after contact with F18 solution (pH 7.5) compared to F18 solution at alkaline pH can be observed. According to Drago et al., it is the simultaneous increase in pH with increased osmotic pressure that makes the environment hostile to bacterial adhesion and proliferation [25].

Within the surface area of the analyzed membrane filtered supernatant, after treatment with F18 extract at neutral pH (Fig. 6J), the presence of bacterial cells was not observed, suggesting that the possible formation of the silica gel layer had an influence on the detachment of *S. aureus* cell aggregates from the test sample surface. Along with SEM images, the EDS analysis, that was performed on this filtering membrane surface, showed the presence of 43.8% silicon (mass percentage) which corroborates the possible formation of silica gel layer. This phenomenon is vastly reported in the literature for bioactive glasses, as one of the steps for the formation of hydroxyapatite [HA], and are expected as their mechanisms of action, as reported by [1–3,15,17].

Relating the obtained results, it can be said that the pH neutralization of the solution with the F18 dissolution products had a strong influence on the decrease of the bioactive glass bactericidal activity in *S. aureus* biofilms. Thus, corroborating with the literature, the obtained results suggest that pH increase may be a fundamental mechanism for bioactive glass bactericidal action. Nonetheless, it cannot be concluded that this is the only mechanism involved in bacterial cell death by this biomaterial. Decreasing pH may alter the solubility of particular ions, which can play an important role in the bactericidal activity [11,27]. The concentration of soluble silica, one of the by-products of bioactive glass dissolution in the aqueous medium, for example, is known to be linked to the basicity of the solution, therefore decreasing pH is expected to reduce the quantity of this solute in the medium, which may result in a decreased osmotic pressure [29].

Regarding the morphological changes observed in the bacterial cells, Drago et al. [30] observed a shrinkage and a reduction in the cell dimensions after direct contact with the S53P4 bioactive glass granules (diameter < 45 μm). Ran, et al., also reported an alteration in cell morphology generated by an alkaline environment exposure, the authors reported using a TSB (Tryptic Soy Broth) medium with pH adjustment with maleic acid and K_2CO_3, and this was linked to a bacterial response to the hostile environment, which led to changes in the cell morphology and structure [31]. Another factor that should be taken into consideration is the increase in the osmotic pressure due to the release of ions into the solution, such as Na^+, Ca^{2+}, which are released from the F18 glass into the aqueous medium. The concentration of solutes in the bacterial cytoplasm is usually higher than that detected in the external environment, resulting in a positive cell membrane pressure. A sudden increase in the concentration of surrounding solutes makes the medium hypertonic, lowering the pressure across the cell membrane, which results in a rapid flow of water from the cell. This water loss leads to wrinkled cells, hence an altered morphology [32].

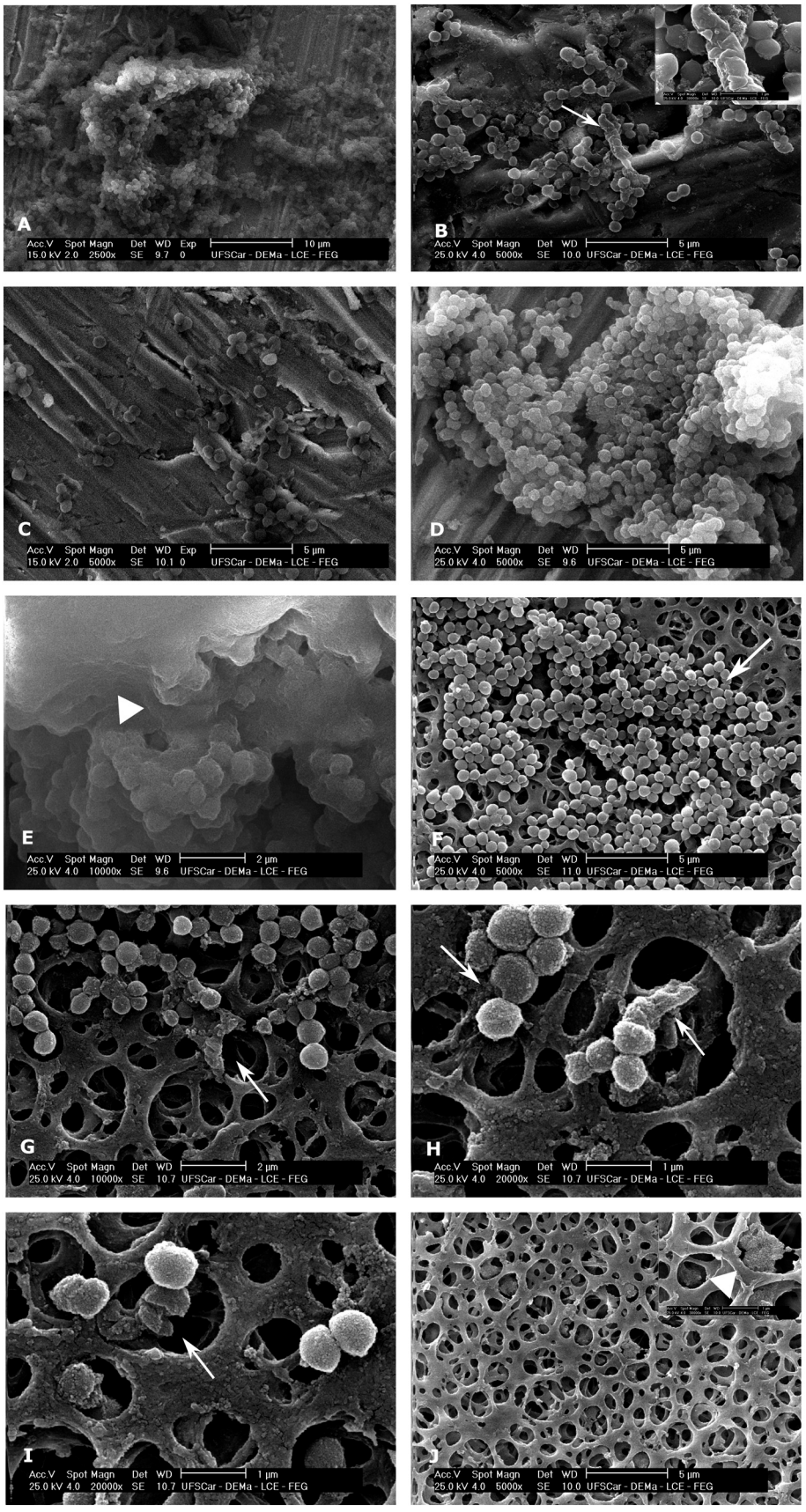

Fig. 6. SEM 24-hour biofilm images of *S. aureus* and membrane filtered supernatant after the pH influence test. Control, sample not coated with biomaterial (A) sample after application of 25 mg/mL F18 (B) dissolution products, 25 mg/mL diluted with distilled water (C) and 25 mg/mL neutralized pH (D) and (E). *S. aureus* cells disaggregated from surfaces, control sample, (F) sample after application of 25 mg/mL F18 dissolution products (G) (H) and (I) sample filtrate with neutralized F18 solution (J). In the upper right corner of the images (B) and (J) 30,000× magnification of the bacterial cell. Arrows indicate bacterial cell *S. aureus*, arrowheads show possible silica gel layer.

5. Conclusions

F18 bioactive glass particles were effective in inhibiting the growth of *Staphylococcus aureus* biofilm after direct contact between the inoculum and the biomaterial for 6 h, which resulted in a reduction of approximately 6 logs of the viable bacterial population. Moreover, the exposure of the bacterial cells to intermediate concentration, such as 12 mg/mL, of both F18 dissolution products and powder form showed high bactericidal activity, reducing the total of viable cells of *S. aureus* and methicillin-resistant *S. aureus* biofilms (MRSA) around 5 logs. An intrinsic relationship between antimicrobial activity and pH increase was also observed. Thus, using the methodology proposed in ISO 22196: 2011 [21], we observed that F18 bioactive glass presents bactericidal activity against *S. aureus* and MRSA biofilms. Therefore, the F18 seems to be a promising biomaterial for preventing and controlling infections by *S. aureus*.

CRediT authorship contribution statement

All authors (Tathiane Ferroni Passosa, Marina Trevelin Souza, Edgar Dutra Zanotto and Clovis Wesley Oliveira de Souza): Conceptualization, Methodology, Writing - reviewing and editing. Tathiane Ferroni Passos, Marina Trevelin Souza: Investigation. Tathiane Ferroni Passos, Marina Trevelin Souza and Clovis Wesley Oliveira de Souza: Formal analysis, Visualization and Writing - original draft preparation. Edgar Dutra Zanotto and Clovis Wesley Oliveira de Souza: Resources, Funding acquisition.

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Ethical approval

Not required.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2020.111475.

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