Universidade Federal do Rio Grande do Sul

Faculdade de Farmácia

Trabalho de Conclusão de Curso de Farmácia

Método alternativo em larvas de *Galleria mellonella* para estudar a infecção e tratamento de biofilmes

Rodrigo Campos da Silva

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“O que sabemos é uma gota, o que ignoramos é um oceano”

Isaac Newton

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Resumo

Biofilmes têm por característica o aumento a tolerância a agentes antimicrobianos e ao sistema imune, o que por fim acaba levando à uma falha na terapia e à cronificação de infecções. Biofilmes desempenham um importante papel em infecções relacionadas à dispositivos médicos e como atualmente não há tratamento eficaz para essas infecções, a única alternativa remanescente é a remoção do mesmo. Estudos *in vivo* desempenham um papel crucial na utilização dos dados de experimentos *in vitro* à uma terapia aplicada. Levando isso em conta, nós descrevemos um método simples que analisa e quantifica a formação de biofilme dentro de larvas de *Galleria mellonella*. Cerdas de escova de dentes foram utilizadas como uma superfície abiótica no intuito de mimetizar um implante médico. Um inóculo padronizado de *Staphylococcus aureus* foi injetado, de forma sistêmica, nas larvas junto com a inserção de uma cerda estéril na última *proleg*. Após incubação, à 37ºC por 24h, as células bacterianas foram removidas das cerdas e quantificadas através das unidades formadoras de colônias (UFC) utilizando um meio seletivo para Staphylococcus. A quantidade de UFC recuperada das cerdas foi aproximadamente 3×10^6, e imagens da microscopia eletrônica de varredura (MEV) confimou a formação de biofilme. Grupos controle, que foram feitos utilizando apenas as cerdas, sem inóculo, não demonstraram células aderidas, como demonstrado pela ausência de UFC e imagens de MEV, o que indica que a microbiota das larvas não estava interferindo nos experimentos. Além disso, um grupo em que foi injetado vancomicina junto do inóculo e da cerda não demonstrou, também, contagens de UFC. Nós apresentamos um método rápido e factível para avaliar a formação de biofilme bacteriano em um modelo *in vivo* e que em um futuro próximo pode ser utilizado para avaliar a eficácia de moléculas que possuam o potencial de impedir a formação de biofilme.

Alternative method in *Galleria mellonella* larvae to study biofilm infection and treatment

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Abstract

In vivo studies are crucial decision-maker step in order to translate *in vitro* data to an applied therapy. Considering this we describe a simple method that analyzes and quantifies biofilm formation inside the *Galleria mellonella* larvae. Toothbrush bristles were employed as an abiotic surface to mimic a medical device. A standardized inoculum of *Staphylococcus aureus* was systemically injected in the larvae together with the insertion of a bristle in the last proleg pair. After incubation adhered cells were detached from bristles and quantified by colony-forming units (CFU) counting using staphylococci-selective medium. About 3 x 10^6 CFU of *S. aureus* were recovered from bristles and scanning electron microscopy images confirmed biofilm formation. Control group did not show adherent bacteria, as demonstrated by non-CFU counting and SEM images, indicating that microbiota do not interfere in experiments. We present a feasible method to evaluate bacterial biofilm formation *in vivo* that in the near future can be used to evaluate antibiofilm compounds.

Key words

Galleria mellonella, bacterial biofilm, experimental method *in vivo.*

1. Introduction

It is estimated that 80% of the microbial infections are biofilm-related, accounting billions of dollars of expense across the world[(1),(2)]. Biofilms play a major role in medical device-related infections, including heart, circulatory devices, urinary catheters, contact lenses, and orthopedic implants[(3)–(5)]. Only in the US, device associated infections account for 26.6% of all health-care associated infections[(6)]. Since there is no available antimicrobial capable of eradicating an already established biofilm, the management to control device-related infections is usually the surgical removal of the implant followed by a new insertion. Therefore, the understanding of biofilm formation and the searching for new treatment options against microbial biofilms are topics of utmost necessity[(3),(7),(8)].

Microbiological translational research is now facing an opportunity to increase knowledge by the use of invertebrate animal models before the mammalian model studies. In this regard, the larvae of *G. mellonella* has been largely used as alternative host model to study several human bacterial and fungi pathogens [(9),(10)], since possesses several technical advantages compared to other invertebrates, such as low laboratorial

maintenance costs, presence of a immune innate system and susceptibility to diverse pathogens [9],[11]–[15]. Herein, we describe a simple method that can facilitate the transition of *in vitro* to *in vivo* testing, potentially reducing the number of mammalian animals used in preliminary evaluations. In 2015, Benthall [11] and collaborators applied toothbrush bristles in a culture medium to form biofilm and then inserted it inside the *G. mellonella* larvae. Differently and more reliable, we propose, for the first time, the use of toothbrush bristles to mimic an implantable polymeric device to evaluate and quantify *Staphylococcus aureus* biofilm formation inside *G. mellonella* body. This method also enables the screening of drugs capable of avoiding biofilm formation and eradicating pre-established biofilms.

2. Methods

2.1 *Galleria mellonella* maintenance

The *G. mellonella* larvae were grow at a controlled environment for the whole cycle in our laboratory and procedures were performed as described before [12].

2.2 Bacterial culture conditions and inoculum standardization

Staphylococcus aureus ATCC 25904 stored in skim milk and 10% glycerol was grown in Muller-Hinton Agar (MHA, Oxoid, UK) for 24h at 37°C. Different inoculum concentrations were tested to be systemically administered in larvae proleg in order to have all live larvae in a period up to 48 h. The standardized inoculum was prepared by diluting 50x a bacterial suspension of optical density at 600 nm of 0.170.

2.3 Bristle material

The commercially available toothbrush bristles (hard bristle, made of Nylon™ a polyamide polymer) were cut in 1 cm pieces with scalpel and sterilized in autoclave for 15 min at 121ºC. To test if the bristle material is prone to bacterial adhesion, the bristles were cultured *in vitro* in the presence of 200 uL of *S. aureus* suspension (OD600 = 0.150) and 800 uL of Brain Heart Infusion Broth (BHI, Oxoid™ UK) during 24h at 37°C. After, the bristles were washed twice in saline solution (2 mL) and analyzed by (i) scanning electron microscopy (SEM) and (ii) by detection of bacterial cells detached from bristle through a sequence of vortex (30 s) sonication (2 min) and vortex (30 s) and grown on Muller Hinton agar (MHA, KASVI®, Italy) after 24h incubation at 37°C.

2.4 *Galleria mellonella* assays

2.4.1 Bristle insertion

One proleg of last pair was punctured with the aid of a 10 uL Hamilton syringe (Sigma Aldrich™, Germany) in order to facilitate the insertion of the 1cm-bristle. One sterilized bristle was completely inserted in the proleg per larvae with the aid of a tweezer.

2.4.2 Galleria *mellonella* infection

Ten microliters of the standardized inoculum were systemically injected in *G. mellonella* larvae through proleg from the last pair by using a 10 uL-Hamilton syringe.

2.4.3 Quantification of bacterial burden during infection in larvae and/or bristle

After infection and/or bristle insertion, larvae were incubated at 37°C for 24h. Then two alive larvae were smashed with 1 mL saline solution and a homogenate was prepared using vortex (30 s). Ten uL were inoculated in staphylococcal selective mannitol salt agar (Merck™, Germany) and incubated at 37°C for 24h for colony-forming-units (CFU) counting by drop plate technique. To quantify bacterial adherence in bristle, two alive larvae had their bristle removed and washed twice with saline solution. The adhered cells were detached in 1 mL de saline by a sequence of vortex (30 s), sonication (2 min) and vortex (30 s) and 10 uL were inoculated in mannitol salt agar and incubated at 37°C overnight for CFU counting. To analyze the bacterial burden in larvae in the presence of the bristle, two larvae possessing the bristles were smashed with the bristle and the sequence of vortex (30 s), sonication (2 min) and vortex (30 s) was performed, following the incubation in mannitol salt agar at 37°C for 24h. The experiments were performed three times in different days.

In summary, the following groups were evaluated:

I. Larval homogenate without bristle (LB): the homogenate was prepared from infected larva;

II. Larval homogenate (burden) with removed bristle (BRB): the homogenate was prepared from infected larvae possessing an implanted bristle, which was removed before smashing;

III. Larval-bristle homogenate (LBB): the homogenate was prepared from larvae which received the inoculum and a bristle.

IV. Bristle from same proleg infection (BSI): only bacteria adhered to the bristle were evaluated. In this case, bacterial inoculum was injected in the right proleg followed by bristle insertion in the same puncture.

V. Bristle from opposite infection (BOI): only bacteria adhered to the bristle were evaluated. In this case, bacterial inoculum was injected in the right proleg followed by the bristle insertion in the opposite (left) proleg.

VI. Bristle from vancomycin-treated larvae (BV): only bacteria adhered to the bristle were evaluated. Bacterial inoculum and bristle were inserted in the same *proleg* and incubated at 37°C for 30 minutes. Then 10 uL of vancomycin (50mg/Kg) was injected in the opposite *proleg.*

2.4.4 Qualitative analysis of *in vivo* bacterial adherence into the bristle – SEM

After 24h of incubation, bristles were removed from larvae and washed twice with saline solution in order to remove non-adherent bacteria. The samples were stored in a cacodylate buffer solution with 2.5% glutaraldehyde. Samples were de-hydrated with acetone gradient and dried using CO_2 critical point technic. Bristles were examined in a JEOL JSM-6060 scanning electron microscope (JOEL, Peabody, MA)[16][12].

2.4.5 *Galleria mellonella* survival curve

To assess larval survival after infection and/or in the presence of a bristle, the larvae groups (20 animals per group) were daily assessed for survival during 5 days [10]. The experiments were performed three times in different days.

In summary, the following groups were evaluated:

I. Control: This group received only the standardized *S. aureus* inoculum – 3 x 10^6 CFU/larvae.

II. Bristle from same proleg infection (BSI): This group had bacterial inoculum injected in the right proleg followed by bristle insertion in the same puncture.

III. Bristle from opposite infection (BOI): This group had bacterial inoculum injected in the right proleg followed by bristle insertion in the opposite (left) proleg.

IV. Bristle from vancomycin-treated larvae (BV): This group had bacterial inoculum and bristle inserted in the same proleg. It was incubated at 37°C for 30 min and then larvae were treated with 10 uL of vancomycin (50mg/Kg) in the opposite *proleg.*

V. Control - bristle with saline (BS): This group had the last proleg stabbed with the syringe for the bristle insertion and received 10 uL saline solution in the opposite proleg.

VI. Infection and one-puncture control: This group received the inoculum and the same *proleg* was stabbed one more time in order to evaluate if the second injury, caused by the bristle insertion in the BSI group, would increase the death rate of the larvae.

VII. Infection and two-punctures control: This group received the inoculum and the same *proleg* was stabbed one more time, after this the opposite *proleg* was stabbed once in order to evaluate if the second injury, caused by the bristle insertion in the BOI group, would increase the death rate of the larvae.

2.4.6 Statistical Analysis

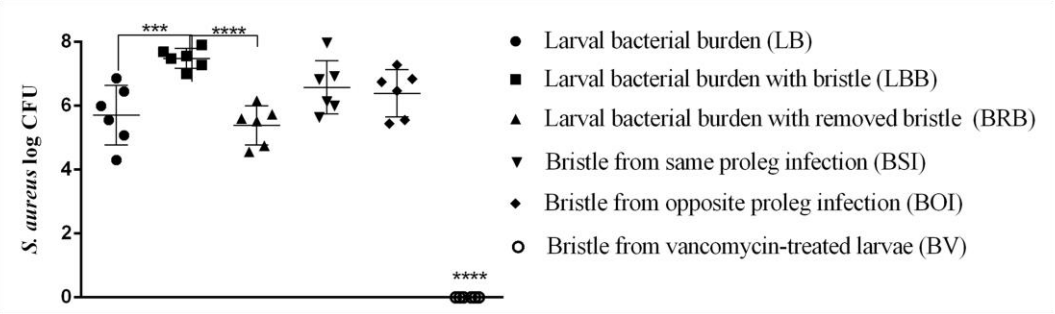
Statistical analysis was performed in Graphpad Prism 6 program and performed as described before [12], considering for all tests $p<0.01$ significant.

3. Results

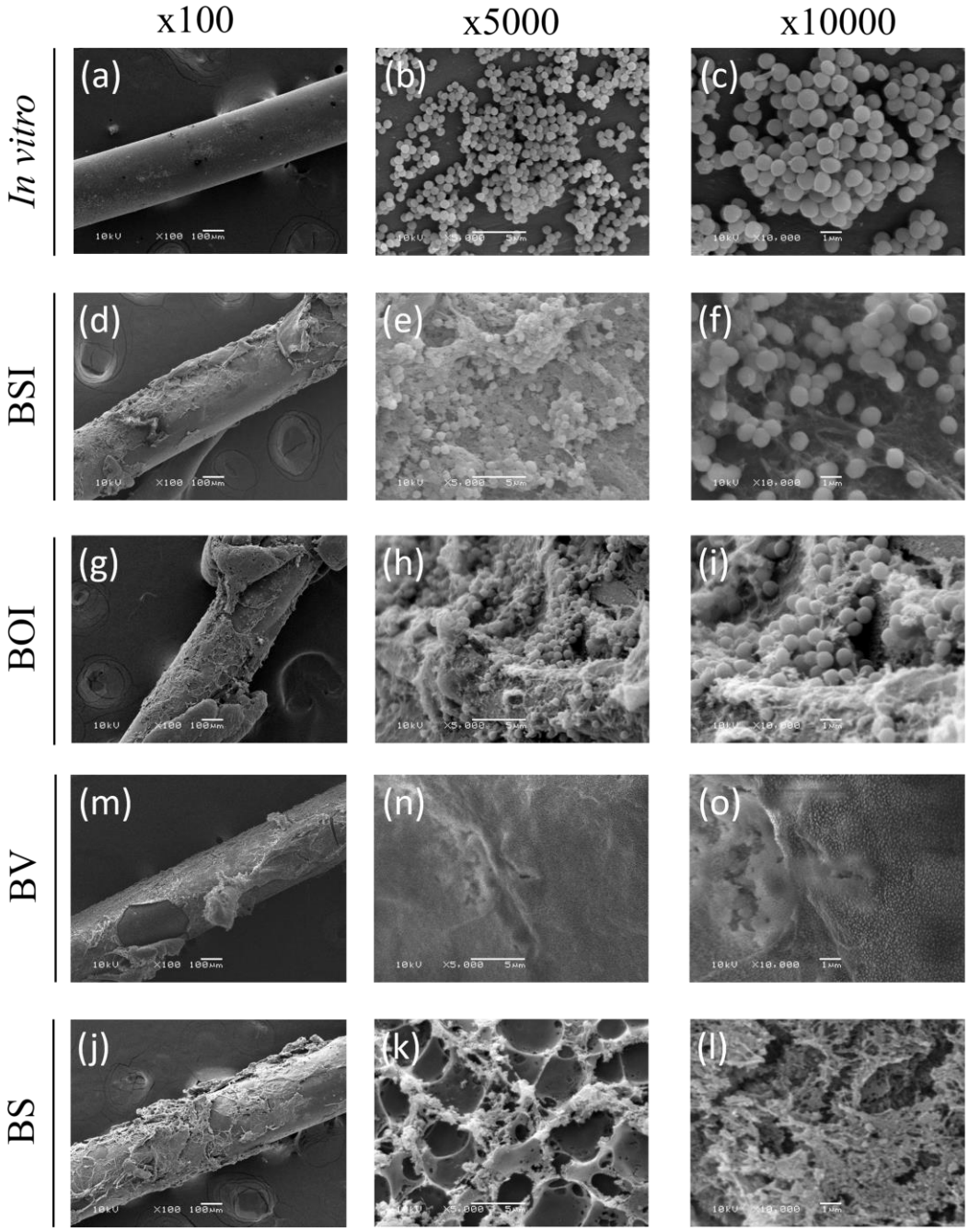
We standardized a *S. aureus* inoculum at $3x10^6$ CFU/mL as enough to infect the larvae, providing a larval bacterial burden (LB) after 24h-infection of 5.7 ± 0.93 log10 CFU (Fig. 1A), and to keep larval survival rate of about 80% for a 120h period (Fig. 2, filled line). Additionally, we certified that toothbrush bristles used in this study is prone to *S. aureus* adhesion *in vitro*, also observed by SEM images (Fig. 1B images a-c). When we determined larval bacterial burden in presence of bristle (LBB), the CFU was significantly higher (7.6 ± 0.31 log10 CFU) than homogenate without bristle, 5.7±0.93 log10 CFU (LB) and larval group with bristle removed previously the counting (BRB), presented 5.5±0.61 log10 CFU (Fig. 1A). These results were expected since in LBB group we obtained the bacterial counting from larvae and bristle. In all experiments the bristles were implanted in one larval proleg from the last proleg pair. It was demonstrated that the quantity of bacteria recovered from biofilm bristle does not differ when bristle and bacterial inoculum were inserted in the same proleg punction (BSI) showing 6.5 ± 0.83 log10 CFU (Fig. 1A and Fig. 1B – images d-f) or in the opposite prolegs (BOI), with 6.4 ± 0.74 log10 CFU (Fig. 1A and Fig. 1B – images g-i). As positive control, the bristle recovered from the vancomycin-treated larvae (BV) was free of bacterial biofilm (Fig. 1A and Fig. 1B – images j-l), while bristles from non-infected

larvae presented an adherent biological matrix from *G. mellonella* larvae without any adherent bacteria (Fig. 1B – images m-o).

A)


S. aureus log CFU
8
6
4
2
0
***
****
****
Larval bacterial burden (LB)
Larval bacterial burden with bristle (LBB)
Larval bacterial burden with removed bristle (BRB)
Bristle from same proleg infection (BSI)
Bristle from opposite proleg infection (BOI)
Bristle from vancomycin-treated larvae (BV)

B)


x100
x5000
x10000
In vitro
BSI
BOI
BV
BS
(a)
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(h)
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(m)
(n)
(o)
(j)
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(l)

Figure 1: A) *Staphylococcus aureus* CFU counting recovered from larvae homogenate and/or bristles. ***= p<0.001 and **** = <0.0001. **B)** Scanning electron microscopy images: (a-c) *in vitro* culture to assess if the bristles are prone to bacterial adhesion; (d-f) bristle and infection in the same proleg infection (BSI); (g-i) bristle and infection in opposite proleg (BOI); (j-l) bristle from vancomycin-treated larvae (BV); (m-o) bristle removed from non-infected larvae (BS), showing adherent biological material from larvae body.

Larval survival analysis indicates that all control groups (*S. aureus*-infected larvae; non-infected larvae (BS); infected-larvae and one puncture (I1P); infected-larvae and two punctures (I2P) did not show any significant difference among them, presenting about 80% of survival up to 120h of observation (Fig. 2). In this sense, it is clear that only insertion of bristle or only infection with the standardized inoculum did not lead to high larval mortality rates. Interestingly, it was evidenced that groups that received bristle after infection, BSI and BOI, had a significant reduction in host survival with statistical significance in relation to controls (Fig. 2).

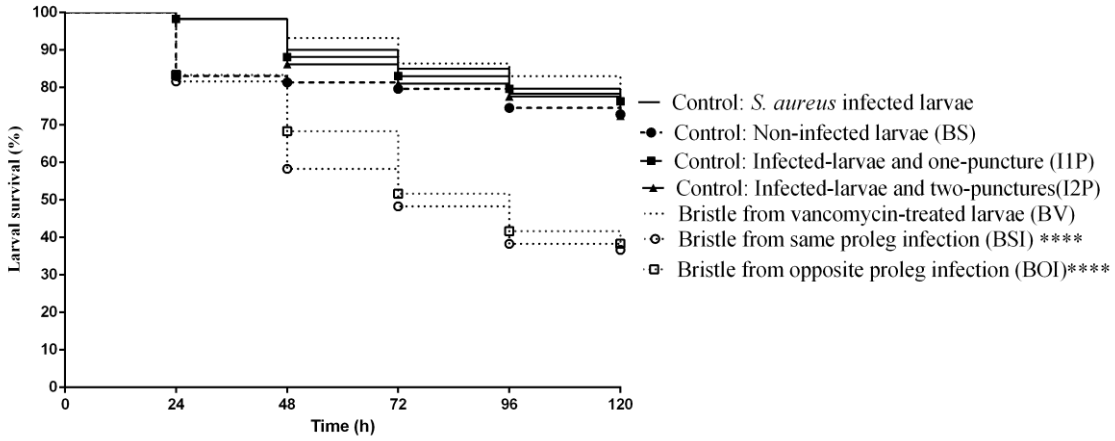

Figure 2: Survival curve of *G. mellonella* larvae up to 120h. All groups were compared with control (3×10^6 CFU *S. aureus* infected larvae). **** represents p-value <0.0001.

4. Discussion

Herein we successfully developed a method capable of analyzing qualitatively and quantitatively the *in vivo* bacterial biofilm formation on abiotic surface with the intention to mimic a medical implant. This method uses an alternative host for infection, the *G. mellonella* larvae and can be extremely useful for preliminary evaluation as a *decision-maker* step for further mammal models investigations.

Importantly, we have shown that bristles serve as abiotic implant since they allow pathogenic bacterial adhesion (Fig. 1B, images a-c) but not the adherence of *G. mellonella*-native microbiota (Fig. 1B, images m-o). We selected *S. aureus* as pathogenic bacterial model due to its clinical importance, but any other microorganism might be investigated. It is noteworthy that only the larvae groups which were infected and received the bristle (Fig. 2, BSI and BOI, independently of proleg inserted) had 39% increased mortality rate compared to controls, in accordance to the clinical data where higher levels of morbi-mortality is observed for biofilm-related infections[8]. When comparing the CFU counting in bristles, both groups were not significantly different (Fig. 1A), demonstrating the bacterial spreading from the infection site throughout the body of the larvae occurs independently of the proleg injected. Moreover, despite BSI and BOI groups did not presented a statistical difference from the other three groups (LB, HRB and LBB), they showed a tendency to display CFU counting similar to the group which larval homogenate was prepared in presence of bristle (LBB), indicating *S. aureus* preference to adhere on the abiotic surface rather than remaining in larval body (Fig. 1A). In practical terms, the inoculum administration and bristle insertion in the same or opposite prolegs allow us to better design future experiments: (i) to test biofilm-eradication molecules it is necessary firstly to insert the bristle and then to induce the infection at the same proleg followed by the treatment at the opposite proleg, while (ii) to test of biofilm-preventing molecules the treatment and bristle insertion can be performed firstly in the same proleg and then infection is performed in the opposite proleg. Corroborating with this idea, the larvae group treated with vancomycin showed bristles without adherent bacterial cells, as observed by counting experiments and by SEM visualization (Fig. 1A and Fig. 1B, BV group images j-l), demonstrating the sensibility of the method to possible active agents.

Important issues must be considered for the method presented herein: (i) it is cheap to be performed; (ii) it analyzes and quantify biofilm formation inside a living organism; (iii) it applies an alternative animal model: *G. mellonella* larvae; (iv) it enables the usage of a high number of animals per group increasing statistical significance and (v) up to date there is no need for an ethic committee. Obviously, invertebrate models, including *G. mellonella,* will not replace vertebrate ones; however, it represents a screening-step between *in vitro* and mammalian *in vivo* evaluations, minimizing the number of vertebrates used in preliminary stages of experimentation. The understanding of biofilm formation and the search for new molecules to control biofilms are of highest importance to modern medicine. In the near future, this method can be used to test antibiofilm molecules capable of preventing biofilm formation or reducing pre-formed biofilms and even to study the efficacy of coated surfaces to protect materials from the initial bacterial adhesion and biofilm development.

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Competing Interests

The authors declare no conflict of interests.

Contribution

Conceptualization, A.J.M., D.S.T., R.C.S.; methodology, R.C.S. and F.R.B.; writing—original draft preparation, R.C.S., F.R.B., A.J.M. and D.S.T.; writing—review and editing, A.J.M. and D.S.T.; supervision, A.J.M. and D.S.T.; funding acquisition, A.J.M. and D.S.T.

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