Chapter 4.2

Maggot excretions inhibit biofilm formation on biomaterials

Maggot excretions decrease biofilms

Gwendolyn Cazander
Mariëlle C. van de Veerdonk
Christina M.J.E. Vandenbroucke-Grauls
Marco W.J. Schreurs
Gerrolt N. Jukema

Abstract

Background: Biofilm-associated infections in trauma surgery are difficult to treat with conventional therapies. Therefore, it is necessary to focus on possible new treatment modalities. Maggots in captured bags, which are permeable for larval excretions/secretions, aid in healing severe infected wounds suspect for biofilm formation. Therefore it was hypothesized that maggot excretions/secretions reduce biofilm formation. Questions/purposes: Biofilm formation of Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella oxytoca, Enterococcus faecalis, and Enterobacter cloacae was investigated on polyethylene, titanium, and stainless steel. The quantities of biofilm formation between the bacterial species and the tested biomaterials were compared, as well as the quantity of biofilm formation after various incubation times. Maggot excretions/secretions were added to existing biofilms to examine their effect. Methods: Comblike models of the biomaterials, made to fit into a 96-well microtiter plate, were incubated with bacterial suspension. The formed biofilms were stained in crystal violet, which was eluted in ethanol. The optical density (at 595 nm) of the eluate was determined to quantify biofilm formation. Maggot excretions/secretions were pipetted in different concentrations to (nonstained) 7-day-old biofilms, incubated 24 hours, and finally measured. Results: The strongest biofilms were formed by S aureus and S epidermidis on polyethylene and the weakest on titanium. The highest quantity of biofilm formation was reached within 7 days for both bacteria. The presence of excretions/secretions reduced biofilm formation on all biomaterials. A maximum of 92% of biofilm reduction was measured.
Conclusions: Our observations suggest maggot excretions/secretions decrease biofilm formation and could provide a new treatment for biofilm formation on infected biomaterials.

Introduction

Infections of medical prosthetic devices in trauma and orthopaedic surgery are difficult to treat with conventional therapies like antibiotics and surgery, [8] and can lead to severe consequences for patients, such as removal of the implant with functional loss of the affected limb afterwards.[18,30] The most frequently isolated bacteria causing these implant- and patient-related infections are Staphylococcus aureus and the coagulase-negative Staphylococcus epidermidis.[1,11] Less commonly found are Pseudomonas aeruginosa, Klebsiella oxytoca, Enterococcus faecalis, and Enterobacter cloacae. In prosthesis-associated infections, the causative bacteria form biofilms composed mainly of a polysaccharide matrix.[7] The most important differences between bacteria in biofilms and planktonic microorganisms are the reduced diffusion of antimicrobial agents in the biofilm matrix as a result of chemical reactions, the change of local environment, e.g. the pH and the limited oxygen concentration.[3,27] Finally, an essential difference is the metabolic state of biofilm bacteria; the genotype and phenotype of bacteria in biofilms have been changed, as well as the protein expression and this results in metabolic quiescence.[27] These alterations can explain the resistance of biofilm bacteria against current antimicrobial therapies[7,27,30] and therefore new treatment possibilities to reduce biofilm formation (BF) on biomaterials are required.

The most important development in the treatment of infectious diseases in the last century was the discovery of penicillin by Fleming in 1928.[33] Around the same time, maggot debridement therapy (MDT) was introduced by William S. Baer, an orthopaedic surgeon who worked in Johns Hopkins Hospital in Baltimore, Maryland.[2] Baer used MDT to treat children with at that time incurable osteomyelitis. Although Baer showed the beneficial effects of maggots of the *Lucilia sericata*, MDT disappeared after the discovery of penicillin, which could heal many sorts of severe infections.[33] Because of increasing antibiotic resistance,[15] larval therapy was reintroduced in the 1980s as a treatment for severely infected wounds.[22] Nowadays, maggots are successfully used in various patient clinics.[13,28] In 2004, MDT was approved by the US Food and Drug Administration (510[k] #33391).[31] In our clinical practice, we use maggots captured in small nylon bags, consisting of a 2-mm thin foam layer of polyvinyl alcohol (Biobag®; BioMonde GmbH, Barsbüttel, Germany).[13] These bags are able to permeate maggot excretions and secretions (ES). We have observed healing of wound infections suspected for BF using maggots in such bags. Moreover, in our previous research, we showed reduction of biofilms formed by P aeruginosa on different biomaterials in vitro by adding maggot ES.[6] However, the earlier study only tested the effect of ES on immature biofilms of P aeruginosa, whereas the current research investigated the influence of ES on mature biofilms of other, more commonly isolated bacterial species. If maggot ES also reduce BF of other bacteria, ES could provide us with

a new treatment possibility for biofilm-associated infections.

Therefore, we asked (1) whether five bacterial species (S aureus, S epidermidis, K oxytoca, E faecalis, and E cloacae) were able to form biofilms on polyethylene (PE), titanium (TI) and/or surgical stainless steel (SSS), and if so, whether (2) the quantity of BF differed among the bacterial species, (3) the quantity of BF depended on the biomaterial surface, and (4) the quantity of biofilms of the biofilm-forming bacteria differed among 3, 5, 7, or 9 days of incubation time. Finally, we hypothesized (5) sterile maggot ES could decrease the growth of the bacterial biofilms on the commonly used biomaterials stainless steel (SSS), titanium (TI), and polyethylene (PE).

Materials and Methods

We investigated BF of five different, clinical isolated species, which were all expected to form biofilms on comblike models of PE, TI, and SSS suspending in a 96-well microtiter plate with nutrient medium and bacteria (Fig. 1). The bacterial species that showed visible BF which could be quantified were used to investigate the second up to the fifth research question, in which the quantity of BF is always the dependent variable. BF was compared among the different biofilm-forming species, between the biomaterials SSS, TI, and PE and among various incubation times on Day 5, 7 and 9. Finally, the effect of maggot ES on BF was examined.

The custom-made, sterile comblike models of regularly available orthopaedic implant materials, consisting of eight prongs were produced according to our design. These models of SSS, TI, and PE (Litos GmbH, Hamburg, Germany) were made to fit into a 96-well microtiter plate.[6] Each prong was considered one test and experiments were performed at least 12 times.
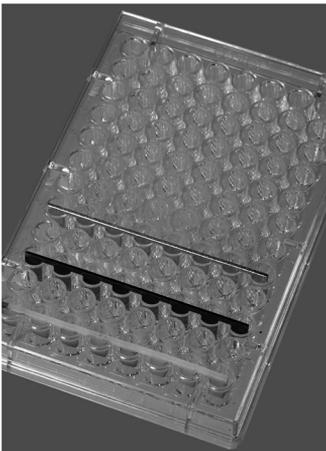

Figure 1 Combs were made of SSS (top), TI (middle) and PE (bottom) hanging in a 96-well microtiter plate.

S aureus, S epidermidis, K oxytoca, E faecalis, and E cloacae were all isolated from infected prosthetic devices of patients in our clinic. BF was tested using a previously described model.[6] Briefly, S aureus and S epidermidis were grown overnight in tryptic soy broth (TSB) medium (Becton, Dickinson and Co, Franklin Lakes, NJ) and K oxytoca, E faecalis, and E cloacae in brain heart infusion (BHI) medium (Becton, Dickinson and Co) at 37° C. A stationary phase culture was made in either TSB or BHI to a density of McFarland 0.5 corresponding with 1.5×10^8 colony-forming units (CFUs) per µL. The inoculum was controlled by inoculating nutrient agar plates (Biotrading Benelux BV, Mijdrecht, The Netherlands) with 10-µL volumes of serial 1:10 dilutions of the bacterial suspension and counting the formed CFUs after incubation overnight at 37° C.

The bacterial suspension was diluted to a final inoculum of 2.5×10^5 bacteria per mL and a volume of 100 µL of this suspension was pipetted into 24 wells of a sterile 96-well flat-bottomed microtiter plate (Greiner Bio-one BV, Monroe, NC). One comb of each biomaterial was placed in 8 of the wells and 4 of these microtiter plates with combs were incubated for 3, 5, 7, or 9 days at 37° C to form biofilms on the devices. Control combs were suspended in nutrient medium.

After the incubation time, at room temperature each comb was washed under slow-running distilled water for 30 seconds to remove planktonic cells and all comb models were stained for 15 minutes with 1% crystal violet (Fig. 2), based on the methods of Pitts[19] and Stepanovic.[26] Each comb was suspended in 8 wells of a new microtiter plate filled with 270 µL ethanol absolute to absorb the crystal violet.[19] The plates were left for 48 hours at room temperature to allow the stained biofilm bacteria to elute with ethanol. All stained biofilm bacteria are soluble in ethanol, however the biofilm on PE could not be eluted entirely in ethanol (maximum time tested was 96 hours), but we standardized the time of elution to 48 hours in order to guarantee a reliable interpretation of the results.
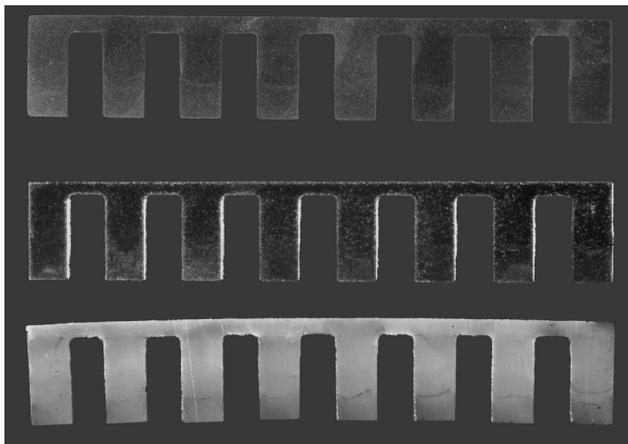

Figure 2 This illustration shows 7 days old biofilms formed by *S. epidermidis* on all combs after staining with crystal violet. Biofilms formed by *S. aureus* appeared equally to that of *S. epidermidis*. From top to bottom were shown TI, SSS and PE.

After gentle shaking of the microtiter plate, a volume of 150 µL of the ethanol and crystal violet solution was pipetted into a new plate and the optical density (OD) of this solution was measured at a wavelength of 595 nm and is representative for the quantity of biofilm formation.[19] Biofilms of each bacterial specie were formed at least in octuplicate, which means one comb was tested with each of the 8 prongs suspended in a separate well with nutrient medium and bacterial suspension.

Sterile maggot ES of Instar-3 larvae (BioMonde GmbH) were collected as described previously.[6] Briefly, the maggots were incubated in sterile tubes for 1 hour at 35° C in darkness. Then, ES were removed by pipette, divided in aliquots and stored at −80° C. One ES pool was collected from at least 1000 maggots, which resulted in approximately 1200 µL ES per hour. The protein concentration was determined using the Pierce Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology, Rockford, IL).

To compute absolute and relative OD, we first subtracted the material-specific background absorbance. The measured OD values of the biofilm experiments were converted into percentages by using the following formula: [OD (biofilm) x 100%]/OD (control), where OD (biofilm) refers to BF measured on Day 3, 5 or 9 (without ES) or to BF after the addition of increasing concentrations of ES and OD (control) is the BF after 7 days on the negative control prongs. In all experiments, the 7-day-old biofilm without ES (the control) was considered 100%. Absolute OD values of the control BF after 7 days without ES are reported in the Results.

To investigate the influence of ES on BF, biofilms were allowed to form on thirteen combs of each of the materials for 7 days, two other combs were suspended in nutrient medium without bacteria as negative controls and represented the material-specific background absorbance. BF on two of the thirteen combs was measured on Day 7. Two other combs were suspended in a new microtiter plate with medium and incubated another 24 hours to allow further growth of the biofilm (the 8-day-old biofilms); nine combs were suspended in a logarithmic range of concentrations of ES, diluted in nutrient medium: 0.31, 0.93, 2.78, 8.33, 25, and 75 µg ES per well. All wells were incubated another 24 hours at 37° C and then we quantified the biofilm as described previously. This experiment investigated possible biofilm inhibition and breakdown by ES. Biofilm inhibition (or prevention) by ES was defined as BF that was lower than 8-day-old biofilms which grew further after Day 7. BF that was lower than 7-day-old BF after incubation in ES was defined as biofilm breakdown. Biofilm reduction is any effect of ES that results in less BF.

The first research question was not analyzed statistically because the results were only obtained by observation. For the second and the last research questions, Student's t test for independent groups was used. The means of the 7-day-old biofilms of the biofilm-forming bacterial species were compared

with each other to analyze whether BF depended on the bacterial specie. Furthermore, the mean BF with ES (per concentration) and the mean of the

Table 1 An overview of the study design

| Research question	Method	Statistical analysis
(1) Do *S aureus, S epidermidis, K oxytoca, E faecalis,* and/or *E cloacae* form biofilms on PE, TI and/or SSS?	All bacterial species were isolated from device-related infections in our clinic and allowed for BF on PE, TI and SSS during 7 days. Biofilm were stained in CV. Then, we observed if visible biofilms were formed on the air-liquid level on the combs. The experiments were performed in octuplicate.	No statistical analysis. Results obtained by observation.
(2) Does the quantity of biofilm formation differ among the bacterial species?	We allow 7 days of BF on comblike models of PE, TI and SSS. The experiments were done in octuplicate.	Student's T test for independent variables A comparison was made between the mean of the 7-day-old biofilm formed by one bacterial specie and the mean of the 7-day-old biofilm by another bacterial specie.
(3) Does the quantity of biofilm formation depend on the biomaterial surface?	BF by each bacterial specie on PE, TI and SSS was allowed for 7 days. The experiments were done in octuplicate.	One-way ANOVA, LSD post hoc test A comparison was made between the mean of the quantities of biofilm formed after 7 days on PE, TI and SSS.
(4) Does the quantity of biofilm formation differ between 3, 5, 7 and 9 days?	We incubated bacterial species for BF on PE, TI and SSS during 3, 5, 7 and 9 days and then measured BF. The experiments were performed in octuplicate.	Repeated measures ANOVA, Bonferroni correction A comparison was made between the mean of the quantities of biofilm on PE, TI and SSS on each time.
(5) Do maggot ES inhibit biofilm formation by different bacterial species on PE, TI and/or SSS?	Biofilms were formed on PE, TI and SSS. Then, maggot ES, diluted in nutrient medium, were added to 7-day-old biofilms in different concentrations, in order to test inhibition of further biofilm growth (comparing to 8-day-old BF) and breakdown of existing biofilms (comparing to 7-day-old BF). All experiments with ES were incubated during 24 hours and done in quadruplicate.	Student's T test for independent variables A comparison was made between the mean BF with ES (per concentration) and the mean of the 7-day-old BF and 8-day-old BF without ES.

BF = biofilm formation; PE = polyethylene; TI = titanium; SSS = surgical stainless steel; ES = excretions and secretions

8-day-old biofilm without ES were compared. For the third research question, one-way ANOVA was used and followed by the least significance difference post hoc test. All analyses met the assumption of homogeneity of variances (Levene's statistic: p>0.05). To analyze whether the BF depended on the material surface, the mean quantity of a 7-day-old biofilm was compared among the materials for each bacterium. The mean quantities of biofilms formed on Days 5, 7, and 9 for each bacterium were analyzed using repeated-measures ANOVA followed by the Bonferroni correction to investigate whether the BF changed among these 3 days. All analyses met the assumption of sphericity (Mauchly's test: p>0.05). All statistical analyses were performed using SPSS® for Windows®, Version 14.0 (SPSS Inc, Chicago, IL). An overview of the study design is shown in Table 1.

Results

All tested bacterial species were able to form visible biofilms on PE, SSS and TI, however biofilms of K oxytoca, E faecalis, and E cloacae could be hardly observed, in contrast to the clearly visible biofilms of S aureus and S epidermidis. Quantities of biofilm formed by oxytoca, E faecalis, and E cloacae were insufficient to be measured. Therefore, we decided to focus on BF by S aureus and S epidermidis and continued the experiments with these two bacterial species.

BF by S aureus was determined and compared to BF by S epidermidis. No differences in quantity were shown on PE (p = 0.582), SSS (p = 0.051), or TI (p = 0.178) (Fig. 3A–B).
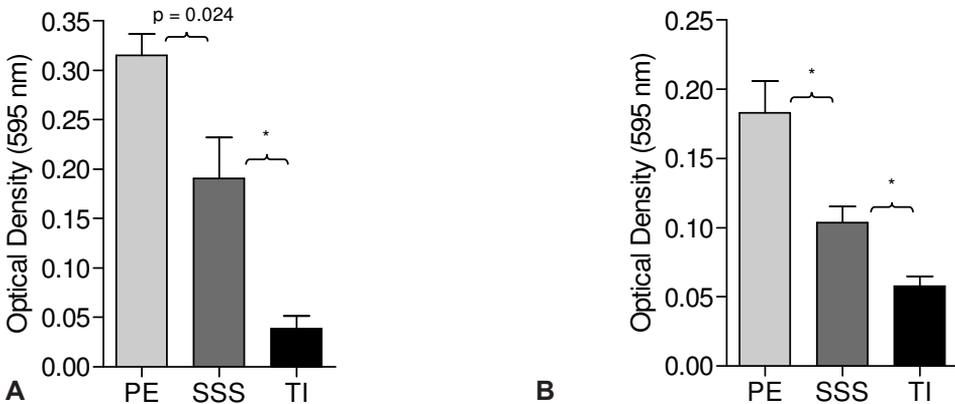
Figures 3A-B The ODs reflect the quantities of 7-day-old biofilms produced by (A) S aureus and   (B) S epidermis on PE, SSS, and TI and answer the second and the third research questions, respectively, whether the quantity of BF differed among S aureus and S epidermidis and whether BF depended on the biomaterial surface. Both bacteria formed equal quantities of BF and showed PE had the highest BF followed by SSS and TI with the lowest quantity of biofilm. * For these comparisons all p-values < 0.001.

BF by S aureus was greater on PE (OD_{595} 0.315) than on SSS (OD_{595} 0.190) (p = 0.024) and TI (OD_{595} 0.0386) (p < 0.001) (Fig. 3A; Table 2) and the same trend was seen for BF by S epidermidis (PE: OD_{595} 0.248, SSS: OD_{595} 0.104 [p < 0.001]; TI: OD_{595} 0.0578 [p < 0.001]) (Fig. 3B; Table 3).

Table 2 Optical density (595 nm) for a 7-day-old biofilm by *S. aureus* on all materials.

Optical density (595nm)	7-day-old biofilm	Background Absorbance
Polyethylene	0.315	0.167
Surgical stainless steel	0.190	0.101
Titanium	0.0386	0.0801

Table 3 Optical density (595 nm) for a 7-day-old biofilm by *S. epidermidis* on all materials.

Optical density (595nm)	7-day-old biofilm	Background Absorbance
Polyethylene	0.248	0.162
Surgical stainless steel	0.104	0.0800
Titanium	0.0578	0.0740

S aureus and S epidermidis were allowed for BF during 3, 5, 7 and 9 days. On Day 3, biofilms of both bacteria occurred in insufficient quantity to be measured. On TI, PE and SSS, most biofilm was formed by S aureus on Day 7 and Day 9 (Day 7 versus Day 9 [all p-values ≥ 0.198]) (Fig. 4A). The amount of biofilm formed by S epidermidis on PE was equal on all times of incubation (Day 5 versus Day 7 [p = 1.000], Day 5 versus Day 9 [p = 0.074]) (Fig. 4B). On Day 7 and Day 9 the largest quantity of biofilms was formed by S epidermidis on SSS and TI (Day 7 versus Day 9 [all p-values ≥0.141]) (Fig. 4B).
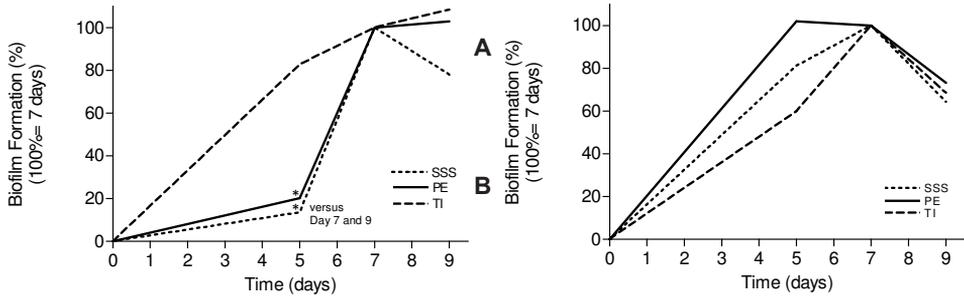
Figures 4A-B   The graphs show the amount of BF after various incubation times and answer the fourth research question, whether quantity of biofilms differed among 3, 5, 7, or 9 days. The 7-day-old BF was defined as 100%. (A) Biofilms by S aureus kept on growing until Day 9 on PE, TI and SSS. (B) The largest amount of biofilm by S epidermidis was formed within 7 to 9 days on SSS and TI. The amount of biofilm on PE was equal on all times of incubation. * For these comparisons all p-values < 0.001. All other comparisons were non-significant.
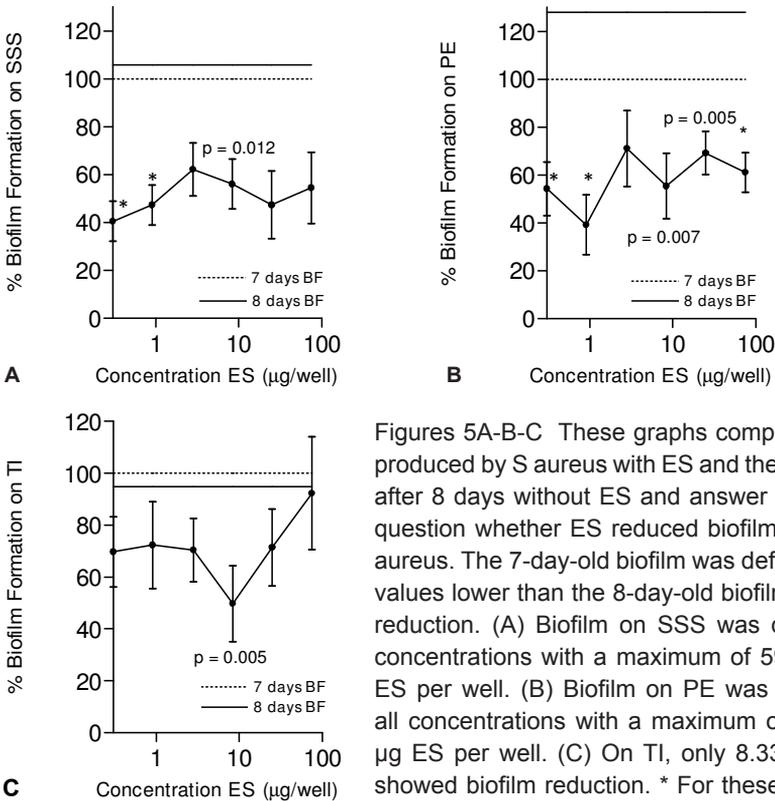
Figures 5A-B-C These graphs compare the mean BF produced by S aureus with ES and the mean control BF after 8 days without ES and answer the last research question whether ES reduced biofilms produced by S aureus. The 7-day-old biofilm was defined as 100%. All values lower than the 8-day-old biofilm showed biofilm reduction. (A) Biofilm on SSS was decreased for all concentrations with a maximum of 59.5% for 0.31 µg ES per well. (B) Biofilm on PE was also reduced for all concentrations with a maximum of 60.8% for 0.93 µg ES per well. (C) On TI, only 8.33 µg ES per well showed biofilm reduction. * For these comparisons all p-values < 0.001. Non-significant p-values were not shown.

The addition of ES to 7-day-old biofilms of S aureus and S epidermidis reduced these biofilms compared with biofilms without addition of ES on all tested biomaterials (Fig. 5; Fig. 6). The biofilm of S aureus on SSS and PE decreased for all concentrations of ES to minima of 40.5% on SSS for 0.31 µg ES per well (p < 0.001) and 39.2% on PE for 0.93 µg ES per well (p < 0.001) as compared to the control biofilm without ES (Fig. 5A–B). On TI, only 8.33 µg ES per well showed a breakdown of biofilm (p = 0.005) (Fig. 5C), but BF on TI was very low overall.

Biofilms of S epidermidis formed on SSS and PE were also reduced for all concentrations of ES (Fig. 6A–B). Biofilms decreased to minima of 32.3% on SSS for 25 µg ES per well (p < 0.001) and 7.6% on PE for 8.33 µg ES per well (p < 0.001) compared with the control. On TI, concentrations of 0.31 µg ES per well (p < 0.001) and 0.93 µg ES per well (p = 0.021) showed less biofilm (Fig. 6C).

A   Concentration ES (µg/well)

B   Concentration ES (µg/well)

C   Concentration ES (µg/well)

Figures 6A-B-C   These graphs compare the mean BF produced by S epidermidis with ES and the mean control BF after 8 days without ES and answer the last research question whether ES reduced biofilms produced by S epidermidis. The 7-day-old biofilm was defined as 100%. All values lower than the 8-day-old biofilm showed biofilm reduction. (A) Biofilm on SSS was reduced for all concentrations with a maximum of 67.7% for 25 µg ES per well. (B) Biofilm on PE was also decreased for all concentrations with a maximum of 92.4% for 8.33 µg ES per well. (C) On TI, only 0.31 µg ES per well and 0.93 µg ES per well showed biofilm reduction. * For these comparisons all p-values < 0.001. Non-significant p-values were not shown.

Discussion

Biofilm-associated infections related with implants cannot easily be treated and frequently the implanted device must be removed with severe consequences for the patient. Therefore, new therapies for these kinds of infections are needed. We previously reported maggot ES reduced BF of P aeruginosa.[6] The current study investigated whether other bacterial species, viz. S aureus, S epidermidis, K oxytoca, E faecalis, and E cloacae, were able to form biofilms on PE, TI, and SSS, and if so, whether the quantity of BF differed between the bacterial species, whether the quantity of BF depended on the biomaterial surface, and whether the largest amount of BF was formed after 3, 5, 7, or 9 days. Finally, we hypothesized sterile maggot ES could reduce BF on the biomaterials.

Several limitations of the study can be noted. First, we performed only a crystal violet assay as a standard method to measure biofilms, but also other methods could be used in vitro[35] and in vivo.[14] We modified the crystal violet assay to our design with the combs of PE, TI and SSS, however the assay was based on the commonly used methods of Pitts[19] and Stepanovic.[26] Second, every experiment was performed with one pool maggot ES, but we did not compare the effectiveness between the various pools. The use of different pools will not likely influence the conclusions of this research, because in our earlier study, we did compare the effectiveness of ES and similar results of biofilm reduction occurred using various pools of ES.[6] Moreover, our study conditions were adapted as much as possible to those in clinical practice. For example, ES were collected under standard conditions in darkness, had an acidity of pH 8, and were incubated at a temperature of 35° C. Furthermore, the protein concentration from each collected ES was measured to standardize the various pools. The biomaterials were produced according to specifications for common patient implants and all bacterial species were isolated from implant infections of patients in our clinic. Therefore, we believe these results were representative and sterile maggot ES reduce not only biofilms of P aeruginosa [6] but also existing and mature biofilms of S aureus and S epidermidis.

We observed biofilms were formed by all tested bacterial species. These bacterial species were expected to form biofilms, as they were all isolated from device-related infections. The possible biofilm-forming capacities of the five tested species on medical devices have been described by other researchers.[4,10,20] BF by K oxytoca, E faecalis, and E cloacae was insufficient to be quantified. Nutrient media other than BHI were used to test BF by these bacteria but did not show BF. The highest amount of biofilm was formed by S aureus and S epidermidis, which are also the most frequently isolated pathogens from orthopaedic surgical wounds in literature.[1]

BF between S aureus and S epidermidis was not different. Few research compared the quantity among these two bacterial species; Hudetz[12] also describes equal BF but a stronger adherence to material surfaces by S epidermidis.

The quantity of BF depended on the biomaterial surface, however studies in literature show different results. Hudetz[12] found that the kind of metal, TI or SSS, plays a minor role in the quantity of BF, whereas MacKintosh[16] showed that biomaterial surface characteristics do influence BF. In this study, TI had the least amount of biofilm in vitro formed by S aureus and S epidermidis. In the previous research less biofilm was formed by P aeruginosa on SSS than on TI.[6] Therefore, we conclude that the quantity of BF depends on the biomaterial, as well as on the bacterial specie creating the biofilm.

After 7 days of BF, the highest amount of biofilm was formed. In the first 7 days, the nutrient medium in the microtiter plate was not changed, because the biofilm bacteria were still viable as the biofilm kept growing. New nutrient medium with or without ES was added after 7 days of incubation in order to maintain the viability of the bacteria. The average time for biofilm maturation is 6 to 8 days.[10,24] Although the 7-day-old biofilms in this study were not examined by confocal microscopy, their maturation process was more advanced than that of the previously tested 24-hours-old PAO1-biofilms which were decreased by ES.[6] Thus, this research shows more evidence for the therapeutic potential of ES in the treatment against biofilm-associated infections which involve mature biofilms.

The biofilm-decreasing capacity of maggot ES seem optimal in low protein concentrations from 0.31 up to 8.33 µg ES per well (3.1 to 83.3 µg ES per mL). In previous studies, protein concentrations of up 20 µg ES per well were also reported as the most effective concentrations for biofilm reduction.[6,32] There are several possible explanations for the fact that the influence of ES at higher concentrations than 20 µg ES per well declined.[6] We hypothesize a cofactor necessary to regulate the process was depleted[17] or there is an non-enzymatic interaction. In future studies concentration ranges below 0.31 µg ES per well will be tested to examine the lower limit amount of ES causing biofilm reduction.

In previous research was found that maggot ES do not possess direct bactericidal or bacteriostatic activity against planktonic organisms.[5] Therefore, ES are not expected to reduce biofilm by destroying the bacteria within the matrix. However, the influence of ES on biofilm bacteria will be investigated in future studies, by examination of total viable counts of bacteria.

Maggot ES broke down existing biofilms, because all measurements were lower than the control BF after 7 days. However, they also seem to inhibit further growth of BF as ES reduced BF to less than 100% in the experiments with a higher amount of biofilm on Day 8 comparing to Day 7 (Fig 5A-B). Therefore, potential therapeutic use of ES could be prevention or inhibition of BF, e.g. flushing surgical wounds before closing, as well as treatment against existing

biofilm-associated infections, especially on orthopaedic medical devices. The aim of further research is to clarify the mechanism(s) of biofilm reduction by maggot ES and explore the substance(s) of ES involved herein.

Acknowledgments

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