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Comparative analysis of biofilm models to determine the efficacy of antimicrobials

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ABSTRACT

Biofilms are one of the greatest challenges in today's treatment of chronic wounds. While antimicrobials kill platonic bacteria within seconds, they are rarely able to harm biofilms. In order to identify effective substances for antibacterial therapy, cost-efficient, standardized and reproducible models that aim to mimic the clinical situation are required.

In this study, two 3D biofilm models based on human plasma with immune cells (lhBIOM) or based on sheep blood (sbBIOM) containing *S. aureus* or *P. aeruginosa*, are compared with the human biofilm model hpBIOM regarding their microscopic structure (scanning electron microscopy; SEM) and their bacterial resistance to octenidine hydrochloride (OCT) and a sodium hypochlorite (NaOCl) wound-irrigation solution.

The three analyzed biofilm models show little to no reaction to treatment with the hypochlorous solution while planktonic *S. aureus* and *P. aeruginosa* cells are reduced within minutes. After 48 h, octenidine hydrochloride manages to erode the biofilm matrix and significantly reduce the bacterial load. The determined effects are qualitatively reflected by SEM.

Our results show that both ethically acceptable human and sheep blood based biofilm models can be used as a standard for in vitro testing of new antimicrobial substances. Due to their composition, both fulfill the criteria of a reality-reflecting model and therefore should be used in the approval for new antimicrobial agents.

1. Introduction

Due to rising numbers of elderly patients and an accompanying increase in multimorbidity, chronic wounds remain a major issue in every medical field. Patients suffering from diabetes, peripheral arterial disease or chronic venous insufficiency are especially predisposed to develop chronic wounds (Hachenberg et al., 2010). Local (subacute) infections with chronic inflammation due to the persistence of various bacteria, including biofilm formation, prolong the healing process and pose a considerable risk of systemic spread. According to current data, at least 78% of chronic wounds are covered with biofilms (Malone et al., 2017).

Since bacterial biofilm colonization of chronic wounds is a medical challenge, the search for local therapies is becoming increasingly important. There are various approaches to remove biofilm colonization from wounds (Johani et al., 2018). Commercially available antiseptic solutions are used which seemed to have proven effective in in vitro settings. However, clinical observation sometimes contradicts the stated performance of antimicrobial substances. Surgical debridement is currently considered the only effective therapy (Malone et al., 2017), but it is not always a feasible option in the outpatient sector.

In in vitro tests, biofilms with only one bacterial species on slippery surfaces (for example plastic or metal metal) (Harrison and Buckling, 2009; Huebner et al., 2010; Watters et al., 2016) or flow chambers

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(Schipper et al., 2009; Kisch et al., 2014) are usually used as models to test the effectiveness of anti-biofilm colonization. To determine the negative effects of biofilm on human cells, some research groups have used single bacterial biofilms combined with 2D cell cultures (Kirker et al., 2009; Secor et al., 2012; Tankersley et al., 2014; Vyas et al., 2016) but usually only biofilm-conditioned media (BCM) is applied to the cell culture in order to avoid a direct, often deadly contact between bacteria and cells (Secor et al., 2011; Tankersley et al., 2014; Kirker et al., 2012). This type of biofilm colonization, even if bacteria form a three-dimensional construct with its typical extrapolymeric substance (EPS), is not comparable to a wound biofilm. The latter generally consists of several microbial specimens, sometimes more than 50, EPS as well as further blood components from the patient (Malone et al., 2017; Johani et al., 2018). To effectively eradicate the bacteria, antibacterial therapy must first overcome the protein and glucose threshold of the extrapolymeric substance and the blood components. However, since antimicrobials often lose their efficacy when confronted with a high protein concentration (Rembe et al., 2020) in the wound microenvironment, they are often unable to penetrate the extrapolymeric substance and destroy the biofilm. In many cases, even highly effective antimicrobial substances demonstrate a reduced therapeutic effect on bacteria organized in biofilm compared to planktonic bacteria (Rembe et al., 2020). Often, a superficial efficacy is achieved, as demonstrated in electron microscopic analysis (Besser et al., 2019, 2020), but penetration is not deep enough into the up-to-2-mm-thick biofilm matrix to reduce the overall bacterial load. Therefore, the need for new and improved antimicrobial substances, possibly transferred from other chemical classes, is of great importance. In order to test these with regard to their clinical efficacy and anti-biofilm effect, models close to the real biofilm matrix are crucial.

There are various possibilities to generate an in vitro model comparable to the human wound biofilm. Very close to the real clinical situation is a biofilm colonization based on human plasma with a buffy coat of the same donor (hpBIOM) [Besser et a. 2018, 2020]. However, the production of such models is costly, time-consuming and not readily available. The experimental models introduced in this work examine possible alternative modifications to the hpBIOM model, which can be used easily and without ethical concerns. The first gradation to the complex model is fresh frozen plasma from blood group AB, which cannot be used clinically, combined with a leukocyte cone from platelet depletion. The latter is a by-product of platelet donation, from a human source and is usually discarded. Another model ties to the standard for the approval of medical products DIN EN 13727 [German standards, 2013]: sheep blood from animal husbandry is used to get closer to the physiological setting. This method avoids the use of human material and thus becomes cost efficient and easily reproducible on a larger scale.

Comparative analysis of these three models could reveal which one is most effective for the standardization of anti-biofilm therapeutics, with the corresponding optimization of time and resources.

2. Methods

2.1. Test organisms and nutrients

The chosen test organisms *Staphylococcus aureus* (DSM-799) and *Pseudomonas aeruginosa* (DSM-939; both DSMZ, Braunschweig, Germany) as well as the *Candida albicans* strain (DSM-1386) were previously tested for biofilm formation (data not shown). The bacterial strains were cultivated on casein/soy peptone agar plates (CSA) and the test yeast was cultivated on malt extract agar (MEA) according to EN 13727 and EN 13624, respectively. The second subculture was used for testing. Each bacterial suspension was adjusted to a 0.5 McFarland standard (approx. 1.5×10^8 cfu/mL) using a densitometer (Grant Bio™ DEN-1B, Grant Instruments Ltd; Cambs SG8 6 GB, England) and added to the plasma and buffy coat of immune cells mixture, respectively, resulting in a final bacterial concentration of 1.5–3 x 10^6 cfu per single biofilm model.

2.2. Preparation of buffy coat based human plasma biofilm model (hpBIOM)

The preparation of the hpBIOM was performed based on the protocol described previously (Besser et al. 2019, 2020). Human plasma (citrate buffered) and buffy coat from the same anonymous donor were obtained from the Institute for Transfusion Medicine, University Medical Center Hamburg-Eppendorf, and Germany. Preparation of the hpBIOM was started one day after donation. Buffy coat was centrifuged at 1610 g at room temperature (RT) for 30 min to remove residual erythrocytes. Next, plasma and buffy coat were mixed in a sterile glass bottle and continuously agitated at 22 °C. After application of the bacterial or fungal solution (resulting in a final concentration of 1.5×10^6 cfu/-model), 18.26 µL $CaCl_2$ (500 mM) per mL plasma was added to the biofilm mix to induce coagulation. The resulting biofilm stock mixture was immediately transferred into 12-well plates (1.5 mL per model/well). The plates were incubated for 14 h on a rotation shaker at 60 rpm and 37.0 °C to polymerize with the organisms and form an extracellular matrix, finally yielding a stable biofilm disc with integrated test organisms.

2.3. Preparation of leukocyte depleted human plasma biofilm model (lhBIOM)

The preparation of biofilm model lhBIOM is similar to that of hpBIOM described above. lhBIOM is always based on fresh frozen plasma (FFP) of blood group AB. The "immunocompetence" of this model originates from the LRS® chamber of leukocyte apheresis (Trima Accel®, Terumo, USA) during platelet donation. The special Terumo BCT design (Trima Accel® LRS® Platelet, Plasma Set", REF number 82300) removes nearly all leukocytes of the donor from the platelet sample, so that its concentration is equivalent to about 40×10^3 leukocytes/µl. Preparation of the IhBIOM was started one day after donation. The content of the LRS® chamber was placed in a tube, washed out with 3 ml plasma (FFP) to remove any residual leukocytes and centrifuged at 1610 g. The layer of erythrocytes was gently removed and the remaining plasma-leukocytes mixture was added to the FFP at room temperature. After mixing, the bacterial suspension was added. The further procedure of model preparation was performed as described above.

2.4. Preparation of sheeps' blood based biofilm model (sbBIOM)

For the preparation of the sbBIOM sheep blood in citrate phosphate dextrose buffer (CBD) from Acila (Mörfelden Waldorf, Germany) was used. The preparation of the sbBIOM was started one day after blood withdrawal. In order to extract the plasma and leucocytes, the blood was centrifuged at 1610 g for 30 min. The middle layer, which is rich in leucocytes and the plasma-supernatant were separated using a pipette. The subsequent steps were carried out in accordance to the procedure described for the preparation of the hpBIOM.

2.5. Antimicrobial treatment of all biofilm models and quantification of bacterial load

For testing and comparing the three different biofilm models, two commercially available antimicrobial wound irrigation solutions with the active agent octenidine-dihydrochloride/phenoxyethanol (0.1% OCT/PE) and sodium hypochlorite (0.2% NaOCl), respectively were used in commercially available concentrations. Each biofilm disc was treated with 300 µL wound irrigation solution for 24, 48 and 72 h, with repeated application of 300 µl every 24 h. After the specified treatment periods, antimicrobial activity was terminated by adding an equivalent volume (300, 600 and 900 µl after 24, 48 and 72 h) of a neutralizing

solution to each well. TLSNt-SDS (6% polysorbate 80, 6% Saponin, 0.8% Lecithin, 2% Sodium dodecyl sulphate, 0.6% Sodium thiosulfate in Aqua dest.) was chosen as neutralizing solution. Sufficient Neutralization of the antimicrobial effects of the wound irrigation solutions was ensured by performing validation tests based on EN 13727 (data not shown).

The plates were placed on a rotation shaker at RT for 5 min. The biofilm models were dissolved using bromelain (Bromelain-POS®, Ursupharm Arzneimittel; Saarbrücken, Germany). Ten tablets were pestled to a fine powder and dissolved in 100 mL PBS buffer (phosphate buffered saline). 1.5 mL bromelain solution was added to each well/biofilm model. The biofilm model was detached from the wall of the well using a pipette tip and punctured with a pipette tip several times to facilitate the bromelain distribution for dissolving the model.

After 3–4 h at 37 °C the biofilm models were completely dissolved. For quantification, the resulting solution was serially tenfold diluted. 50 µL of each dilution was plated (spread technique) and incubated for 48 h at 37 °C under aerobic conditions. Colony forming units (in cfu/mL) were determined using a manual colony counter (schuett count, schuett-biotec GmbH; Goettingen Germany).

2.6. Quantitative suspension method (QSM)

To compare both antimicrobial agents regarding their effect on planktonic bacteria, they were also evaluated in a quantitative suspension method (QSM) based on DIN EN 13727 under conditions with high organic load. Thereby, tests were performed with a burden of 3 g/L bovine serum albumin [bovine serum albumin fraction V (Serva Electrophoresis GmbH, Heidelberg, Germany)] diluted in trypton salt broth (1.0 g/L tryptone, 8.5 g/L NaCl, pH 7.0 ± 0.2) and 0.3 ml/L sheep erythrocytes (ACILA Dr. Weidner GmbH, Darmstadt, Germany). The bacterial test suspensions of *S. aureus* and *P. aeruginosa* were adjusted to 1.5×10^8 cfu/mL (0.5 McFarland standard) initial concentration in trypton salt broth. For the test conduction, 100 µl high burden suspension were added to 800 µl of each agent (A and B) and incubated for 2 min at RT, followed by addition of 100 µl bacteria test suspension. After an exposure of 1, 5 and 15 min 100 µl test solution were transferred into 900 µl neutralization solution for 5 min ±10 s to terminate antimicrobial activity. Surviving bacteria (in cfu/mL) were quantified on agar plates as described for the biofilm models.

2.7. Isolation and purification of bacterial EPS

After dissolving the biofilm models as described previously, 24 discs of the same model were pooled, taken up in 6 mM phosphate buffer (pH 7), dispersed for 5 min, and the biofilm matrix was destabilized by vortexing with a hydrated highly acidic cation exchange resin (1 h at 2700 RPM). After sedimentation of the resin, centrifugation of the supernatant was performed at 12,000g and 4 °C for mechanical separation of the EPS. The supernatant was sterile filtrated (0.22 µm cellulose acetate) and dialyzed with 5 L aqua bidest each overnight. Subsequently, the EPS is separated from the dialysate by precipitation with ice-cold ethanol, filtered off via a suction filter and dried in a vacuum desiccator using phosphorus pentoxide. The EPS extract obtained is resuspended in Tris-HCl buffer (pH 7.5) for wet chemical quantification or dry weighed for gas chromatography-assisted characterization.

2.8. Wet chemical and gas chromatographic characterization of EPS

The analysis of the different bacterial EPS components was performed using wet chemistry. Total protein content was detected by photometric Lowry assay (Peterson, 1977). Quantification was conducted in relation to serum albumin. Total EPS glucose was also detected photometrically; in this case by phenol-sulfuric acid assay (Dubois et al., 1956), with quantification in terms of glucose. The uronic acids specific for EPS were detected photometrically using hydroxybiphenyl according to Blumenkrantz (Filisetti-Cozzi et al., 1991). Quantification was performed in respect to glucuronic acid. To check for potential contamination of the EPS extract with fragments of the "outer membrane", comparative measurements of 2-keto-3-deoxyoctonate (KDO), the representative compound of the membrane-integrated LPS of Gram-negative bacteria and thus a marker for cell rupture, were performed on the EPS and the corresponding cell-free membrane extracts. After disruption of the cell pellets obtained from the EPS isolation step, membrane isolation was carried out by shock freezing in liquid nitrogen and French press (1300 bar) by ultracentrifugation (100,000×*g*, 60 min, 4 °C). This was followed by photometric detection of the KDO according to Karkhanis et al. (1978), quantifying the content in the EPS with respect to the pure substance.

Identification of the Pseudomonas-typical EPS-composing component alginate was conducted as previously described (Michalowski, 2012). Briefly, methanolysis of the dry EPS raw extract (16 h at 85 °C in 2 M methanolic HCl), to present it as methylglycosides, was performed avoiding non-reproducible lactone formation on the uronic acids. Subsequently, derivatization of the methyl glycosides to highly volatile TMS derivatives was carried out with a silylation mixture of BSTFA, TMCS, and TMSI. After dilution of the cooled reaction mixture with hexane, gas chromatographic separation of the monomeric analytes is carried out using helium as carrier gas in a 30 m capillary column on CP-Sil 8 CB (split injection, 4 min at 150 °C–220 °C at 4 °C/min, detection via FID). Erythritol and alginate from brown algae Macrocystis pyrifera act as internal standards for reference.

2.9. Qualitative evaluation by scanning electron microscopy (SEM)

To add a visual aspect of the effects of both antimicrobial agents on biofilm morphology and structure, scanning electron microscopy (SEM) was performed. After neutralization of the antimicrobial agents, the models were fixed with a glutaraldehyde/PVP-solution containing 2.5% glutaraldehyde, 2% polyvinylpyrrolidone (PVP) and 0.5% NaNO2 in 0.1 M cacodylate buffer for 1h at 4 °C. After washing in 0.1 M cacodylate buffer they were stored at 4 °C until preparation of freeze fracture fragments with liquid nitrogen. Samples were incubated in a solution containing 2% arginine-HCL, glycine, sucrose and sodium glutamate for 18 h at RT to stain the glycocalyx. After rinsing with aqua dest. and 0.1 M cacodylate buffer and immersing in a mixture of 2% tannic acid and guanidine-HCL for 5.5 h at RT, they were rinsed again and incubated over night at 4 °C. For continuing staining, samples were placed in a 1% OsO_4 solution for 30 min at RT followed by three rinsing steps with 0.1 M cacodylate buffer and storing over night at 4 °C. In a last step samples were dehydrated with isopropyl alcohol and acetone and dried in liquid CO_2 in a critical point dryer (BAL-TEC AG, Balzers, Liechtenstein). With a sputter coater (BAL-TEC AG, Balzers, Liechtenstein), samples were sputtered with gold palladium and afterwards analyzed by Zeiss Sigma SEM (Zeiss, Oberkochen, Germany).

2.10. Statistical analyses

Data is expressed as means ± standard error of the mean (SEM) based on triplicates derived from three different anonymous blood donors. Bacterial reduction rates (in Δlog10 cfu/mL) were calculated and analyzed using the statistics program GraphPad PRISM (Version 8.2.1; GraphPad Software Inc., La Jolla, USA). Statistical analysis contained a two-way ANOVA, followed by Holm-Sidak posthoc test for evaluation of multiple comparisons. A p-value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Identification and characterization of the EPS

In the EPS extract, about 80% by weight neutral sugars, protein and uronic acids could be detected by calculation using wet chemical

analysis methods (Table 1). The comparatively high levels of sugar and uronic acids identified suggest biofilms with mucoid Pseudomonas strains, since non-mucoid strains have a significantly lower content, i.e. by a ratio of 10–100 (cf. Strathmann, 2003). The detected protein content appears rather excessive, since it probably also includes human plasma proteins which could not be completely separated in the course of EPS purification (precipitation). KDO was not detectable in the EPS extract. If cell rupture had occurred in the course of EPS isolation, a maximum of 8.5 µg KDO would have been detectable in the extract. In fact, a KDO content of <0.9 µg was determined, with which, purely by calculation, membrane fragments of a maximum of 10.6% of the cell amount used can be concluded. The light microscopic controls on the EPS-free cell suspension did not reveal any evidence of cell fragments (data not shown).

The partial gas chromatographic characterization of the EPS matrix, here limited to determination of the uronic acid fraction, is shown in Fig. 1. The analytes were identified with respect to an internal standard (erythritol, elution after 5.9 min at the latest, data not shown). The obtained relative retention times result from comparison to the standard substance algal alginate. Methanolysis and silylation do not yield uniform cleavage or derivatization products, but several anomeric forms of the analyte, depending on the position of the equilibria.

3.2. Antimicrobial efficacy on planktonic bacteria (QSM)

On planktonic bacteria OCT/PE eradicated both tested pathogens *P. aeruginosa* and *S. aureus* within 60 s of exposure (Fig. 2 a/b). No further growth could be detected after 5 and 15 min. The same occurred for the hypochloric acid solution with a complete eradication within the first minute. Due to these obvious results, no further long-term analyzes were performed.

3.3. S. aureus biofilm resistance of the hpBIOM, lhBIOM and the sbBIOM

The untreated *S. aureus* control biofilms hpBIOM, lhBIOM and sbBIOM show a constant bacterial count of approx. 10^7 cfu/ml with minimal growth at the time points 24, 48 and 72h (Fig. 3a). After the 2nd application of OCT/PE (24 h), bacterial quantity in the hpBIOM decreases significantly by approximately one third (2.98 ± 1.33 $\log_{10}$). After 72 h, the antimicrobial agent exerts its full effect by eradicating *S. aureus* completely. The hypochlorous acid achieves a lower cfu reduction in the hpBIOM: about 80% of the inoculated bacteria are still active after 72 h (6.88 ± 3.89 $\log_{10}$). The lhBIOM shows equivalent results, with OCT/PE reducing biofilm after 24 h (by 2.58 ± 2.05 $\log_{10}$) and achieving nearly complete reduction of the bacteria in the biofilm after 48 h (Fig. 3a). After application of the hypochlorous acid, no effect on the bacteria is observed within 72 h (7.82 ± 0.35 $\log_{10}$). In the biofilm model based on sheep's blood (sbBIOM), OCT/PE does not succeed in completely eradicating bacteria, however the same course as in the hpBIOM can be observed. After 48 h the cfu reduction is significantly decreased yielding remaining bacterial counts of 1.02 ± 0.75 $\log_{10}$ and 0.21 ± 0.39 $\log_{10}$ after 72 h. Also for the application of hypochlorous acid, similar results as in the human material based models can be shown. It induced no reduction in bacterial counts after 24 h (8.98 ± 0.33 $\log_{10}$), after 48 h (8.64 ± 0.13 $\log_{10}$) of after 72 h (8.29 ± 0.34 $\log_{10}$).

Table 1
Wet chemical analysis of bacterial EPS dried extract derived from 24 human biofilm models lhBIOM for characterization of *P. aeruginosa* biofilm.

Total cell count (24 biofilm models)	4,3E+10 CFU
KDO membrane extract (24 biofilm models)	8,5 µg
EPS-extract (24 biofilm models)	168800 µg (dry weight)
Glucose	18676,0 µg
Protein (BSA)	107204,1 µg
Uronic acid (Glucuronoc acid)	7969,6 µg
2-Keto-3-desoxyoctonat (KDO)	<0,9 µg
Sum	**133849,7 µg (79,3% w/w)**

3.4. P. aeruginosa biofilm resistance of the hpBIOM, lhBIOM and the sbBIOM

Overall the *P. aeruginosa* biofilm proved more resilient than the *S. aureus* version. All control biofilm models show an increase in bacterial counts by approximately 1.5 log steps within the 72-h investigation period (10^9 to > 10^{10}; Fig. 3b). In the hpBIOM, the OCT/PE containing antimicrobial solution causes a 60% reduction of the bacterial load within 48 h (3.81 ± 0.97 $\log_{10}$) and a complete eradication after 72 h. In contrast, the hypochlorous acid does not achieve a reduction in the hpBIOM.

In the lhBIOM, the same trend, however no complete reduction, can be observed with regard to OCT/PE (reduction to 1.33 ± 0.95 $\log_{10}$ after 72h) as well as to the hypochlorous acid. The latter shows only a minimal reduction of 0.5 $\log_{10}$ steps after 48h (9.02 ± 0.12 $\log_{10}$) and 72h (9.41 ± 0.10 $\log_{10}$) compared to the untreated control.

In the biofilm model based on sheep's blood (sbBIOM), OCT/PE reduces the bacterial count significantly by 2 $\log_{10}$ steps (7.78 ± 0.10 $\log_{10}$) after 24 h, by about 7 $\log_{10}$ steps after 48 h (2.46 ± 1.12 $\log_{10}$), before achieving complete eradication of *P. aeruginosa* after 72h. For the hypochlorous acid, equivalent results as in the human models are observed (Fig. 3b). Here, it does not succeed in influencing the bacteria within the biofilm to any significant extent (reduction after 24h: 9.67 ± 0.13 $\log_{10}$, 48h: 9.52 ± 0.23 $\log_{10}$; and 72h: 9.36 ± 0.31 $\log_{10}$).

3.5. Scanning electron microscopy (SEM)

In SEM, treatment with hypochlorous acid or OCT/PE solution induces observable changes in the structure of the biofilm models. In the untreated, 72 h old biofilm control, surface structure appears smooth and fine-pored (Fig. 4a/d). The glycocalyx staining shows a cover-like structure of EPS. In contrast, the biofilm is fissured after 72 h OCT/PE application (Fig. 4c/f). Exposed bacterial colonies as well as isolated residual erythrocytes are visible. The solution has corroded the previously homogeneous surface, thus creating openings for deeper penetration. The biofilm structure after application of the hypochlorous acid is slightly coarser than that of the untreated control, but only a few holes occur wider and/or deeper (Fig. 4b/e). The electron microscopic structure and also the effects of the wound irrigation solution used differ only slightly between the hpBIOM, the lhBIOM and the sheep model (data not shown).

4. Discussion

The term "biofilm" is not used consistently in medicine: it is used for the bacterial colonization of implants, such as endoprostheses or stents. It is also an established term for the persistent colonization of chronic wounds with bacteria, which has proven to be highly resistant to therapy. While a typical implant-coating biofilm on synthetic surfaces can be easily constructed in vitro, so far no widely established 3-dimensional model exists, that depicts the biofilm of a (chronic) wound including the (host) immunocompetence aspect. The special feature of such model is that the bacterial species "arrange" themselves symbiotically in the biofilm, i.e. they protect each other by forming a matrix, the extracellular polymeric substance (EPS) (Fig. 4). The fast EPS build-up within the biofilm models used here could not only be visualized by SEM (Besser et al., 2020), but also confirmed by wet chemistry and gas chromatography analysis and quantification. The detected alginates do not occur in human cells or in blood plasma. They are essential components of the EPS matrix of mucoid Pseudomonas strains and particularly responsible for the formation of a three-dimensional, heterogeneous biofilm architecture (Davies 1999; Wingender et al.,

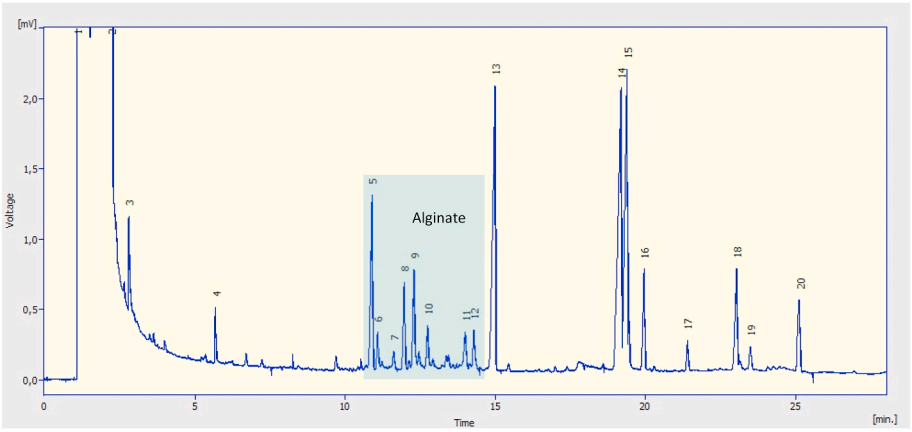

Fig. 1. Gas chromatographic detection of alginates in the bacterial EPS of the biofilm of *P. aeruginosa* in hpBIOM. Assignment of peak numbers: 1 to 3 = running medium front, 5 to 12 = mannuronic and guluronic acid (alginate; highlighted in grey), 4, 13 to 20 = not identified.

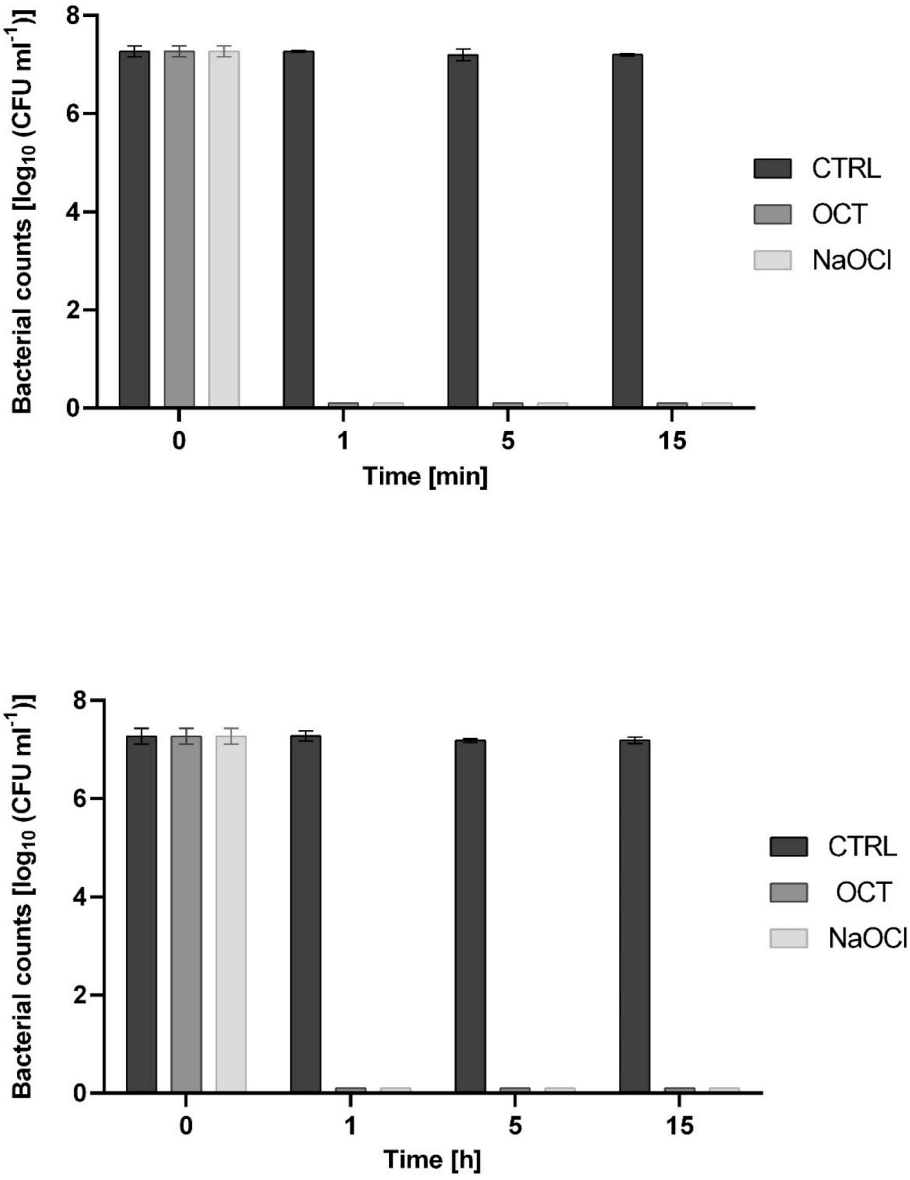

Fig. 2. Reduction of *S. aureus* (**a**) and *P. aeruginosa* (**b**) in planktonic form determined by QSM after application of two antimicrobial agents (OCT/PE and NaOCl) compared to an untreated control (CTR). Remaining bacterial counts (in $\log_{10}$ cfu/mL) are outlined over the course of 15 min of treatment (Values expressed as mean ± SEM. *$p \leq 0.05$ vs. CTR).

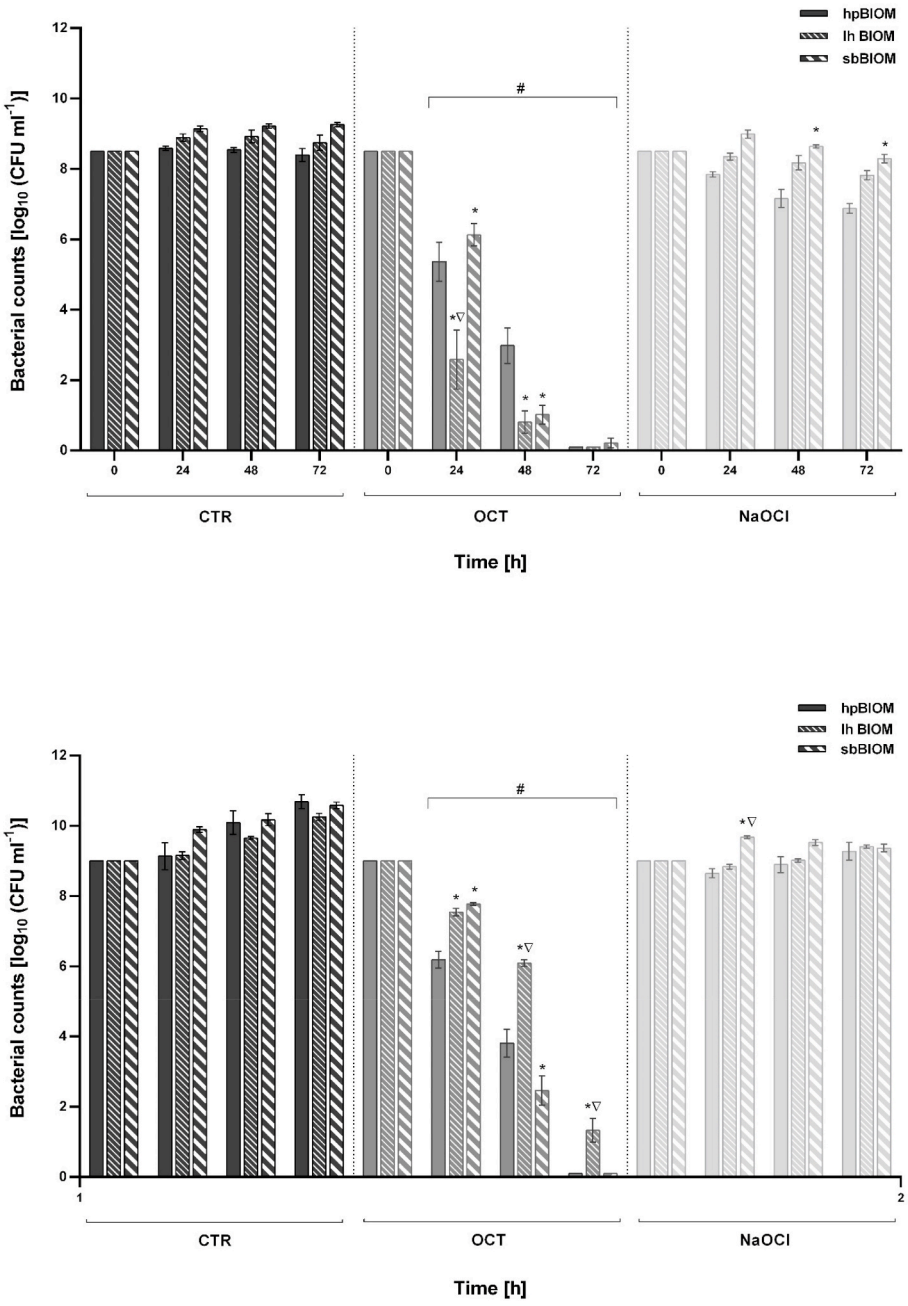

Fig. 3. Comparison of the biofilm models hpBIOM, lhBIOM and sbBIOM including *S. aureus* (**a**) or *P. aeruginosa* (**b**) with and without application of 0.3 mL of the antimicrobial agents OCT/PE and NaOCl compared to an untreated control (CTR). Reduction rates of included bacteria (in $\log_{10}$ cfu/mL) are outlined over the course of 72h of treatment after an initial biofilm maturation period of 12h. (Values expressed as mean ± SEM. $*p \leq 0.05$ vs. hpBIOM+ [agent], $\bigtriangledown p \leq 0.05$ vs. sbBIOM+ [agent], $\#p \leq 0.05$ vs. corresponding CTR or NaOCl-value).

2001). This matrix not only prevents the patient's immune system from reacting adequately, but also impedes disinfectants and antimicrobial substances from penetrating the biofilm and killing the bacteria living within it. Antimicrobials lose their efficacy as only the superficial and planktonic bacteria are killed while the bacteria in deep layers of the biofilm continue to exist and multiply. In fact, the biofilm of a (chronic) wound is 100–1000 times more resistant to antimicrobials in daily use and to the host immune cells than planktonic bacteria, i.e. those floating in fluid [Besser et al., 2020; Rembe et al., 2020]. Therefore the need to identify and manufacture new types of antimicrobials is unaltered. Such novel or innovative compounds should not only address the bacterial membranes and proteins, but also be capable of degrading the EPS formed in biofilms.

The predescribed biofilm models are mostly two-dimensional or liquid based (Harrison and Buckling, 2009; Huebner et al., 2010; Watters et al., 2016). Besides the hpBIOM described here, only two other 3D models exist as to the knowledge of the authors: a liquid-filled chamber with several bacterial species (Lipp et al., 2010; Agostinho et al., 2011; Woods et al., 2012) and a collagen gel matrix with serum proteins containing up to two bacterial species (Werthén et al., 2010). Concerning wound biofilms, the comparability and durability of both models is low. In most cases, only the biofilm-conditioned medium (BCM) is used for test purposes in order to avoid direct contact between bacteria and (human) cells [Secor et al., 2011; Tankersley et al., 2014; Kirker et al., 2012], as this leads to rapid cell death.

The models analyzed here contain the immunocompetence of the human or animal from whom the blood was drawn. This extends the duration of the test, as the immune cells fight the microbes and inhibit their proliferation allowing the model to last up to 90 h. In addition, they mimic an organic wound relevant matrix with components such as cytokines, EPS or bacteriotoxins influencing the structure and built-up of biofilms and their resistance against antimicrobials [Besser et al. (2020); Rembe et al. (2020). It is well-known that even a physiological protein load of the (test) environment can significantly change the efficacy of

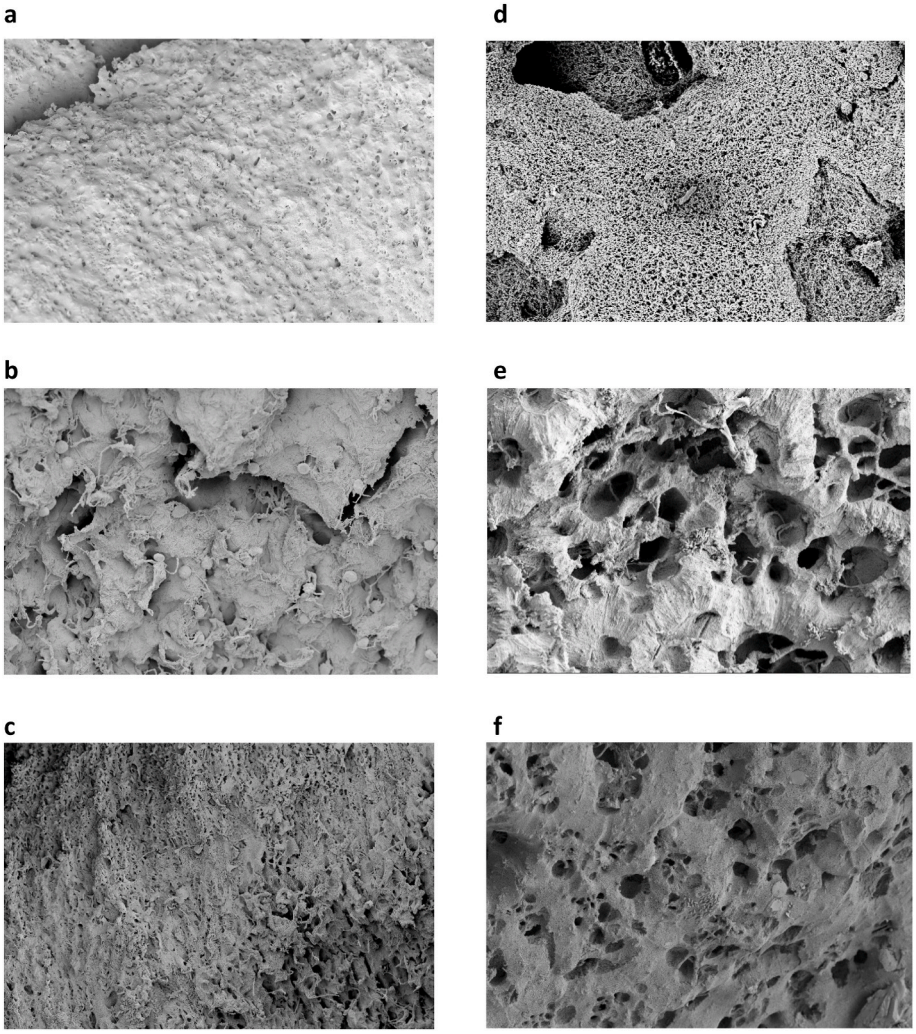

Fig. 4. Scanning electron microscopy (SEM) visualisation of biofilm surface alteration in the hpBIOM (**a-c**) and lhBIOM (**d-f**) (here *P. aeruginosa*) under control conditions (**a, d**) and after 72h of treatment with the antimicrobial agents OCT/PE (**b, e**) and NaOCl (**c, f**). Densely connected surface structure of the untreated biofilm models (**a, d**). After treatment with OCT/PE, surface structure appears rugged and 'broken-open' with several holes as potential new entry points for antimicrobials. (**b, e**) After treatment with NaOCl biofilm surface remain denser and connected though lots of small holes are visible (**c, f**). With regard to the quantitative analyses the changes in EPS structure seem insufficient for antimicrobial agent penetration.

the antimicrobials being employed [Rembe et al., 2018]. Therefore test conditions such as osmotic pressure, pH value, oxygen content and temperature, consistency, etc. ought to be adjusted as closely as possible to the physiological conditions of a growing biofilm (den Reijer et al., 2016; Liu et al., 2020; Lone et al., 2015).

The analyses of a biofilm model based on sheep's blood (sbBIOM) prepared according to DIN EN 13727 (DIN EN, German Standards 2012) showed very similar results compared to the human models. Since the sbBIOM can be used in accredited laboratories for the approval of medical devices without further official authorizations, it seems a suitable option, as the model reflects the "resistance" of biofilms in human wounds. In addition, it appears to be irrelevant whether the immune cells originate from the same individual (hpBIOM or sbBIOM), and even a variation of the blood group has no effect (lhBIOM) according to the current state of investigations. This was unexpected, but enormously facilitates model generation and proves more cost efficient. This is an essential aspect regarding the testing of existing and upcoming antimicrobials and medical devices for biofilm control. Additionally, it is ethically justifiable, as no blood products are used in a research setting instead of a clinical context.

Regardless of the kind of model, the two chosen test substances, a hypochlorous acid (NaOCl) and an alcohol-tenside mixture (Octenisept®), show significant differences in their anti-biofilm efficacy. A superiority of the latter is shown, even if it only develops its full efficacy after 48 h (and not after seconds as in planktonic bacteria). However, it should be emphasized that NaOCl and octenidine-dihydrochloride have been used as examples of different extents in potencies of antimicrobials. Other common antimicrobials can be expected to fall somewhere in-between these two extremes. Many agents appear to be unable to effectively break through the EPS of biofilms to reach the embedded bacteria within [Phillips et al., 2015; Besser et al., 2020; Rembe et al., 2020]. Currently, the most effective and recommended therapy for biofilm colonized wounds is the sharp and surgical debridement (Malone and Swanson, 2017; Schultz et al., 2017). In modern medicine with new and increasingly effective pharmaceuticals, this is unacceptable, particularly as there are risks associated with every (surgical) procedure. It seems to require a different chemical composition or maybe a mixture. Since the EPS consists of a three-dimensional construct of proteins and types of sugar molecules, protease and/or sugar splitting of the enzymes seem to be necessary [Percival et al., 2017]. To test such new substrates, in vitro (wound) biofilm models close to the physiologic reality, such as the variations introduced here are necessary.

5. Conclusion

To detect efficient antimicrobials for anti-biofilm treatment strategies in (chronic) wounds, test methods that reflect the real clinical situation are needed. The three models analyzed here are suitable, with the sheep model probably having the greatest practicability due to regulatory restrictions for accredited laboratories. Taking into account the discussed aspects, it seems evident that novel or innovative pharmacochemical compounds, targeting deposited biofilm EPS as well as

bacterial cell components and alter the biochemical microenvironment, are most suitable. For this purpose, the here investigated 3 dimensional wound biofilm model variations were developed, evaluated and compared, to drive and facilitate research, development and adequate evaluation.

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Declaration of competing interest

All authors declare no conflict of interest.

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