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A novel chronic wound biofilm model sustaining coexistence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* suitable for testing of antibiofilm effect of antimicrobial solutions and wound dressings

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Abstract
Chronic wounds are a large burden to patients and healthcare systems. Biofilm infections in chronic wounds are crucial factors leading to non-healing of wounds. It is important to study biofilm in wounds and to develop effective interventions against wound biofilm. This study presents a novel in vitro biofilm model mimicking infected chronic wounds. The novel layered chronic wound biofilm model uses woundlike media and includes both *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which have been identified as the most important pathogens in wounds. The model sustains their coexistence for at least 96 h. Microscopy of the model revealed microbial growth in non-surface attached microcolonies as previously observed in vivo. The model was used to determine log₁₀-reduction for the use of an antimicrobial solution and antimicrobial dressings (containing silver or honey) showing moderate-to-low antibiofilm effect, which indicates better concordance with the observed clinical performance of this type of treatment than other widely used standard tests.

KEYWORDS
biofilm, coexistence of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, model, test of antibiofilm effect

1 | INTRODUCTION

Chronic wounds constitute a large burden on patients, society and healthcare systems, affecting 2% of the population in the developed countries with an estimated 2%–3% of the healthcare budgets related to chronic wounds globally, equalling US $13–15 billion annually.¹–³ Bacteria inflict on the healing of chronic wounds,⁴ with *Pseudomonas aeruginosa* being identified as a major pathogen⁵ together with *Staphylococcus aureus*, but the underlying mechanisms on the pathogenicity of wound biofilm are not well understood.⁶ Furthermore, the
presence of bacterial biofilms in wounds has been linked to the non-healing of chronic wounds. In particular, *P. aeruginosa* and *S. aureus* are frequently found together in chronic infections and their co-occurrence has been linked to increased virulence and worse patient outcome. Unfortunately, it has proven difficult to develop in vitro models allowing the co-culture of these two species, which makes it difficult to study their interactions and to develop effective treatment.

Many commercially available dressings and treatments claim antimicrobial and often antibiofilm effects, but strong clinical evidence is unrealistically fast and easy eradication of biofilm. As a result, we describe the development and performance of an in vitro model allowing the user to test for antimicrobial effect of wound dressings and antimicrobial solutions. That is, it should be of a suitable size to allow for addition of solutions and placing of dressings, preferably with an air headspace and a flat surface.

1. has a physical format that allows the user to test for antimicrobial effect of wound dressings and antimicrobial solutions. That is, it should be of a suitable size to allow for addition of solutions and placing of dressings, preferably with an air headspace and a flat surface.

2. sustains coexistence of *P. aeruginosa* and *S. aureus* for up to 96 h, allowing the user to test the antibiofilm performance of dressings and antiseptics for 24–48 h on mature 24–48-h biofilm reflecting clinical practice. As coexistence is seen in vivo, coexistence in vitro can be seen as an indication of similar growth conditions in the model as in vivo.

3. exhibits a three-dimensional (3D) structure resembling observations from clinical specimens, including non-surface-attached microcolonies.

4. simulates wound matrix by using complex growth medium, including serum, blood and animal tissue digest, and introduces a surrogate subcutaneous layer with a large fat content.

Biofilm formation and response to treatment in the novel model was compared to a comparable in vitro model using simple tryptic soy broth (TSB) as growth medium.

## METHODS

### 2.1 Bacteria

*S. aureus* DSM 110939 and *P. aeruginosa* PA14 were used as model organisms for this study. *S. aureus* DSM 110939 was originally isolated from a prosthetic knee infection and is well characterized. PA14 was originally isolated from a burn wound and has been applied in numerous wound studies. Bacterial cultures for inoculation of biofilm models were prepared as follows: *S. aureus* DSM 110939 and *P. aeruginosa* PA14 were inoculated, each on separate plates of tryptic soy agar (TSA) and incubated overnight at 37°C. Liquid bacterial culture was prepared by inoculation of a single colony from the TSA plates into 25 ml of TSB medium followed by incubating overnight at 37°C with shaking at 150 rpm.

### 2.2 In vitro models

#### 2.2.1 Semi-solid agar model with tryptic soy broth

A semi-solid agar base (400 μl of 25% strength TSB medium containing 0.5% agar, corresponding to approximately 2.1 mm depth) was prepared in 1 ml wells of 4-well titre plates (Nunc™, Thermo Fisher Scientific) and allowed to solidify. Approximately 26.6 μl of *P. aeruginosa* containing approximately 25–100 colony-forming units (CFU) in tempered semi-solid agar (0.5% agar, 25% TSB medium) was added onto the top of the agar base resulting in an approximately 140-μm-thick bacterial layer. After solidification, another 140 μm layer (26.6 μl) semi-solid agar containing approximately 75–200 CFU...
S. aureus prepared in a similar manner was added to allow formation of a two-layer, two-species biofilm (Figure 1A). For single-species version of the model, only the relevant species was added onto the agar base: For the single-species P. aeruginosa model, a 140-μm-thick layer containing P. aeruginosa was added first and then covered with a 140-μm-thick layer without bacteria, whereas for the single-species S. aureus model, a 140 μm agar layer without bacteria was added first and then covered by a 140 μm layer with S. aureus. The models were incubated at 25°C.

2.2.2 | Layered chronic wound biofilm model

The novel layered chronic wound biofilm (CWB) model consisted of two layers mimicking a dermis layer on top of a subcutaneous fat layer (Figure 1B). First, a surrogate subcutaneous fat layer consisting of 2% peptone (Oxoid) (w/v), 10% pig fat (w/v), 0.5% bacteriological agar (w/v) and 68% sterile saline (v/v), 2% laked horse blood (Thermo Fisher Scientific) (v/v) and 20% cattle serum (SSI Diagnostica) (v/v) was cast (450 μl) in each 1 ml well of a 4-well plate. A device supporting 2-ml Eppendorf tubes achieved the formation of a void in this layer (Figure 2A). After solidification, 200 μl of dermis layer preparation was poured onto the subcutaneous fat layer resulting in a total thickness of approximately 4 mm with a void approximately 3 mm in depth. The surrogate dermis layer contained 5% laked horse blood (Thermo Fisher Scientific) (v/v) and 50% cattle serum (SSI Diagnostica) (v/v), 2% peptone (w/v), 0.5% bacteriological agar (w/v) and 45% sterile saline (v/v). The mentioned device supporting 2-ml Eppendorf tubes ensured that the dermis layer did not cover the void in the subcutaneous fat layer, thus mimicking a breach of the dermis. After solidification, the device was removed prior to the addition of bacteria. Both bacteria were prepared in subcutaneous fat layer agar. Approximately 140 μm layer (32.2 μl) containing approximately 25–100 CFU P. aeruginosa was added onto the top of the layered chronic wound biofilm model. After solidification of the Pseudomonas layer, another 140 μm (32.2 μl) fat layer agar containing approximately 75–200 CFU S. aureus was added to allow formation of a two-layer, two-species biofilm (Figures 1B and 2B). For single-species versions of the model, only the relevant species were added onto the agar base as described for the semisolid agar model with TSB. Subsequently, the models were incubated at 25°C.

2.3 | Microscopy

The biofilm model was gently washed by adding 500 μl milli-Q water. The gel was then removed from its mould, placed on a microscope.
slide and left to dry for 1 h. A mix of Syto™9, Sytox™Orange Dead Cell Stain and wheat germ agglutinin (WGA) Alexa Fluor® 594 conjugate (Invitrogen) was prepared and added to the gel. Sytox™Orange was used to indicate permeable membranes. Therefore, cells which appear yellow or orange in colour have permeable membranes and are assumed to be dead or dying. The use of WGA, a lectin binding specifically to the N-acetylgalactosamines in the peptidoglycan layer of bacterial cell walls, served as a morphology-independent marker to distinguish Gram-negative PA 14 from Gram-positive S. aureus. With these stains, live P. aeruginosa appeared green, dead P. aeruginosa appeared orange, live S. aureus appeared green with a slight red “halo” around it, and dead S. aureus appeared yellow with a red halo. The stained gel was left to incubate in the dark for 15 min. A razor blade was used to cut thin vertical slices of 2–3 mm thickness from the gel. These were visualized in a Zeiss LSM 880 inverted confocal laser scanning microscope (Carl Zeiss GmbH, Germany), and the images were subsequently processed using the IMARIS software (Bitplane AG, Switzerland).

2.4 | Harvest and enumeration of the biofilm model

At the end of the biofilm incubation time, the surface of the agar in each well was carefully rinsed three times with 0.5 ml sterile saline. Then the biofilms were harvested by transferring the content of each single well to a 2-ml Precellys lysis tube containing 1 ml of cold saline and 10 Precellys ceramic beads (2.8 mm), followed by bead beating (3 × 5 s, 6000 rpm) using a Precellys tissue homogenizer (Bertin-Instruments) and 10 min sonication (40 kHz). Subsequently, bacteria were quantified using 10-fold dilution series and plating on TSB agar (P. aeruginosa) and Azide blood agar (Sigma-Aldrich) (S. aureus), with the exception of microbial counts upon treatment with wound dressings, where both species were counted on TSB agar. Both S. aureus and P. aeruginosa grew on TSB agar and their colonies could be differentiated by their distinct morphologies: Colonies of S. aureus were small with clearly defined edges and white-yellowish, while colonies of P. aeruginosa PA 14 were large with irregular margins and greenish.

2.5 | Treatment of biofilm models

2.5.1 | Treatment with rinsing solution

After establishing both single-species and two-species 48-h mature biofilms in the layered CWB model, the models were treated by addition of 250 µl of commercially available rinsing solution containing 0.1% polyhexamethylene biguanide (PHMB) (Prontosan® Wound Irrigation Solution, B. Braun, Germany) for a duration of 10 min every 24 h. After the end of the treatment time, the supernatant PHMB solution was removed and the model was washed three times with 0.5 ml saline after which it was returned to the incubator at 25°C. The models were harvested at time points 96, 120 and 216 h after inoculation.

2.5.2 | Testing of wound dressings

Three commercially available wound dressings termed Silver 1, Silver 2 and Honey 1 were tested for their antimicrobial performance using the layered CWB model with inoculation of both bacterial species. Pieces of 1.6 cm diameter were aseptically cut from the centre part of the dressings and placed on top of the model agar after previous establishment of 48-h mature two-species biofilm. Wound dressings were aseptically removed using forceps after 48 h of treatment, just prior to harvest and enumeration.

2.6 | Neutralization of antimicrobial compounds

Dey-Engley neutralizing solution (Sigma-Aldrich) was used for neutralization of active antimicrobial compounds in models treated with PHMB solution and silver-containing wound dressings. Dey-Engley neutralizing solution (500 µl) was used to rinse the surface of the biofilm model three times. Furthermore, 1 ml Dey-Engley solution was added in the bead-beating step instead of 1 ml saline.

Neutralization of models treated with wound dressings containing honey was done by six repeated washing steps with 0.5 ml sterile saline (0.9% NaCl) added to and subsequently removed from the biofilm model surface. Subsequent bead-beating was performed with addition of 1 ml saline.

2.7 | Statistical analysis

Treatment effects of PHMB and wound dressings were analysed by one-tailed t-tests compared to untreated controls of the same model type and at the same age (Microsoft Office Excel) with a significance level of α = 5%.

3 | RESULTS

As mentioned in the introduction, we aimed to achieve an in vitro biofilm model of chronic infected wounds containing non-surface attached microcolonies of both P. aeruginosa and S. aureus showing stable coexistence of both species over several days, thereby allowing for testing of antimicrobial treatments such as wound dressings or solutions. To achieve this, we combined

1. The use of a 4-well format with a well diameter of approximately 16 mm—a size and format that allows application of wound dressings or antimicrobial solutions while still being easy to handle during harvest and culturing.
2. Woundlike growth medium, which has been shown to support mixed species biofilm.
3. Addition of 0.5% agar to the growth medium and sequential addition of P. aeruginosa and S. aureus, which simulates the spatial distribution of the two bacteria in 3D structure biofilms:
Single-species biofilm in the model with TSB medium and the novel layered CWB model. Comparable, high numbers of CFU were found for both bacteria in both models (n = 8, mean ± SD). Two independent operators analysed n = 4 each. a: Staphylococcus aureus in TSB model, b: Pseudomonas aeruginosa in TSB model. c: S. aureus in CWB model, d: P. aeruginosa in CWB model. CFU, colony-forming unit; CWB, chronic wound biofilm; TSB, tryptic soy broth.

Knowledge on the distribution of P. aeruginosa and S. aureus in wounds based on investigation of clinical specimens collected from infected chronic wounds showing that S. aureus predominantly was found at the wound surface, whereas P. aeruginosa seemed to be “buried” in deeper layers.

3.1 | Biofilm model development

Initially, single-species biofilm was investigated in the layered CWB model with the described layered woundlike growth medium using 4-well plates and was compared to a similar biofilm model using TSB medium (Figure 3). For both P. aeruginosa and S. aureus, comparable numbers of CFU were found in both models throughout the investigated 9 days of biofilm formation.

Inoculation of the layered model containing TSB medium with both bacterial species did not result in stable coexistence of both bacterial species, as S. aureus did not reach bacterial numbers of even 10^5 CFU/ml, while P. aeruginosa CFU/ml was unaffected by coculturing with S. aureus (Figure 4). These findings are in concordance with studies that have shown P. aeruginosa to produce antimicrobial exoproducts effective against S. aureus.

The layered woundlike growth medium contained laked horse blood, cattle serum, and peptone, which is similar to wound-simulating media used in several studies. According to Thaarup and Bjarnsholt, a suitable medium simulating wound fluid should consist of a meat-based broth combined with mammal serum and red blood cells, because the wound bed is recognized to contain mainly damaged host tissue, plasma and red blood cells. In contrast to the layered model containing TSB medium, the woundlike growth medium resulted in a CWB model with stable microbial numbers for both species for 96 h. Beyond this time point, an apparent detrimental effect of P. aeruginosa did result in a significant decline of S. aureus (Figure 4). Thus, it seems that the medium composition aided in development and maintenance of a biofilm model with two species for at least 96 h. This finding is in agreement with observations made by Cendra et al. who observed that addition of Bolton broth sustained growth of S. aureus together with P. aeruginosa. It also means that the layered CWB model can allow for testing of antimicrobial products for up to 48 h on mature 48-h biofilm of both P. aeruginosa and S. aureus. Beyond 96 h, a large variation in S. aureus cell count was observed due to large run-to-run variance. The inconsistent killing of S. aureus by P. aeruginosa could be a combined effect of several factors: (1) S. aureus could be more protected in some microcolonies because of certain metabolic states or biofilm extracellular matrix; (2) biological variation in production of anti-Staphylococcus compounds by Pseudomonas even in biofilm models prepared under identical conditions; (3) the distance between S. aureus and P. aeruginosa microcolonies might influence the killing.

3.2 | Microscopy

Confocal laser scanning microscopy was used to investigate the distribution of the bacteria in the novel CWB model, both at 48 h, where
both bacterial species were shown to coexist in equal numbers by culturing, and at 120 h, where the numbers of culturable *S. aureus* cells were shown to start declining.

Surprisingly, bacteria were found as deep as 2000 μm below the surface of the model, while in theory only the top 280 μm had been inoculated with bacteria (Figure 5A,D). Migration of bacteria deep into the model might occur due to the very porous structure of the model as only 0.5% of agar was used for model preparation, probably assisted by the presence of air bubbles and water channels. At 48 h, both microbial species were present as single cells (Figure 5B), large aggregates (Figure 5C) and as non-surface-attached microcolonies (Figure 5D). At 120 h, the overview micrograph (Figure 6A) shows a combination of orange–yellow and green areas, where the orange–yellow areas are indicative of dead bacteria and living bacteria are green. The great majority of dead bacteria were *S. aureus* (Figure 6B,D), with only subpopulations of microcolonies surviving, while *P. aeruginosa* was found alive and in large aggregates (Figure 6C). In addition to the morphological differences between the two species, the used WGA Alexa Fluor 594 conjugate improved our ability to differentiate between PA 14 and *S. aureus*: WGA is a lectin, which binds specifically to the N-acetylglucosamines in the peptidoglycan layer of bacterial cell walls. Due to the outer membrane of Gram-negative PA 14, this WGA-conjugated stain only binds to the Gram-positive *S. aureus* and gives them a red “halo”. The overall
3.3 Biofilm model testing

As our basic performance requirements for the novel model were achieved with the presented design of the layered CWB model, we went on to test antimicrobial solutions and wound dressings using the model. As an initial benchmark to evaluate the response of the model to antimicrobial treatment, we subjected the model to repeated daily 10 min treatments with a commercial PHMB solution (Table 1).

Data show a particularly marked effect on \( S. aureus \) in the layered CWB model with eradication of the bacterium after 3 days of repeated treatment with PHMB even in the single-species version of the model. Lower \( S. aureus \) numbers after 2 days of PHMB treatment (total biofilm age 96 h) in the two-species model compared to the single-species model might indicate a synergistic anti-\( Staphylococcus \) effect of PHMB and \( Pseudomonas \).

On the other hand, \( P. aeruginosa \) did survive even after 7 days of repeated PHMB treatment, albeit reduced by approximately 3.1 \( \log_{10} \) units compared to control without PHMB treatment.
These data indicate that the model exhibits resilience to antibiofilm treatment which is also experienced in the clinic. Some in vitro models show large log10-reduction of bacteria to an extent that does not correlate well with clinically experienced resilience of biofilm infections in chronic wounds. A recent review on the documentation of the antimicrobial effect of topical antimicrobials, including PHMB, documents that there is a tendency of markedly larger log reductions being reported from in vitro studies than from animal model studies and human trials. Thus, the layered CWB model data seem to offer more useful information on the possible clinical effect of antimicrobials on biofilms in infected chronic wounds. It is important to note that we do not recommend the use of the two-species novel layered CWB model beyond a biofilm age of 96 h for the evaluation of antimicrobial products.

### 3.4 Testing of wound dressings

We investigated the biofilm reduction of two silver- and one honey-containing wound dressings using the layered CWB model. These dressings were placed onto 48-h biofilm and their effects were evaluated after 48 h of treatment. The data are presented as log10 reductions in Table 2. It is evident that limited reductions in bacterial numbers were achieved on P. aeruginosa (approximately 1–2 log10 CFU), while S. aureus did not seem to be affected by the used products. Two products were wound dressings containing silver as antimicrobial agent, which has been reported to be less effective against S. aureus compared to P. aeruginosa. Notably, the dressings were not moistened upon application as they would be by exudate in a clinical situation. As a lack of water will deter diffusion of active compounds from the dressing towards the wound model matrix containing the bacteria, the observed antimicrobial effect might be underestimated in this experiment.

### DISCUSSION

#### 4.1 Further model development and refinement

#### 4.1.1 Wound bed temperature

Although numerous relevant factors such as microbial growth rates, diffusion rates of chemical substances like antimicrobial agents, and
the water solubility of gases such as oxygen are known to vary with temperature, the sensitivity of the in vitro models to incubation temperature has not been investigated. Only limited information on clinical wound bed temperatures is available, currently indicating wound bed temperatures in the range from 31 to 36°C.\textsuperscript{37} Collection of further clinical data of wound bed temperatures on a broader collection of wound types would be valuable in future efforts to mimic clinically relevant temperatures more closely in in vitro models.

4.1.2 | Model matrix

The idea behind the two-layer model has been an attempt to show the impact of different nutritional conditions on the bacteria. The complexity of the extracellular matrix and the tissue in the dermal layer and in the subcutaneous layer cannot be reproduced in this model as the model lacks the structure of the tissue and the cellular response to the invading microorganisms. Nevertheless, the differences in nutritional products expected in the dermal layer and subcutaneous layer have been the inspiration to the two-layer model. But not only the bacteria may behave differently due to the composition of the two-layer model, antimicrobial products as those tested in the present study may also exhibit differences in penetration, binding and inactivation.

Besides this lack of cells and tissue, the presented model uses plant-based agar to achieve a woundlike semi-solid structure, thereby introducing an obvious artefact compared to clinical wounds. Importantly, microscopy showed microbial microcolonies comparable to observations made on ex vivo specimens.\textsuperscript{9,38} Nevertheless, the use of matrix material of animal origin such as collagen\textsuperscript{23,39} might be warranted\textsuperscript{28} to minimize bias due to differences in interaction of the matrix with antimicrobial compounds.

4.1.3 | Investigations of the microbial metabolism by transcriptome analysis

As any reductionistic approach, an in vitro model of infected chronic wounds will only present the microorganisms to be part of the environment and context experienced by the bacteria in vivo.\textsuperscript{17} As bacteria have rather different response to antibiotics and other antimicrobials dependent on, for example, their mode of growth (biofilm vs. planktonic) and other factors in their microenvironment, it might be warranted to perform close investigations of the microbial metabolism and their transcriptome in vitro compared to clinical wound specimens. Such investigations would give us needed insights into how well (or poorly) in vitro models mimic important factors influencing microbial gene expression and metabolism, and consequently, microbial susceptibility to antimicrobial treatment. Furthermore, it might trigger further refinement of in vitro models to achieve closer resemblance between bacterial biofilm grown in the laboratory and existing in the wounds of patients.

5 | CONCLUSION

In conclusion, we here present a novel layered chronic wound biofilm model that sustains prolonged coexistence of \textit{P. aeruginosa} and \textit{S. aureus}. The model allows for direct testing of antimicrobial solutions and wound dressings on mature 48-h two-species biofilm for up to 48 h. The layered CWB model exhibits microbiological resilience against antimicrobial solutions and wound dressings, which might give industry and clinicians a better indication of the clinical antibiofilm performance of such treatments. The observed relatively low antibiofilm effect of antimicrobial dressings containing silver and honey in the new in vitro model seems to be in better accordance with the relatively low level of clinical evidence for superiority of antimicrobial dressings and solutions in the treatment of chronically infected wounds.\textsuperscript{13,15,40,41}

We wish to point out that the complexity of the model may be increased in the future to achieve even better resemblance between in vitro and in vivo. We believe that a stepwise development of a validated model is important and that the contribution of the two-layer model is a crucial step.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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