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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 $\log_{10}$-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 pathogen[5] together with *Staphylococcus aureus*, but the underlying mechanisms on the pathogenicity of wound biofilm are not well understood.[6] Furthermore, the

Chen and Lorenzen served as co-first authors.

presence of bacterial biofilms in wounds has been linked to the non-healing of chronic wounds.[7,8] In particular, *P. aeruginosa* and *S. aureus* are frequently found together in chronic infections[5,9,10] and their co-occurrence has been linked to increased virulence and worse patient outcome.[11] 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 scarce.[12–15] Development of novel and effective active compounds and dressings is hindered by lack of suitable in vitro models that reproduce antibiofilm effects as experienced in clinical practice.[16,17]

Industry standards, such as AATCC100[18] or ASTM E2799-12,[19] evaluate antimicrobial effect against planktonic bacteria and microbial biofilm attached to an abiotic surface, respectively. Such assays might be valuable at the early screening stages in the search for novel active compounds, but as they do not truly mimic an infected chronic wound, obtained data cannot be expected to translate well to clinical applications. Numerous researchers have developed more sophisticated closed, static models, such as the Lubbock chronic wound model,[20] a porcine explant model,[21] the semi-solid model,[22] and a collagen model,[23] giving the developers tools to assess and develop their candidate compounds or treatments. However, these models are single-species biofilm[21–23] or growing for only 24 h.[20] DeLeon et al. further developed the Lubbock chronic wound biofilm model by omission of solid surfaces for biofilm attachment.[24] Kucera et al. transferred modified Lubbock chronic wound biofilm to agar plates to allow for testing of dressings on multispecies biofilm.[25] Recent efforts have succeeded in establishing two-species biofilm models containing the two most prominent wound pathogens *P. aeruginosa* and *S. aureus*.[26] Unfortunately, this system seems not to be suitable for testing wound dressing with alleged antibiofilm effect as their two-species biofilm is dependent on the microenvironment established at the air–liquid interface on a semi-immersed cover slide. Oates et al.[27] succeeded in the development of a four-species basally perfused biofilm model, allowing testing of antiseptics and dressings in their set-up, while using monoacetate filters as growth support for the bacteria. A comprehensive review on in vitro models and their properties and limitations may be found in Thaarup et al.[28] and Brackman and Coenye.[6]

This study aims to contribute to the development of in vitro chronic wound biofilm models that may be useful in research and development of novel treatments with antibiofilm effect as requested by Thaarup et al. Our goal is to be able to gain data that are a more realistic reflection of the effect and such treatments can be expected to achieve in the clinic. Therefore, we wished to avoid

- biofilm formation on solid surfaces,
- growth conditions that only allow formation of single-species biofilm,
- unrealistically fast and easy eradication of biofilm.

As a result, we describe the development and performance of an in vitro biofilm model that

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,[9] 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.

2 | METHODS

2.1 | Bacteria

S. aureus DSM 110939[29] and *P. aeruginosa* PA14[30] 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) (Sigma) 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 temperated 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

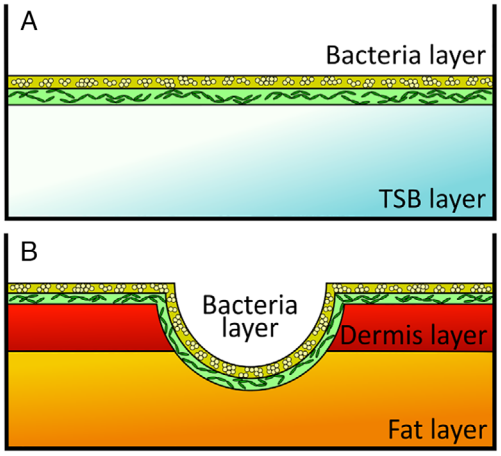

FIGURE 1 (A) The two-species biofilm with TSB medium. This model was composed by one layer with 25% TSB media and 0.5% agar. TSB agar medium containing *Pseudomonas aeruginosa* and *Staphylococcus aureus* was added on the top for bacteria layers. (B) The layered chronic wound biofilm model with two-layer, two-species biofilm. This chronic wound model was composed of two layers, each with different composition. The bottom layer (yellow) represented a subcutaneous fat tissue layer, and the top layer (red) represented a dermis tissue layer in human skin. Thin layers of agar using the fat tissue layer composition containing *P. aeruginosa* and *S. aureus* were added on the top for bacteria biofilm layers. TSB, tryptic soy broth

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 agar 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

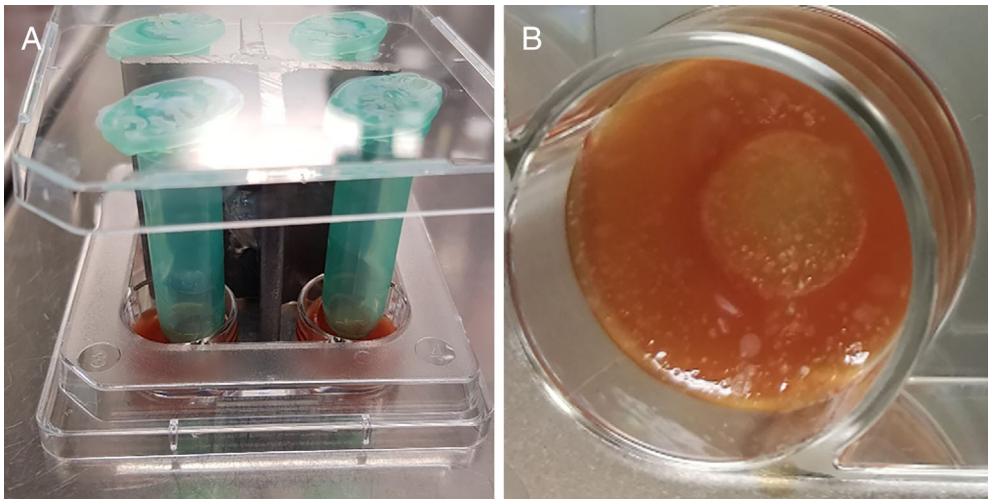

FIGURE 2 (A) The formation of the void in the layered chronic wound biofilm model. A lid, binding four 2-ml Eppendorf tubes with a holding rack, was used to make the void in the model. Both the lid and the holding rack were disinfected using 70% ethanol prior to use. (B) Close-up of the layered chronic wound biofilm model with a mimicked breach of the dermis in the centre exposing the surrogate subcutaneous fat layer below

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*-acetylglucosamines 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*.[31] 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 $\alpha = 5\%$.

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.[20,24,26]
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:

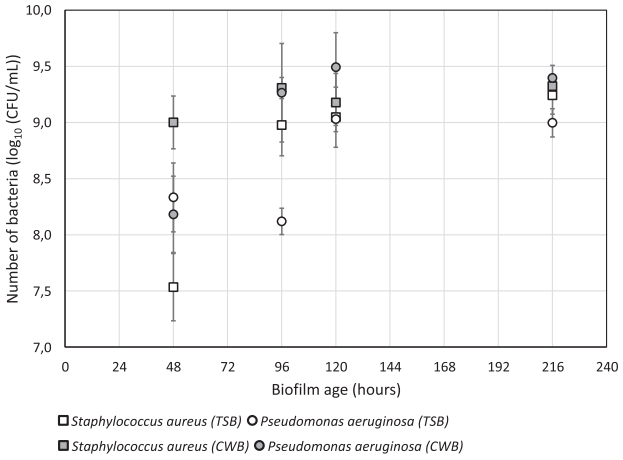

FIGURE 3 Single-species biofilm in the model with TSB medium and the novel layered CWB model. Comparable, high numbers of CFU were found for both bacteria in both models ($n = 8$, mean ± SD). Two independent operators analysed $n = 4$ each. □: *Staphylococcus aureus* in TSB model, ○: *Pseudomonas aeruginosa* in TSB model, ■: *S. aureus* in CWB model, ●: *P. aeruginosa* in CWB model. CFU, colony-forming unit; CWB, chronic wound biofilm; TSB, tryptic soy broth

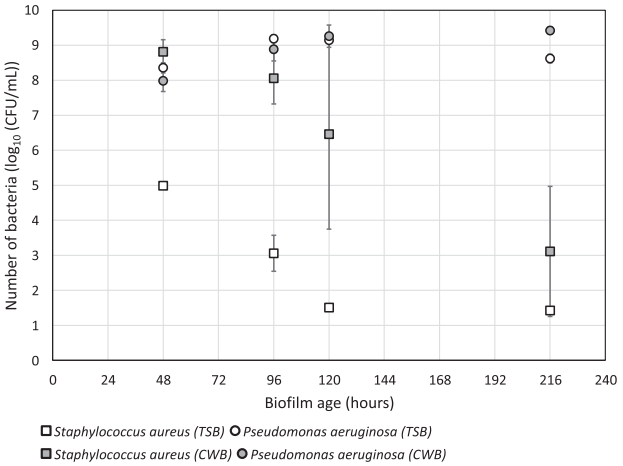

FIGURE 4 Two-species biofilm in the TSB model and the CWB model. *Pseudomonas aeruginosa* grew in numbers comparable to the single-species models. *Staphylococcus aureus*, on the other hand, did not seem to establish appreciable biofilm in the TSB model, whereas it was decimated in the presence of *P. aeruginosa* after 5 days in the layered CWB model after initial high numbers comparable to the single-species models (TSB model: $n = 8$; two operators analysed $n = 4$ each; CWB model: $n = 16$; one operator analysed $n = 4$, another operator analysed $n = 4$ in three independent experiments. Mean ± SD). □: *S. aureus* in TSB model, ○: *P. aeruginosa* in TSB model, ■: *S. aureus* in CWB model, ●: *P. aeruginosa* in CWB model. CWB, chronic wound biofilm; TSB, tryptic soy broth

a. The semi-solid biofilm approach using 0.5% agar was shown to allow for growth of non-surface-attached microcolonies similar to observations from clinical specimens.[22]
b. 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.[9]

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*.[24,32–34]

The layered woundlike growth medium contained laked horse blood, cattle serum, and peptone, which is similar to wound-simulating media used in several studies.[20,24,25] 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.[28] 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.[26] 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

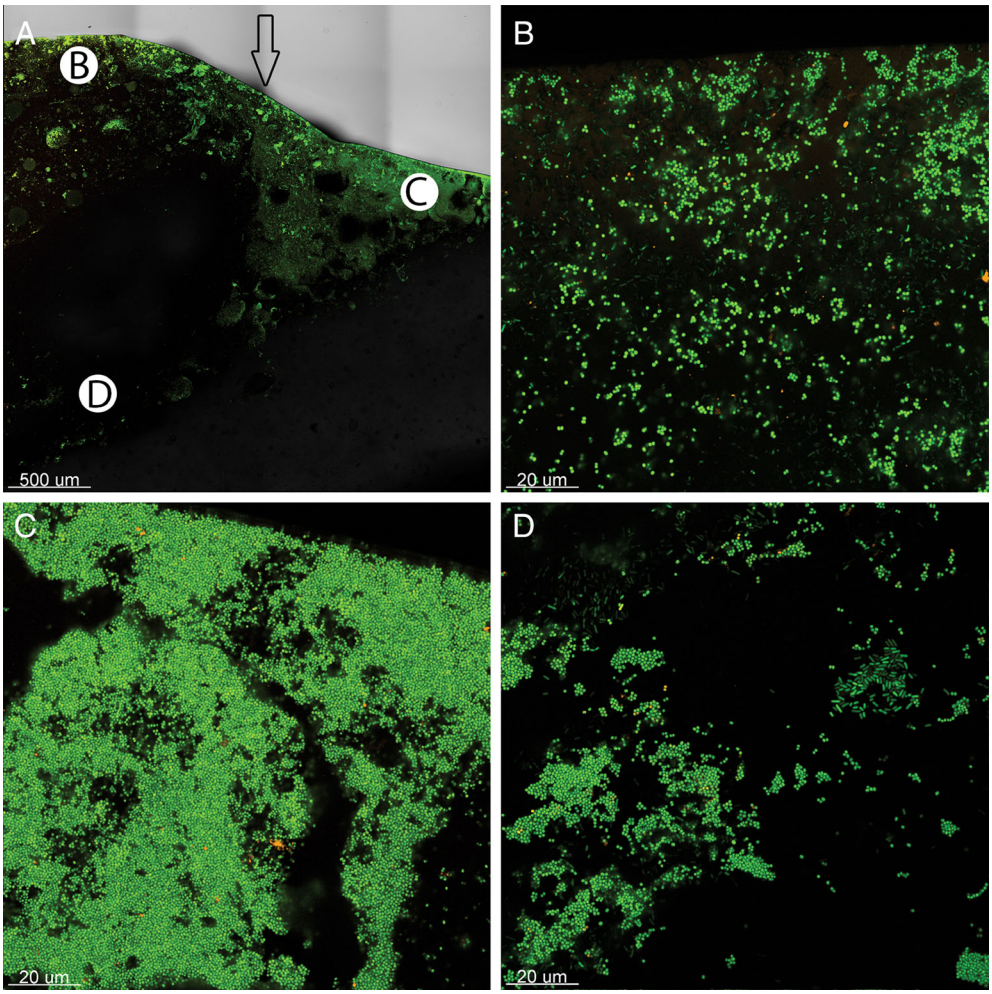

FIGURE 5 Images from day 2 (48-h biofilm) of the layered chronic wound biofilm model. (A) Overview of the model. Live bacteria were seen deeper into the model than 280 μm, which was the initially inoculated range. An arrow indicates where the void of the model begins, and letters denominate where the subsequent images are located. The majority of the bacteria found were alive (green). (B) Top 100 μm of the model. A mix of both *Staphylococcus aureus* (cocci) and *Pseudomonas aeruginosa* (rods) were found. (C) The top 100 μm in the void of the model. Here, large aggregates of *S. aureus* were seen. (D) 2000 μm deep into the model. Non-surface-attached microcolonies of *P. aeruginosa* (rods) and *S. aureus* (cocci) were found. A mix of Syto™9, Sytox™Orange Dead Cell Stain and WGA Alexa Fluor® 594 conjugate was used for staining of bacteria. Sytox™Orange was used to indicate permeable membranes meaning that cells which appear yellow or orange in colour have permeable membranes and are assumed to be dead or dying. WGA 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. WGA, wheat germ agglutinin

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 surface, 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".[31] The overall

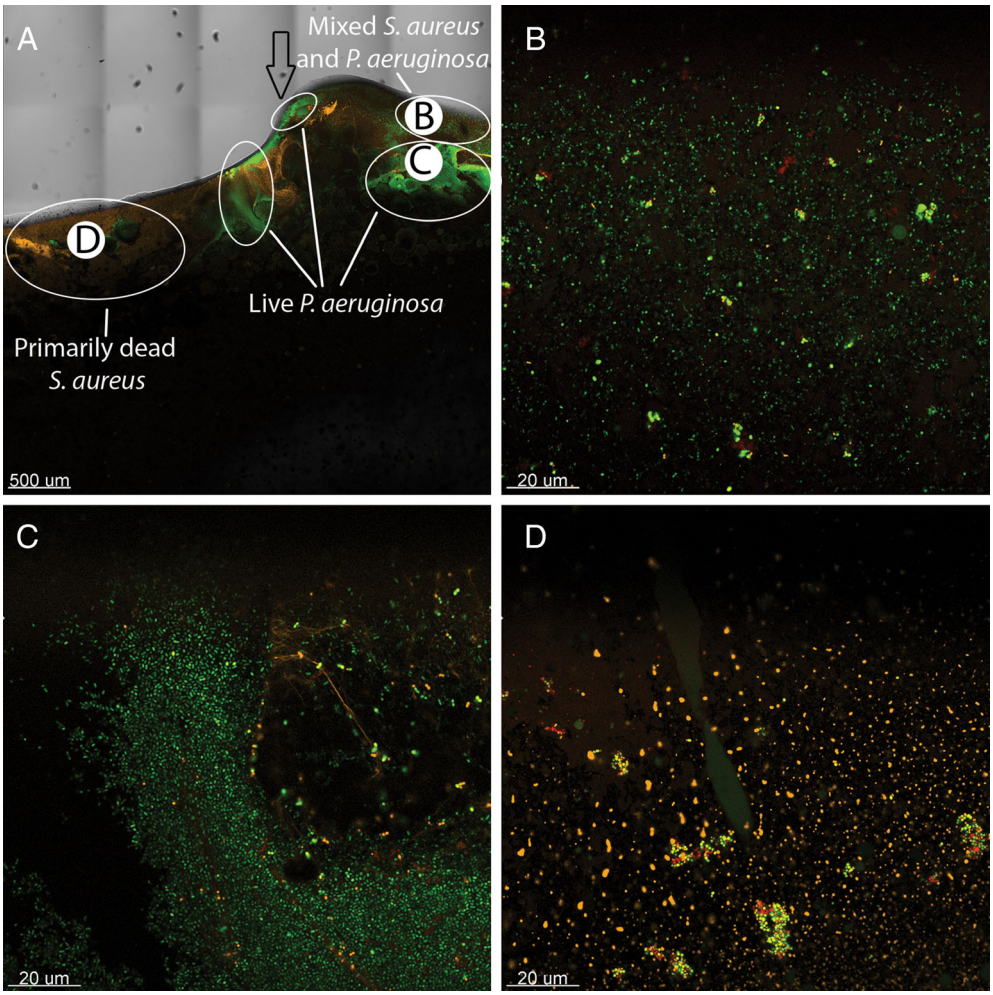

FIGURE 6 Images from day 5 (120-h biofilm) of the layered chronic wound biofilm model. (A) Overview of the model. A large variety of locations were zoomed into and general trends have been circled. Although difficult to see, live bacteria were still present up to 2000 μm deep into the model. An arrow indicates where the void of the model begins, and letters denominate where the subsequent images are located. (B) Top 100 μm of the model. A multitude of live *Pseudomonas aeruginosa* was seen together with a few clusters of live and dead *Staphylococcus aureus* (bright green/yellow cocci). (C) 400–500 μm into the model. Only *P. aeruginosa* was seen, whereof the majority were alive. (D) In the void of the model, about 100 μm deep. Large amounts of dead *S. aureus* were found, together with a few clusters of mixed live and dead *S. aureus*. A mix of Syto™9, Sytox™Orange Dead Cell Stain and WGA Alexa Fluor® 594 conjugate was used for staining of bacteria. Sytox™Orange was used to indicate permeable membranes meaning that cells that appear yellow or orange in colour have permeable membranes and are assumed to be dead or dying. WGA 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. WGA, wheat germ agglutinin

finding was that microscopy confirmed our data obtained by culturing, namely initial coexistence of microcolonies of both species changing to *S. aureus* dying off.

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.

TABLE 1 *Pseudomonas aeruginosa* and *Staphylococcus aureus* recovered from single- and two-species layered chronic wound biofilm model subjected to repeated daily 10 min

	Total biofilm age including 48 h prior to treatment (hours)	*P. aeruginosa*		*S. aureus*	
		log_{10} CFU/ml	log_{10} reduction	log_{10} CFU/ml	log_{10} reduction
Single species	96	7.1 (0.6)***	2.2 (0.7)	5.9 (0.2)***	3.4 (0.2)
	120	5.2 (0.4)***	4.3 (0.5)	1.4 (0)***	7.8 (0.3)
	216	5.3 (0.3)***	4.1 (0.3)	1.4 (0)***	7.9 (0.1)
Two species	96	6.2 (0.4)***	2.6 (0.5)	2.6 (1.3)***	5.5 (1.5)
	120	5.5 (0.6)***	3.7 (0.7)	1.4 (0)***	5.1 (2.7)
	216	6.3 (0.4)***	3.1 (0.4)	1.4 (0)***	1.7 (1.9)

Note: Treatment with rinsing solution containing PHMB for up to 7 days after initial 48 h to establish mature biofilm. log_{10}-reduction numbers calculated compared to untreated controls as shown in Figures 3 and 4. (Mean [SD], $n = 8$). Comparison of PHMB-treated biofilm to untreated controls of the CWB model as presented in Figures 3 and 4 using one-tailed *t*-tests resulted in $p < 0.001$ for all comparisons. ns, not significant ($p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Abbreviations: CWB, chronic wound biofilm; PHMB, polyhexamethylene biguanide.

TABLE 2 *Pseudomonas aeruginosa* and *Staphylococcus aureus* recovered from two-species layered chronic wound biofilm model

	P. aeruginosa		*S. aureus*	
Treatment	log_{10} CFU/ml	log_{10} reduction	log_{10} CFU/ml	log_{10} reduction
Untreated control	8.9 (0.3)		8.1 (0.7)	
Silver 1	7.0 (1.4)**	1.8 (1.4)	7.5 (0.7)*	0.5 (1.0)
Silver 2	7.9 (1.1)*	1.0 (1.2)	7.9 (0.2)^{ns}	0.1 (0.8)
Honey 1	8.0 (0.2)***	0.9 (0.4)	8.3 (0.1)^{ns}	−0.2 (0.7)

Note: Biofilms were allowed to establish for 48 h. At this time, wound dressings were placed onto the surface of the model for 48 h. After a total of 96 h, biofilms were harvested and CFUs enumerated. log_{10}reductions for the wound dressings were calculated relative to untreated control. Mean (SD), $n = 8$, two operators analysed $n = 4$ each. Comparison of models treated with dressings to untreated controls of the CWB model as presented in Figure 4 using one-tailed *t*-tests. ns, not significant ($p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Abbreviations: CFU, colony-forming unit; CWB, chronic wound biofilm model.

These data indicate that the model exhibits resilience to antibiofilm treatment which is also experienced in the clinic. Some in vitro models show large log_{10}-reduction of bacteria[35] 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,[15] 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 log_{10} reductions in Table 2. It is evident that limited reductions in bacterial numbers were achieved on *P. aeruginosa* (approximately 1–2 log_{10} 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*.[36] 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.

4 | 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.[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.[9,38] Nevertheless, the use of matrix material of animal origin such as collagen[23,39] might be warranted[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.[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 P. aeruginosa* and *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.[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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