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Coculture of *P. aeruginosa* and *S. aureus* on cell derived matrix - an *in vitro* model of biofilms in infected wounds

Zahra Gounani*[1,2], Didem Şen Karaman[3], Arun P. Venu[4], Fang Cheng[4, 5], Jessica. M. Rosenholm*[1]

1. Pharmaceutical Sciences Laboratory, Faculty of Science and Engineering, Åbo Akademi University, Turku 20520, Finland
2. Physics, Faculty of Science and Engineering, Åbo Akademi University, Turku 20500, Finland
3. Biomedical Engineering Department, Faculty of Engineering and Architecture, İzmir Katip Çelebi University, İzmir, Turkey
4. Cell Biology, Biosciences, Faculty of Science and Engineering, Åbo Akademi University, Turku 20520,
5. School of Pharmaceutical Sciences (Shenzhen), Sun Yat-sen University, Guangzhou, 510006, China

Corresponding authors:

Zahra Gounani

Current address:

Physics/Åbo Akademi University

Gadolinia (2nd floor)

Porthaninkatu 3, FI - 20500 Turku, Finland

E-mail: zahra.gounani@abo.fi

Jessica M. Rosenholm

Pharmacy/Åbo Akademi University

BioCity (3rd floor)

Tykistökatu 6A, FI - 20520 Turku, Finland

E-mail: jessica.rosenholm@abo.fi

Abstract

Polymicrobial biofilms are major complications of various chronic infections. Therefore, *in vitro* biorelevant polymicrobial biofilm models are essential tools for medical studies. This study presents a dual species biofilm of *Pseudomonas aeruginosa* and *Staphylococcus aureus* developed on cell-derived matrices (CDMs), in order to simulate the microenvironment of *in vivo* biofilms. *P. aeruginosa* and *S. aureus* are two of the most frequent pathogens in polymicrobial biofilms of wound infections. Although they are commonly isolated from polymicrobial biofilms, their interaction is antagonistic; and there is severe battle between them for nutrients and space. We introduced a nutritious formulation supporting co-cultures of *P. aeruginosa* and *S. aureus* in order to study the interaction of these gram-positive and gram-negative bacterial species. Quantitative analyses demonstrated that the enrichment of tryptic soy broth (TSB) with NaCl and glucose facilitate dual-species biofilm formation of *P. aeruginosa* and *S. aureus* when it is mixed with fetal bovine serum (FBS). Furthermore, the dual species biofilm was incubated on CDMs. Characterization of the model by fluorescent and electron microscopies revealed realistic features of chronic multi-species biofilms, including competitive distribution pattern of two bacterial species and small-colony variants (SCVs) morphology of *S. aureus.*

Keywords; Dual-species biofilm, Cell derived matrix, Biofilm model, *Pseudomonas aeruginosa*, *Staphylococcus aureus*.

1. Introduction

Wound management is a well-established clinical practice; however, chronic wounds remain one of the major health issues and count for significant morbidity and mortality all over the world. Chronic wounds include diabetic ulcers, pressure ulcers, venous leg ulcers and burn wounds, and these affect 6.5 million people only in the U.S annually (Han and Ceilley, 2017). It is estimated that chronic wound care cost 2-3% of the healthcare budget in developed countries, and wound healing accounts for a market exceeding US$27 billion (Frykberg and Banks, 2015; Han and Ceilley, 2017). Chronic wounds are formed when the wound healing process fails to progress orderly and stall in one phase, usually the inflammatory phase (Frykberg and Banks, 2015). It occurs as a result of molecular events such as excessive ROS production, increased stimulation of proteases, and secretion of inflammatory cytokines; all of which cause an extended inflammatory stage (McCarty and Percival, 2013). Microbial biofilms are also considered as one of the main causes of wound chronicity, although it is yet to be proved by the medical community (Sun et al., 2008). Biofilms are often polymicrobial, i.e. consisting of different species, such as *Staphylococcus*, *Pseudomonas, Enterococcus, Proteus* and several others (Lipsky and Hoey, 2009).

In vitro and *in vivo* models are essential elements of medical studies and drug development research (Sahlgren et al., 2017). *In vivo* models provide more realistic data, but has the disadvantages of being expensive and are subject to ethical dilemmas associated with animal research (Mathes et al., 2014). *Ex vivo* models provide close *in vivo* resemblance, but the variability of the tissues between different donors, availability limits, and complexity of the tissue restricts their applications (Mathes et al., 2014). Therefore, *in vitro* models are one of the key requirements to obtain predictive data before *in vivo* and clinical evaluation (Mathes et al., 2014). An appropriate *in vitro* biofilm model should offer *in vivo* relevant characteristics while being rapid, non-expensive and easy to set up (Kucera et al., 2014) There are previous

studies reporting on multispecies biofilm models, for example, Sun *et al*. developed an outstanding multispecies model form three different bacterial species of *Pseudomonas aeruginosa, Enterococcus faecalis, and Staphylococcus aureus* (Sun et al., 2008). In another study, the authors used a similar model to study the dynamics of population of each species during biofilm formation by selective culture and qPCR (Kucera et al., 2014). However, these studies developed multi-species biofilms on solid surfaces which are lack of *in vivo* features and microenvironment. Werthen *et al*. developed mono-species biofilm model on a matrix of collagen (Werthe et al., 2010). Although they avoided using solid surface to grow biofilms, their model did not include the complex features of polymicrobial biofilms.

In this study, a dual species biofilm of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was generated on cell-derived matrices (CDMs) as a major component of wound bed, to provide a more biorelevant biofilm model. CDMs are decellularized extracellular matrices (ECM) which are comprised of highly organized assemblies of macromolecules including collagen, fibronectin, laminin, glycosaminoglycans, proteoglycans and matricellular proteins (Fitzpatrick and McDevitt, 2015). ECM is the largest constituent of the skin layers and play a major role in the wound healing process (Magana et al., 2018). ECM create structural and functional integrity for the wound bed, and influence all physiological and structural interactions of wound components and biofilm residents (Zeng et al., 2015). CDMs have been recently developed and used successfully in designing cell cultural substrates and tissue engineered products (Fitzpatrick and McDevitt, 2015). Using CDMs as substrate for growing biofilms, we expected to recreate a more relevant microenvironment of *in vivo* biofilms.

P. aeruginosa and *S. aureus* both are opportunistic species and the most common residents of polymicrobial biofilms in chronic wounds (DeLeon et al., 2014). The co-presence of *P. aeruginosa* and *S. aureus* cause delayed and improper wound healing and trigger worse outcomes for patients (Hotterbeekx et al., 2017). *P. aeruginosa* and *S. aureus* are also the most

isolated bacterial species in cystic fibrosis (CF) patients (Laura M. Filkins et al., 2015). Despite the fact that they are common co-colonizing pathogens in most infections, *in vitro* cocultures of *P. aeruginosa* and *S. aureus* is problematic, as *P. aeruginosa* eradicate *S. aureus* already in the first hours of incubation (DeLeon et al., 2014). The killing effect of *P. aeruginosa* on *S. aureus* has been attributed to different extracellular factors secreted by *P. aeruginosa,* including anti-Staphylococcal 4-Hydroxy-2-Heptylquinoline N-Oxide (HQNO), Pyocyanin and LasA protease (Hotterbeekx et al., 2017). To develop a cost-effective and appropriate growth media in which co-cultured biofilm formation of *P. aeruginosa* and *S. aureus* is supported, we modified tryptic soy broth (TSB) with FBS, NaCl and glucose. The existence of dual species in the biomimetic biofilm model was characterized by different imaging methods. The presented method to provide dual species biofilm growth on CDMs can be ascribed as a pioneering effort to create phenotype of complex *in vivo* biofilms with the observable traits, including the biochemical and physiological properties.

2. Material and Methods

2.1. Growth kinetic of bacterial species in different broths

Pseudomonas aeruginosa (PAO1 DSM *19880)* and *Staphylococcus aureus* (ATCC 25923) were stored at -70°C and restored on tryptic soy agar plates (TSA, Sigma Aldrich Co., St. Louis, MO). Single colonies of each bacterial species from agar plates were grown in tryptic soy broth (TSB), Mueller Hinton broth (MHB) and Luria-Bertani (LB) for 20 h at 37 °C (in 50 ml falcon tubes containing 10 ml media, the caps of which were loosed for aeration). The day after, overnight culture was diluted (10: 10000) and allowed to grow for another 24 hours. Growth kinetic of both bacterial species were determined in different media, taking 100 µl of culture at 0, 2, 4, 8, and 24 h for colony forming units (CFU) numeration on the agar plates by dilution plating method. All experiments were carried out with two replicates. For each

replication 5-6 dilutions were cultured on corresponding agar plates. The total number of colonies (log 10 CFU ml^{-1}) was counted after 20 h of incubation at 37 °C.

2.2. Dual species biofilm formation

Dual species biofilm was grown in a medium made by mixing TSB and fatal bovine serum (FBS) (1: 1). TSB was used as it is or TSB supplemented with 1% glucose (TSBG), 2.5% NaCl (TSBN), 1% NaCl (TSBn), 1% glucose + 1% NaCl (TSBGn), 1% glucose + 2.5% NaCl (TSBGN). To prepare overnight culture, single colony of each bacteria was grown in TSB (10 ml) overnight (20 h) at 37 °C and 180 rpm. Then overnight culture was diluted (1000-fold) in TSB and incubated at 37°C and 180 rpm for 4 hours. Microtiter polystyrene 96-well assay plates (Thermo Scientific, Nunclon) and matching peg-lids (Thermo Scientific, Nunclon) were used to grow biofilm. Each well was filled up with 149 µl of TSB or enriched TSBs with NaCl and glucose as described earlier. Aliquots from 4 hours culture of *S. aureus* (10 µl) and *P. aeruginosa* (1 µl) was added to each well to a final volume of 160 µl/well. Final concentration of both *S. aureus* and *P. aeruginosa* in each well was ~10^7 CFU/ml. Non-inoculated media was used as negative control. All experiments were carried out in four replicates. Plate was then incubated at 37°C for 20 h. The day after, peg-lid was removed. Liquid culture containing planktonic bacteria was transferred to another plate for CFU counting. Both biofilms formed on pegs and plate wells were washed with PBS 3 times, to remove loosely attached bacteria. The biofilm in plate wells were dispersed in 160 µl/well fresh media before CFU counting. Peg-lid was transferred to new plate containing 160 µl/well fresh media (recovery plate). To retrieve bacterial cell of biofilm to liquid media, first grown biofilm around pegs was gently scrapped using sterile pipette tip and then peg-lid with recovery plate was sonicated for 3 minutes. To enumerate CFU, 100 µl aliquots of each well was taken to a 10 fold serial dilution and 3×10 µl of five last dilution was plated on MacConkey agar (MCK) plates, as selective media for *P. aeruginosa* and mannitol salt agar (MSA) plates, as selective media for *S. aureus.*

MCK and MSA plates were incubated at 37°C for 24 h and 48 h, respectively. After completion of incubation time, colonies were counted and log10 CFU/ml values were plotted versus time.

2.3. Developing cell derived matrices (CDMs):

CDMs was prepared based on a method reported before with minor modification(Kaukonen et al., 2017). Glass cover slips (diameter, 11 mm) were placed in 24 well plates after sterilization with 70% ethanol. Sterile solution of gelatin (0.2% w/v in sterile PBS) was pre-warmed at 37 °C and added to each well (800 µl) and incubated for 1 h at 37 °C or 24 h at 4 °C. Then gelatin solution was aspirated, and cover slips were washed with PBS gently. Gelatin coating of cover slip then was crosslinked using aqueous solution of glutaraldehyde (1% v/v, 500 µl) for 30 min at room temperature (RT). The crosslinking solution was then removed, and cover slips were washed with PBS two times. Remaining glutaraldehyde were quenched by 500 µl glycine solution (in PBS, 1 M) for 20 min at RT. After removing glycine solution, coverslips were washed with PBS and incubated in pre-warmed Dulbecco's modified eagle's medium (DMEM) media (800 µl) at 37 °C for 1 hour. Then fibroblast cells were cultured on cover slips (5×10^4 per well) at 37 °C. The cells were allowed to grow until they formed a confluent monolayer. Then, growth media was removed and replaced with supplemented DMEM media with 50 µg ml^{-1} ascorbic acid. Ascorbic acid stock was prepared freshly (by dissolving ascorbic acid powder in growth media) and was filtered using a 0.45-µm filter. The ascorbic acid treatment was done every other day and continued for 14 to 21 days. After completion of ascorbic acid treatment, the media was removed, and cells were washed by PBS. Fibroblast cells were then denuded by an extraction solution (500 µl per well), made by mixing 1 ml of NH_4OH, 250 µl of Triton X-100 and 48.75 ml of PBS. The extraction solution was pre-warmed before using and incubated with cells about 2 minutes, until cells were all detached. Then the extraction solution was removed gently from the wells and cell were washed with PBS twice. The final step of CDMs preparation, is removing cellular DNA by DNase I. A solution of DNase I was

prepared in deionized water (10 µg ml $^{-1}$) and was added to each well (500 µl). The plate was incubated at 37 °C for 30 min. Finally, DNase solution was removed and CDMs were washed with PBS twice. CDMs were stored in PBS buffer containing 1% (vol/vol) penicillin–streptomycin at 4 °C until further use (up to 4 weeks).

2.4. Dual species biofilm formation on CDMs

To develop dual species biofilm on CDMs, enriched TSB with 1% glucose + 2.5% NaCl (TSBGN) mixed with FBS (50: 50) was used. CDMs were placed in 24-well plates and were washed with PBS two times. 800 µl of mixed media inoculated with 10^7 and 10^6 CFU/ml of *S. aureus* and *P. aeruginosa* was added to each well. Wells without CDMs filled with inoculated media and non-inoculated were used as positive and negative control. The plates were then incubated at 37 °C for 20 h before CFU enumeration. The experiments were performed in four replicates. After 20 h, planktonic growth removed from the wells and biofilm was washed by PBS three times to remove loose bacteria. Then Biofilm was dispersed in media The CFU number of biofilms on CDMs were determined by culturing serially diluted of bacterial biofilm on selective media of MCK and MSA for *P. aeruginosa* and *S. aureus,* respectively.

2.5. Fluorescent staining of dual-species biofilm on CDMs:

Live/dead viability staining assay was employed to visualize live and dead cells in dual-species biofilm on surface of CDMs. Grown biofilm on surface of CDMs was washed with PBS three times. A mixture of syto 9 (2 µgml^{-1}) and propidium iodide (10 µgml^{-1}) in PBS (500 µl) was added to each sample and incubated for 30 minutes in RT before imaging. Images were obtained using Zeiss LSM780 confocal laser microscopy (Jena, Germany), by Plan-Apochromat 63x/1.40 Oil immersion objective, and excitation with 488 nm and 543 nm lasers.

2.6. Immunofluorescence staining of CDMs:

Extracellular matrix are usually visualized by immunofluorescence staining of their fibrillary proteins(Kaukonen et al., 2017). Here, collagen and fibronectin organizations were immunostained to visualize CDMs. CDMs were fixed in formalin 10% (500 µl) for 15 min at room temperature. Then fixative was removed and CDMs were washed with PBS twice. Horse serum 30% (v/v in PBS) was added to CDMs as blocking solution and left for 15 min at room temperature. Horse serum was replaced with primary antibody solution (10 µg ml^{-1} in horse serum 30%) and incubated for 1 h at room temperature. We used collagen (rabbit anti-collagen I, Novus NB600-408) and fibronectin (rabbit anti-fibronectin, Sigma Aldrich F3648) antibodies. Then primary antibodies were replaced with secondary antibody, Alexa Fluor 488 (green, donkey anti-rabbit), and incubated for another 1 hour at room temperature. Then, secondary antibody solution was removed and CDMs were washed with PBS three times and with deionized water once. For CDM covered by biofilm, after removing secondary antibody a solution of propidium iodide (PI) 10 µl/ml was added to each well and plate was incubated for 30 minutes at room temperature before washing with PBS and deionized water. The excess water was drained by touching a piece of filter paper. Finally, CDMs were mounted on glass slide by Mowiol (7 µl) and were let dry for 24 hours before imaging. Slides were imaged by a Zeiss LSM780 (Jena, Germany) using a Plan-Apochromat 63x/1.40 Oil immersion objective, and excitation with 488 nm and 543 nm lasers.

2.7. Scanning electron microscopy (SEM)

SEM analysis was performed to study the surface morphology of CDMs and dual-species biofilm on the surface of CDMs. CDMs with or without biofilm were washed 5 times with PBS very gently. Then all samples were fixed with formalin 10% for 15 min at room temperature. After removing formalin, CDMs were washed with PBS three times and with deionized water

once and left to dry overnight. Dried samples were mounted on sample holders, and sputter coated with carbon. Scanning electron microscopy (SEM) was performed with a LEO Gemini 1530® (Zeiss, Oberkochen, Germany) with a Thermo Scientific UltraDry Silicon Drift Detector (SDD).

3. Results and Discussion

Our goal with this study was to develop an *in vivo* relevant model of wound biofilms by growing dual-species biofilm on cell derived matrixes (CDMs). Our criteria for the model were as follows: 1) developing a dual-species biofilm model involving both gram-negative and gram-positive bacteria; 2) growing the biofilm in a growth media made of inexpensive and readily available materials in bacterial cell labs; 3) using CDMs as substrate for growing biofilms to provide an *in vivo* like microenvironment for biofilms.

3.1. Growth kinetics of bacterial species in different broths

We first followed the growth profile of each bacteria in different common bacterial growth media (Fig 1). Growth curve of bacteria was obtained by CFU counting at different time points as shown in Fig. 1. In all three media, *P. aeruginosa* showed higher growth rate. Then, a wide range of media mixtures were screened to grow dual species biofilms; however, they all failed in growing *S. aureus* in the presence of *P. aeruginosa* (data not shown). *P. aeruginosa* tend to outcompete or kill *S.aureus* in *in vitro* cocultures (Filkins *et al,* 2015; Hotterbeekx *et al,* 2017).

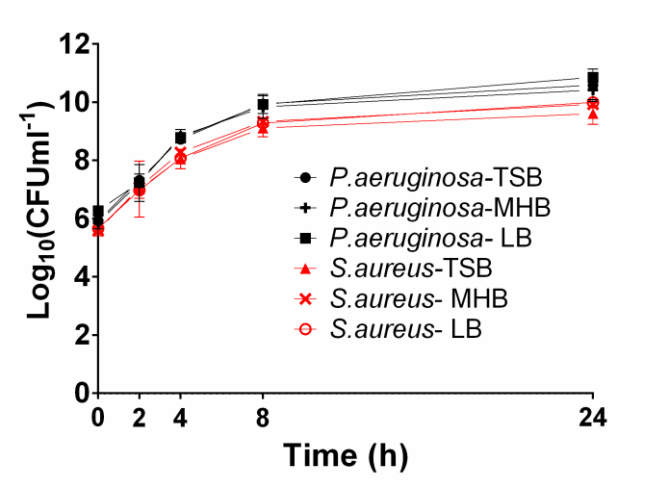

Figure 1: Growth kinetic of *P. aeruginosa* and *S. aureus* in three different media. Results revealed that growth rate of *P. aeruginosa* was slightly higher than *S. aureus* at the 24 h time point, while their growth rates were almost the same in other time points. Error bars indicate standard deviation (SD) from the mean of the 2 biological replicates.

3.2 Media formulation for co-growth of *P. aeruginosa* and *S. aureus*

We finally chose TSB, as it is supporting growing media for *S. aureus*, and mixed it with FBS (1:1)(Laura M Filkins et al., 2015), as we assumed that having some component from wound area may support growth of dual species biofilms (Sun *et al,* 2008). We also enriched TSB with NaCl and glucose to increase the chance of *S. aureus* to survive in the presence of *P. aeruginosa* (Møretrø et al., 2003; Waldrop et al., 2014). Finally, A 1:1 mixture of FBS and TSB or TSB supplemented with 1% glucose, 2.5% NaCl, 1% NaCl, 1% glucose + 1% NaCl, 1% glucose + 2.5% NaCl were examined as growing media for dual species biofilm. Since biofilm assays are often carried out in microtiter plates, either on the surface of plate wells or using peg-lids; both biofilms formed on peg-lids and in plate wells were taken for CFU enumeration.

P. aeruginosa and *S. aureus* were inoculated in a volume ratio of 10:1, which lead to an equal or higher CFU of *S. aureus* than *P. aeruginosa* in inoculation. We inoculated growth media with a 10-time higher volume of *S. aureus* than *P. aeruginosa*, since CFU counting experiments showed that *P. aeruginosa* grows about 1 log of CFU higher than *S. aureus* in both overnight

and 4 hours cultures. Co-culturing of two species in different enriched TSB indicated (Fig. 2) that *P. aeruginosa* forms biofilms in both plate wells and peg-lids in all five media formulations. In contrast, biofilm formation of *S. aureus* was not observed in TSB+FBS unless with addition of NaCl and glucose, which increased biofilm formation of *S. aureus* in both concentrations of 1% and 2.5%. TSB supplemented with 2.5% NaCl and glucose 1% mixed with FBS produced maximum biofilm growth of *S. aureus* (Fig. 2). The results are in agreement with previous studies, suggesting that the addition of NaCl and glucose to growth media promote biofilm formation by *S. aureus* (Waldrop *et al,* 2014; Lee *et al,* 2014). Glucose and NaCl increase biofilm formation of *S. aureus* by inducing expression levels of *icaA* gene (Lee *et al,* 2014; Møretrø *et al,* 2003; Rode *et al,* 2007). *IcaA* gene is strongly correlated to biofilm formation in staphylococcus species. *IcaA* encodes protein that cooperate in the synthesis of poly N-acetyl glucosamine, which mediates intercellular adhesion and accumulation of bacterial cells on the solid surfaces (Lin et al., 2015). There are reports of the negative effect of NaCl on *P. aeruginosa* growth and biofilm formation (Michon et al., 2014); however, we did not observe significant reduction of biofilm formation by *P. aeruginosa* in different media containing 0 to 2.5% NaCl. We also considered planktonic growth profile of each bacteria to have a full view of what takes place in each well. Planktonic growth of both species occurred in all tested media including simple TSB + FBS. Previous studies demonstrated that *P. aeruginosa* PAO1 can inhibit and disperse *S. aureus* biofilm through protease activity, while it has no bactericidal effect on its planktonic cell growth (Park et al., 2012). The reason why *S. aureus* survive as planktonic in TSB+FBS and not in biofilm could also be explained by the fact that biofilm-resident cells have limited access to nutrition and/or oxygen, and thereby grow more slowly than planktonic cells (Donlan, 2001). It might make *S. aureus* biofilm cells more susceptible to *P. aeruginosa*. The higher inoculation volume of *S. aureus* than *P. aeruginosa* seemed to be necessary for *S. aureus* to survive in dual-species biofilm, which indicate that the

CFU number of *S. aureus* must be equal or more than *P. aeruginosa* for growing a dual species biofilm. *S. aureus* killing by *P. aeruginosa* is a multifactorial phenomenon including nutritional, genetic and environmental parameters, (Laura M. Filkins et al., 2015; Orazi and O'Toole, 2017), thereby comprehensive studies are needed to elucidate interaction of these two important species.

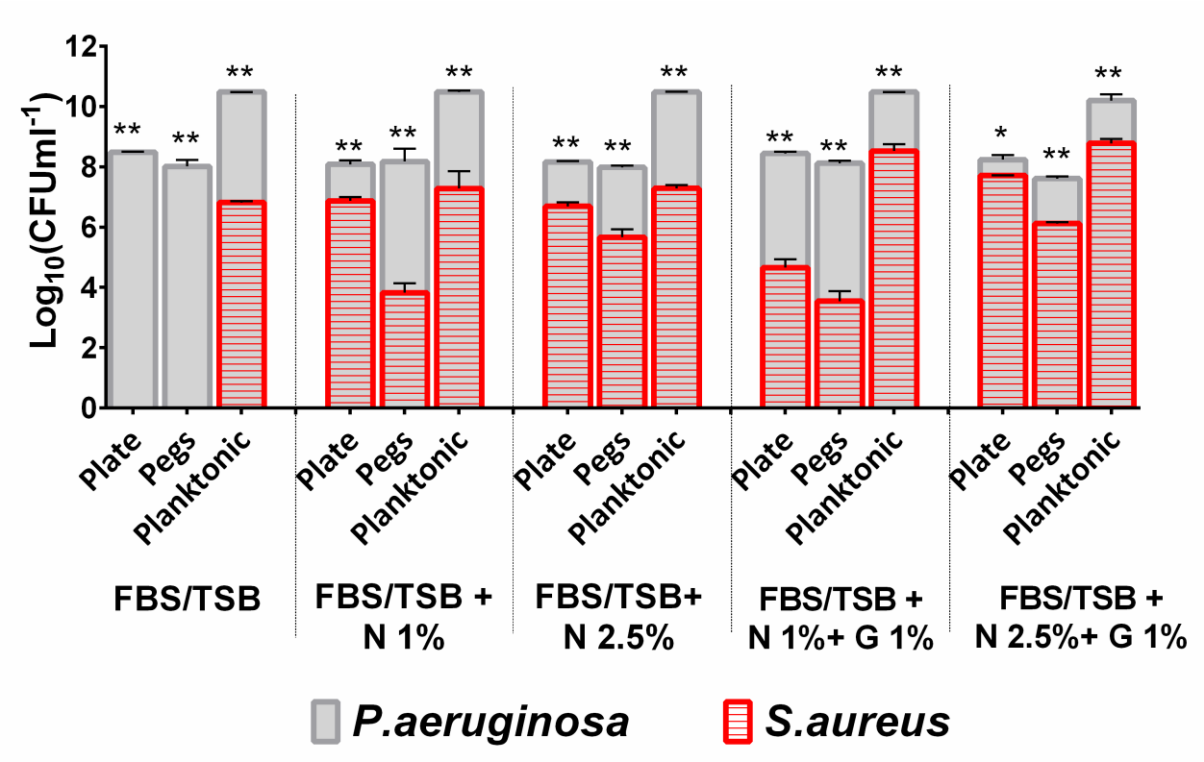

Figure 2: CFU counts of *P. aeruginosa* and *S. aureus,* co-cultured in FBS/TSB and FBS and TSB supplemented with NaCl and glucose in different concentration after incubation for 24 h at 37 °C. The concentration ratio of TSB/FBS for all growth media formulations was 50: 50. Error bars indicate standard deviation (SD) from the mean of the 4 replicates. Two-way ANOVA and Tukey's multiple comparison tests were applied to analyze significance levels,

* ($P<0.01$) and ** ($P<0.001$) indicate significant difference between *P. aeruginosa* and *S. aureus* CFU numbers.

3.3 Dual-species biofilm on CDM

Cell source is one of the main determinants of the composition of CDMs (Fitzpatrick and McDevitt, 2015). Different cell lines have been used to produce CDMs including fibroblasts, chondrocytes and mesenchymal stem cells (Lu et al., 2011). Fibroblast cells are major cell types involved in deposition of ECM and have a crucial role in the wound healing process (Tracy et al., 2016). Therefore, we used human dermal fibroblasts (HDF) cells as source cell to obtain a more relevant wound bed.

Dual-species biofilm was generated on CDMs in 24 well plates using FBS + TSB supplemented with 2.5%NaCl and 1% glucose (Fig. 3). Dual-species biofilm grown in wells without CDMs was used as control. Results did not reveal significant difference between growth rate of two bacterial species on surface of both CDMs and plates well (Fig. 3). The quantity of bacterial species co-cultured on CDMs after 24 hours was 1.6×10^9 and 2.9×10^9 CFU/ml for *P. aeruginosa* and *S. aureus*, respectively (Fig. 3).

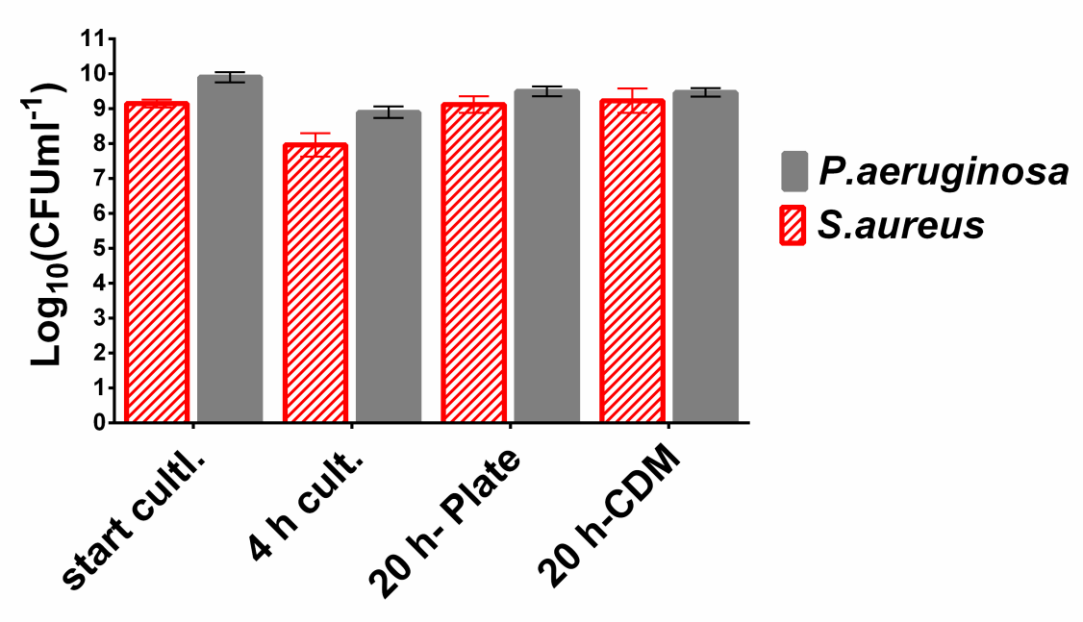

Figure 3. *P. aeruginosa* and *S. aureus* biofilm and planktonic populations. Start culture and 4 hours culture of both species was done in TSB media at 37°C and 180 rpm. Dual species biofilms were grown in TSB enriched mixed with FBS (50:50) at 37°C. No significant difference between *P. aeruginosa* and *S. aureus* CFU counts was observed. No significant different of biofilm formation on CDM in compare to plate surface was obtained. Error bars indicate standard deviation (SD) from the mean of the 4 replicates. Two-way ANOVA and Tukey's multiple comparison tests were applied to analyze significance levels.

3.3 Characterization of the biofilm model

To visualize growth of bacterial species on the surface of CDMs, we examined biofilm with live/dead assay (Fig. 4). The images showed both rod-shape *P. aeruginosa* and spherical-shape *S. aureus* in the biofilm. The green fluorescence color, (indicating alive cells) was more frequently observed for both species' cells than red fluorescence (indicating dead cells). As presented in Fig. 5, CDMs organization and morphology was analyzed by immunofluorescence staining. The fibrillary morphology of collagen type I and fibronectin can be seen in both images of CDMs without and with biofilm. The thickness of the dual-species biofilm, formed on CDMs, was about 6 µm, measured by Z-stack (Fig 6). The Z-stack planes revealed that *P.*

aeruginosa were more abundant in deeper layers of the biofilm and they could be seen inside the CDMs texture, while *S. aureus* cells were found more in the surface layers of the biofilm. This is in agreement with what have been described for wound samples from patients with non-healing diabetic ulcers in previous studies (Fazli *et al,* 2009; Peters *et al,* 2012). Although *P. aeruginosa* and *S. aureus* are two of the most frequent inhabitants of chronic wound biofilms, *P. aeruginosa* cells mostly distribute inside the wound bed while *S. aureus* cells are more located within the wound surface (Kirketerp-Møller et al., 2008; Mulcahy et al., 2015). This specific pattern of distribution could be attributed to several parameters. *P. aeruginosa* produce virulence factors which could disable activity of polymorphonuclear neutrophils, thereby helping *P. aeruginosa* to escape from killing by the immune system and penetrate into deeper layers of the wound bed (Kirketerp-Møller et al., 2008). On the other hand, *P. aeruginosa* has polar type IV pili which make it able to migrate to deeper layers of the wound, where access to nutrition and space might be less competitive (Fazli et al., 2009).

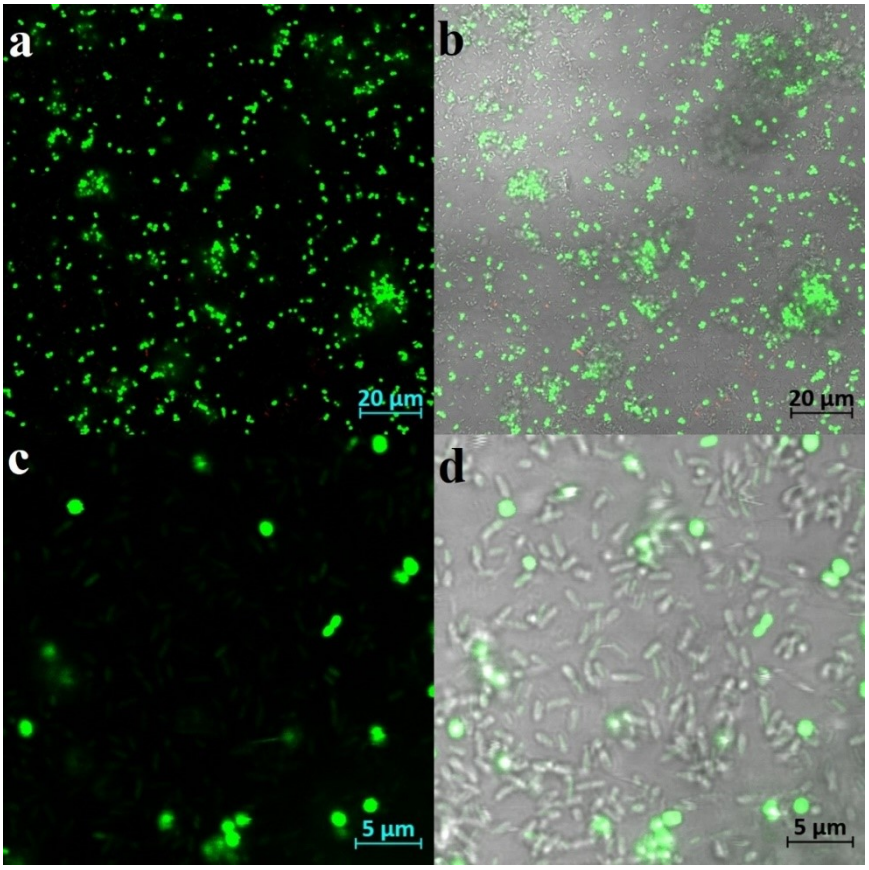

Figure 4: Fluorescence (a) and merged bright field (b) images of dual-species biofilm. Spherical *S. aureus* and rod-shaped *P. aeruginosa* of the surface of CDMs. Samples were stained with syto 9 as a membrane-permeant DNA-binding stain which indicate live cells as green and Propidium iodide as a red-fluorescent and nucleic acid-binding stain which penetrate damaged membrane and indicate dead cells. Images c and d show higher magnifications of a and b, respectively.

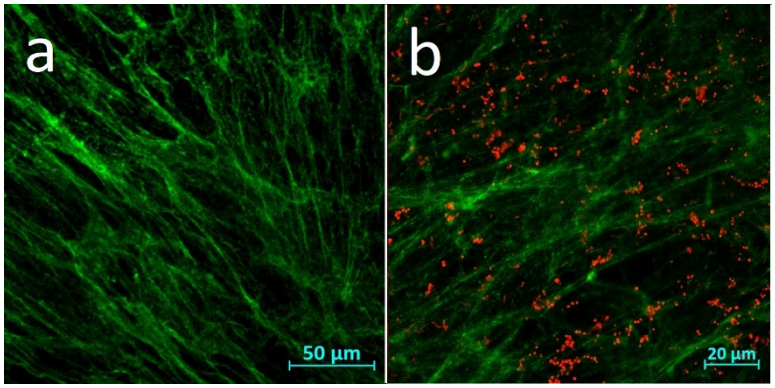

Figure 5: CDMs visualized by immunostaining of collagen I and fibronectins labeled in green by Alexa Fluor 488 (a). Covered CDMs by dual-species biofilm (b), CDMs is visualized by immunostaining of both collagen I and fibronectins labeled in green by Alexa Fluor 488 and biofilm residents were visualized by propidium iodide (red dots).

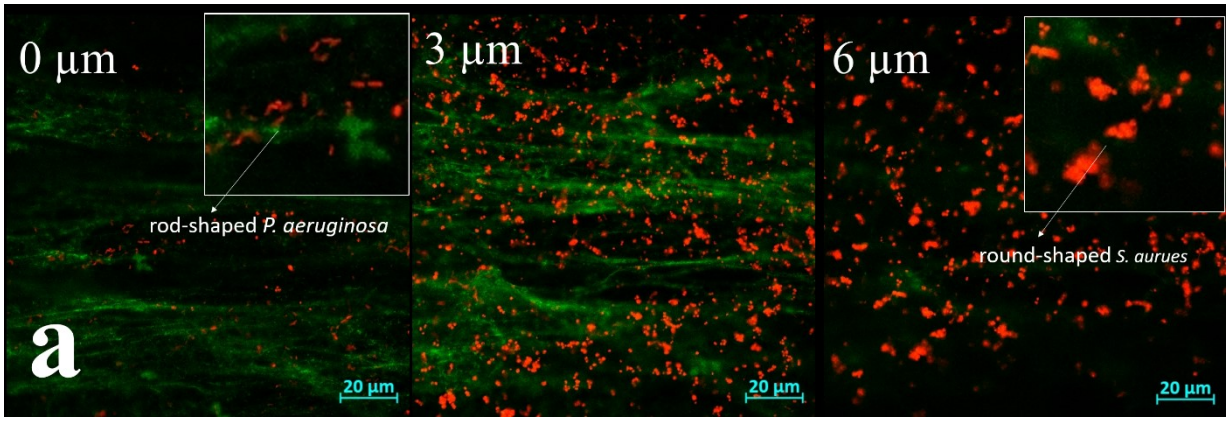

Figure 6: Selected pictures of z-stack planes of dual species biofilm on surface of CDMs. *P. aeruginosa* cells are more frequent in the bottom layer of biofilms, while *S. aureus* cells were seen more in surface layer of biofilm.

SEM images indicate a rough and fibrillary morphology for the surface of CDMs, in agreement with previous studies (Kaukonen *et al,* 2017) (Fig. 7 a). Rod-shaped *P. aeruginosa* with a length of 1-5 µm and width of 0.5-1 µm (Paterson and Kim, 2009) and spherical *S. aureus* with a diameter of 0.5-1 µm (Harris et al., 2002) were distinguishable in the SEM images (Fig. 7 b and c).

S. aureus formed discrete compact colonies with 5-15 µm diameter (analyzed using ImageJ image processing program, 1.47 v, National institutes of Health), while *P. aeruginosa* colonies were more spread over the biofilm (Figure 7 b and C). The inter-species interactions in the biofilm does not limit to inhibition or killing effects, but it also includes the impact on distribution pattern of bacterial species. *P. aeruginosa* induce *S. aureus* biofilm dispersal to compact colonies by making the environment less favorable for *S. aureus*. In the presented dual-species biofilm, S. *aureus* colonies also showed some of the properties of small colony variants (SCVs), including small and smooth colonies on mannitol salt agars and slow growing (data are not shown) (Hotterbeekx *et al,* 2017). Heterogeneous size of colonies and branched and multiple cross walls for *S. aureus* cells were observed in SEM images (Kah et al., 2003; Lin et al., 2016). One possible explanation for appearance of SCVs, may be due to anti-staphylococcal exoproduct of *P. aeruginosa*, HQNO and pyocyanin, which reduce growth of *S. aureus* by inhibition of oxidative respiration of bacteria (Hoffman et al., 2006; Hotterbeekx et al., 2017). SCVs are a survival mechanism of *S. aureus* that help it to escape from being killed by *P. aeruginosa*. SCVs are recovered from chronic infection of soft tissues like skin, bronchitis and mostly form cystic fibrosis (CF) chronic infection (Kriegeskorte et al., 2015). SCVs are very challenging for treatment, since they are very difficult to be detected by routine microbiological methods, poorly sensitive to specific antibiotics and capable to acquire classical mechanisms of resistance to antibiotics, as normal *S.aureus* cells (Garcia et al., 2013). Therefore, a reproducible biofilm model representing these abnormal phenotypes will be a useful tool to study their properties and their treatments.

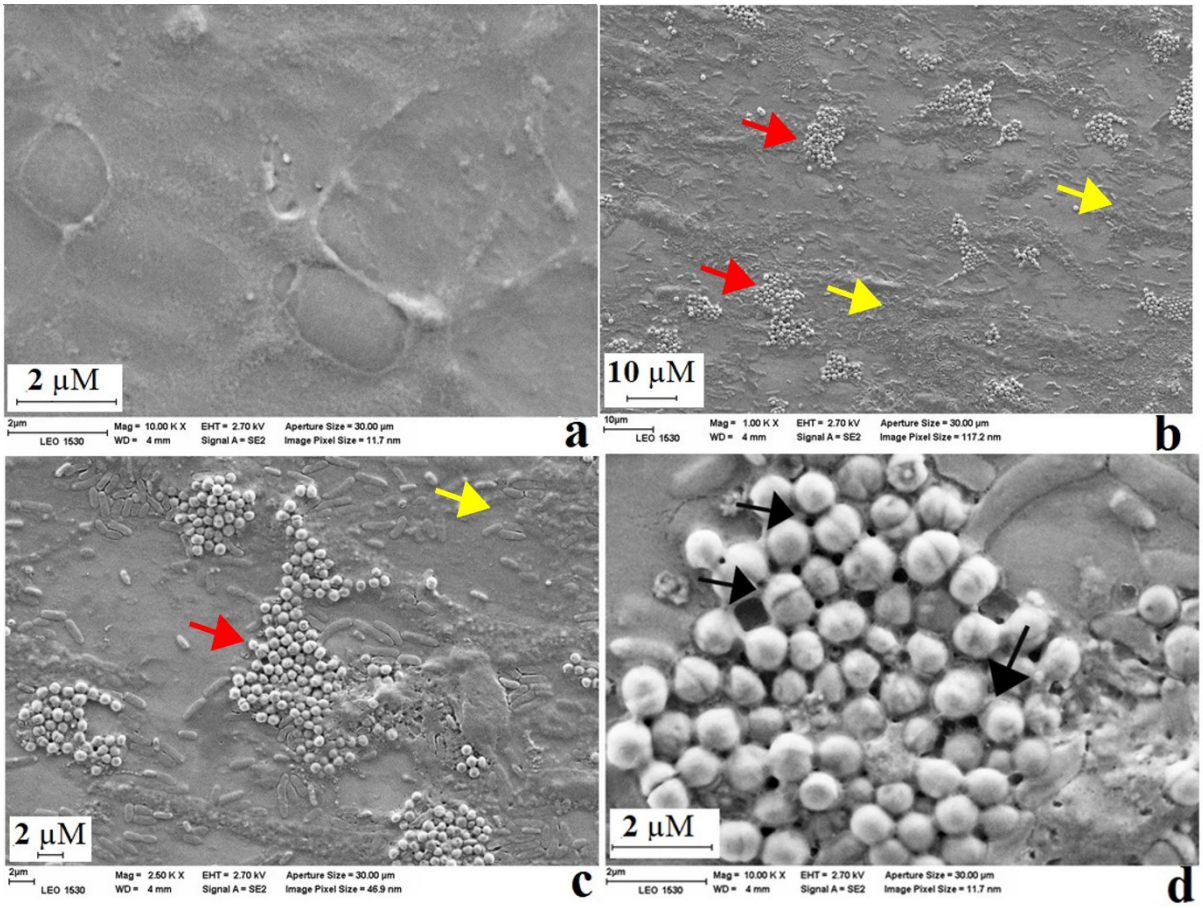

Figure 7: Scanning electron images of surface of CDMs (a) and the biomimetic dual species biofilm model (b and c). Red arrows show *S. aureus* and yellow arrows shows *P. aeruginosa*. The images show the rough surface of CDMs and complex structure of dual-species biofilm including SCV colonies. Arrows in image d indicate intercellular sticky substance among cells and irregular cross cell walls present in SCVs as it was described by Lin et al. (Lin et al., 2016).

4. Conclusion

In summary, we successfully established a dual-species biofilm on CDMs as an *in vitro* model of wound infections. Using CDMs as a substrate for growing a dual species biofilm, the model showed similar distribution patterns of two species in a chronic wound biofilm. The complex distribution pattern of *P. aeruginosa* and *S. aureus* could compromise successful treatment of biofilms existing in wounds, cystic fibrosis, as well as dental and surgical implant infections. In these cases, high-throughput *in vitro* models could be practical alternatives to study the

interaction of biofilm residents. The obtained three-dimensional model has the potential to be employed for high-throughput screening of new antibacterial compounds and wound dressings. The model could further be useful in understanding the dynamic interaction of bacterial cells and biofilms with ECM components, which can provide valuable contribution to wound healing studies.

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Conflicts of interest

The authors declare no competing financial interest.

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