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In vivo model of *Propionibacterium* (*Cutibacterium*) spp. biofilm *in Drosophila melanogaster*

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ABSTRACT

Objectives: Acne vulgaris is a common inflammatory disorder of the pilosebaceous unit and *Propionibacterium acnes* biofilm-forming ability is believed to be a contributing factor to the disease development. *In vivo* models mimicking hair follicle environment are lacking. The aim of this study was to develop an *in vivo Propionibacterium* spp. biofilm model in *Drosophila melanogaster* (fruit fly).
Methods: We created a sterile line of *D. melanogaster* able to sustain *Propionibacterium* spp. biofilms in the gut. In order to mimic the lipid-rich, anaerobic environment of the hair follicle, fruit flies were maintained on lipid-rich diet. *Propionibacterium* spp. biofilms were visualized by immunofluorescence and scanning electron microscopy. We further tested if the biofilm-dispersal activity of DNase I can be demonstrated in the developed model.
Results: We have demonstrated the feasibility of our *in vivo* model for development and study of *P. acnes*, *P. granulosum* and *P. avidum* biofilms. The model is suitable to evaluate dispersal as well as other agents against *P. acnes* biofilm.
Conclusions: We report a novel *in vivo* model for studying *Propionibacterium* spp. biofilms. The model can be suitable for both mechanistic as well as interventional studies.

1. Introduction

Microorganisms in their natural environment exist in a biofilm organization, a complex 3D-structure which is defined as surface attached bacterial aggregates embedded in an extracellular matrix [1,2]. The matrix is composed of hydrated extracellular polymeric substances (polysaccharides, proteins, nucleic acids and lipids) [1,2]. The biofilm formation is associated with clear benefits for microorganisms such as multicellular interactions, horizontal gene transfers, as well as physical and chemical protections from various environmental stresses including antimicrobial compounds [1–5].

Acne vulgaris is the most common skin disease worldwide affecting more than 80% of adolescents and young adults [6] with the estimated global prevalence of 231 million patients in 2019 [7]. Several factors are believed to be pivotal in the pathophysiology of acne including the disbalance of skin microbiome [8]. *Propionibacterium acnes* is a dominant skin commensal and considered to be an important factor in the pathogenesis of acne [9]. A recent taxonomic reclassification proposed to rename *Propionibacterium* as *Cutibacterium* [10] is not generally accepted and not formally bound [11]. To avoid confusion, it is taxonomically eligible to continue to use the genus name *Propionibacterium,* consequently, this denomination is used throughout this article. *P. acnes* colonizes the pilosebaceous unit (PSU) with its anaerobic and lipid-rich microenvironment providing ideal growth conditions [12–14]. The ability of *P. acnes* to form biofilm has been described both *in vitro* and *in vivo*. Several studies directly showed *P. acnes* biofilms in skin biopsies from various skin diseases [12,15–19].

The acne research has long been plagued by the lack of a suitable *in vivo* model. Several animal models have been described including the rhino mice, the Mexican hairless dog, the nude mice and the rabbit ear model [20–23], but none of them mimics the conditions inside the PSU [24,25]. For example, unlike humans, others mammalians do not produce sufficient sebum [26]. Non-mammalian *in vivo* models have, on the other hand, been proposed to study host-bacteria interactions including biofilm formation [27]. *Drosophila melanogaster* (fruit fly) is a powerful model to study the host response to biofilm. Easy to manipulate and inexpensive to sustain, the fruit fly is a complex invertebrate with a high degree of similarities with the mammalian innate immune systems [28,29].

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In this study we report a successful development of an *in vivo* model to investigate *Propionibacterium* spp. biofilm in a germ-free line of *Drosophila melanogaster.*

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. acnes KPA171202 (type IB, ST5, CC5 [30]), *P. granulosum* DSM 20700 and *P. avidum* DSM 4901 (DSMZ) were used as reference strains. Bacteria were grown on Columbia blood agar base (Thermo Fisher Scientific) supplemented with 5% v/v of horse blood (Håtunalab AB) for 72 h followed by culture in brain heart infusion broth (Sigma-Aldrich) supplemented with 2 g/L glucose (BHI_g) for 48 h. For biofilm growth, these pre-cultures were used at 5% v/v to inoculate 10 mL of BHI_g in T-25 cell culture flask (Sarstedt) for 72 h. In some experiments, the culture media was supplemented with 5% v/v of lipids solution containing soybean oil, egg phospholipids and glycerol (Intralipid®, Fresenius Kabi). All bacterial cultures were performed under anaerobic conditions with Oxoid Anaerogen bags (Thermo Fisher Scientific) at 37 °C and 250 rpm for liquid cultures.

2.2. Germ-free fruit fly line

2.2.1. Creation and maintenance

Wild-type (WT) *Drosophila melanogaster* genotype W^{1118} iso; 2-iso; 3-iso was used in this study. A germ-free (GF) line was created in a sterile environment as followed. Fruit flies were starved on 15 g/L agar medium (Fischer Scientific) for 6 h and washed in a cell strainer (Corning). Fruit flies were air-dried and transferred to a vial containing BHI agar supplemented with 5 g/L glucose, 10 g/L sucrose and 60 g/L of yeast extract ($BHIA_{gsy}$) as well as antibiotics and incubated for 24 h. The following antibiotics were used: 20 μg/mL ciprofloxacin, 100 μg/mL kanamycin, 100 μg/mL ampicillin and 100 μg/mL erythromycin (Sigma-Aldrich). After fruit flies removal, eggs were collected and washed. All washing steps were performed in a sterile cell strainer for 2 min in 2.7% v/v sodium hypochlorite followed by 70% v/v ethanol and sterile water for 10 min. Washed eggs were further transferred onto autoclaved Bloomington food (BDSC semi-defined medium recipe [31]) with 10% w/v less agar and supplemented with antibiotics. Newly hatched fruit flies underwent the same treatment for three generations. To maintain the sterility, GF fruit flies were transferred and crossed on autoclaved Bloomington medium plus antibiotics within a biosafety cabinet. Fruit flies incubation was performed at 25 °C environment with 60% humidity.

2.2.2. Validation of the germ-free status

2.2.2.1. Bacterial culture from fruit flies and medium. Four fruit flies (GF or WT) were homogenized with a sterile plastic pestle in 100 μL of sterile buffered peptone water. Colony-forming units (CFU) were calculated by plating serial 10-fold dilutions of fruit flies homogenate. A 50 μL of the homogenate were used to inoculate 5 mL of BHI_g and assess the bacterial growth with optical density measurement at 560 nm. Washings from the vials as well as incubation medium were proceeded in a similar manner. Bacterial cultures were incubated at 37 °C in aerobic and anaerobic conditions. All experiments were performed in triplicate.

2.2.2.2. DNA extraction and bacterial 16S rRNA gene amplification. As a rule, 10 fruit flies (GF, WT or re-infected) were homogenized in 100 μL of enzymatic lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 1.2% v/v Triton X-100). DNA was extracted by a modification of the DNeasy Blood and Tissue kit protocol (Qiagen). Chemical lysis of the homogenate was achieved by treatment with 10 μL of proteinase K, 10 μL of achromopeptidase (1.10^5 U/mL), 40 μL of lysozyme (20 mg/mL) and 40 μL of AL buffer. Mechanical lysis was accomplished by using NucleoSpin Bead Tube Type B (Macherey-Nagel) for 3 min at 50 Hz.

The V3—V4 region (± 450 bp) was amplified with universal *Eubacteria* primers [32] 5′- CCTACGGGNGGCWGCAG -3' (16S—341F) and 5′- GACTACHVGGGTATCTAATCC -3' (16S-805R). *P. acnes* 16S rRNA (127 bp) was amplified using 5′-AGCTTGTTGGTGGGGTAGTG -3' (16S_Pg-Pa-F) and 5′- GTGCAA-TATTCCCCACTGCT -3' (16S_Pg-Pa-R) [33,34]. Amplification products were visualized on a 1% w/v agarose gel containing 1X GelGreen Nucleic Acid Stain (VWR).

2.3. Oral infection of Drosophila melanogaster with Propionibacterium spp.

2.3.1. Fruit flies preparation before infection

Newborn to four days-old GF fruit flies from both sexes were passed two times for 24 h on $BHIA_s$ (BHI Agar supplemented with 10 g/L sucrose) containing antibiotics as described above. In order to get rid of antibiotics, fruit flies were transferred to antibiotic-free $BHIA_s$ and kept for 24 h. As a rule, 30 fruit flies/vial were used for each infection.

2.3.2. Oral infection of Drosophila melanogaster with preformed biofilm

Bacteria isolated from preformed biofilm are shown to exhibit enhanced adhesion and biofilm formation compared to planktonic cells [35,36]. In our preliminary experiment, we found that infection with preformed biofilm of *Propionibacterium* spp. resulted in a more robust biofilm formation in the gut of fruit flies.

The preformed biofilm was removed from T-25 cell culture flasks and centrifuged for 3 min at 3500 rpm at room temperature and resuspended in 100 μL BHI_s. The biofilm was further transferred onto a sterile 24 mm glass fiber filter (Fisher Scientific) placed on 9 mL agar in a fruit fly vial. Fruit flies were exposed to a monospecies biofilm for six days with a new supplement of the biofilm every 24 h. To study the effect of lipids on the biofilm formation in the fruit fly gut, the feeding solution was supplemented with 10% v/v lipid solution (Intralipid®, Fresenius Kabi).

2.4. Biofilm visualization

After six days of infection with *Propionibacterium* spp. biofilm, fruit flies were transferred on $BHIA_s$ and kept for 24 h for shedding unattached bacteria. Fruit flies were anesthetized on ice, fixed in formalin and embedded in paraffin. The paraffin blocks were sectioned (4 μm) and mounted on SuperFrost Plus GOLD white adhesion slide (Fisher Scientific). Samples were observed without staining, before and after deparaffinization using a bright-field microscope.

2.4.1. Propionibacterium spp. visualization by immunofluorescence

After deparaffinization, rehydration and antigen retrieval, immunofluorescence staining was performed as previously described [17]. Fruit flies sections were stained with the following antibodies: anti-*P. acnes* mouse monoclonal, anti-*P. granulosum* chicken polyclonal IgY1 and anti-*P. avidum* rabbit polyclonal (Agrisera). Biofilm matrix was stained by FilmTracer SYPRO Ruby

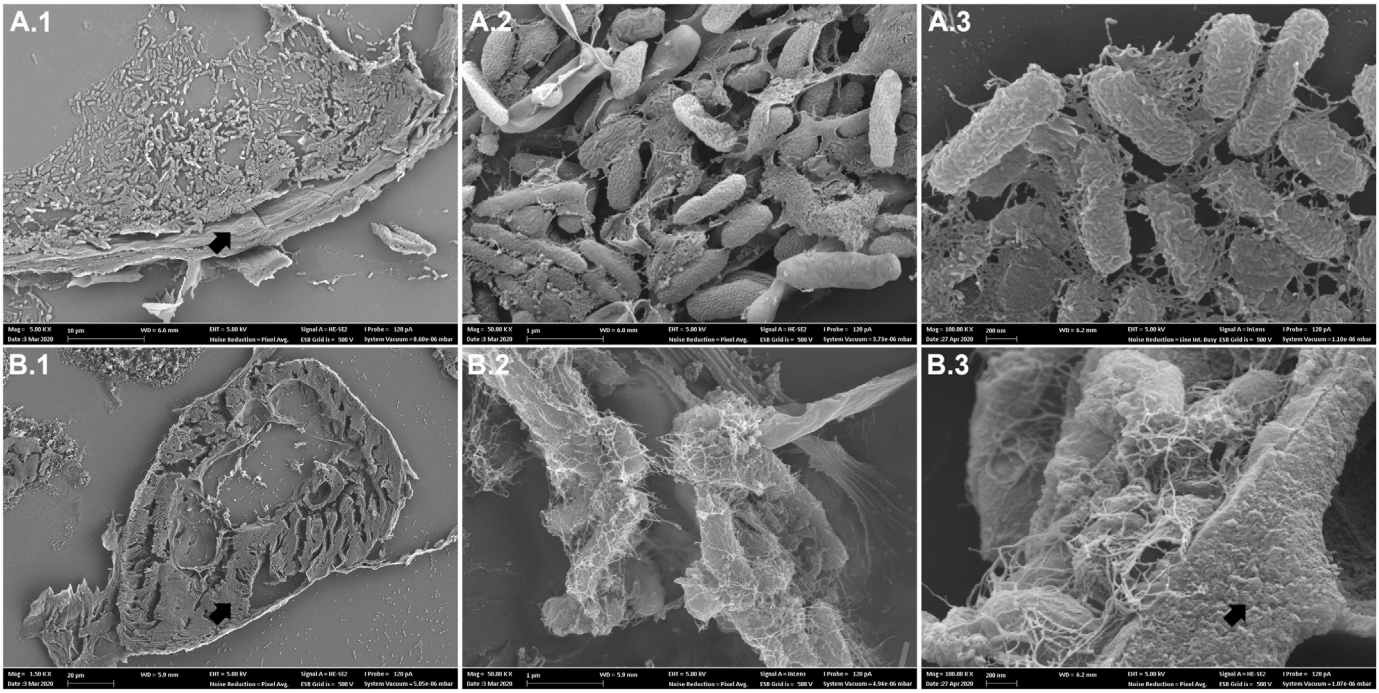

Fig. 1. SEM images of *Propionibacterium acnes* and *Propionibacterium avidum* biofilms in the fruit fly. Serial images of **(A)** *P. acnes* and **(B)** *P. avidum* biofilms in different magnifications. Bacteria are embedded in fimbriae-like structures. Arrows show the fruit fly gut wall.

Biofilm Matrix Stain (Thermo Fisher Scientific). All samples were labelled with 4 μg/mL DAPI (Sigma-Aldrich). The sections were analyzed on a Zeiss Axio Imager M2 microscope (Carl Zeiss Vision).

2.4.2. Electron microscopy of Propionibacterium spp. biofilm in fruit flies

After deparaffinization and rehydration, slides were washed in ultra-pure milli-Q water, dehydrated through ethanol series (v/v: 50%, 70%, 90% and 100%) followed by automated drying in the Leica EM CPD300 Critical Point Dryer. After coating with 5 nm of platinum with a quorum Q150T-ES sputter coater samples were analyzed with a Carl Zeiss Merlin Field Emission Scanning Electron Microscope (Umeå Core Facility for Electron Microscopy).

2.4.3. Propionibacterium acnes biofilm dispersion by bovine DNase I

A common commercial as well as clinical strategy to disperse biofilms is to target extracellular DNA (eDNA) with matrix-degrading enzymes [2,3,37–45]. We, therefore, tested if the bovine DNase I (Sigma) can disperse *P. acnes* biofilm in our fruit fly model. In this experiment fruit flies with a six days old biofilm were exposed for three or five days to 0.0065 nmol/μL of DNase I diluted in 37 g/L of BHI, 5 g/L of glucose, 100 g/L of sucrose, 60 g/L of yeast extract and 3 mM $MgCl_2$ (BHI_{gsym}). Every 24 h, 100 μL of enzyme were transferred onto a sterile 24 mm glass fiber filter placed in a sterile vial on 9 mL agar. A control group consisted of fruit flies treated with BHI_{gsym} only.

After the treatment, fruit flies were sacrificed, embedded in paraffin and sectioned at a thickness of 4 μm. Individual sections were observed with a bright-field microscope and a total number of biofilm positive sections was counted. Biofilm positive sections were defined as sections containing large microbial structures attached to the gut wall in the abdominal part of the fly (Fig. S1).

3. Results and discussion

3.1. Germ-free Drosophila melanogaster a viable host for Propionibacterium spp.

GF lineage of the *D. melanogaster* line was created and assessed by microbial culture and 16S rRNA-PCR analysis. The bacterial load yielded an average of 4.3×10^8 CFU per WT fly. No growth from the GF fruit flies or maintenance vials was observed in aerobic or anaerobic conditions (Fig. S2). The GF status was further confirmed by 16S rRNA PCR (Figure S3 A).

The reproducible growth of *P. acnes* as well as amplification of *P. acnes* 16S rRNA from re-infected GF fruit flies was indicative of *P. acnes* viability in the fruit fly gut (Figure S3 B).

3.2. Propionibacterium spp. are able to form biofilm in the fruit fly model

P. acnes, *P. avidum* and *P. granulosum* developed biofilm-like structures inside the fruit fly gut (Fig. S1, Fig. S4, Fig. S5). Additionally, the biofilm matrix was further visualized with FilmTracer™ SYPRO™ Ruby Biofilm Matrix Stain. High resolution imaging by SEM revealed characteristic fimbriae structures consistent with biofilm (Fig. 1). In line with earlier reports *in vitro* [46], *P. avidum* biofilm were surrounded by a dense matrix in the fruit fly gut. On the contrary, some *P. acnes* strains (*e.g.* KPA171202) were described to not develop extracellular polymeric structures *in vitro* [46]. In our *in vivo* model, *P. acnes* was embedded into extracellular matrix, consistent with our earlier findings *in vitro* [47]. *Propionibacterium* spp. aggregates attached to the gut surface and embedded at fimbriae-like structures were considered as a final prove of the biofilm formation (Fig. 1). All flies maintained on a lipid-rich diet were positive for *P. acnes* biofilm.

Table 1
Effect of DNase I treatment on *Propionibacterium acnes* biofilm in the fruit fly model.

	3 day treatment				5 day treatment				
	Flies number	Sections number (%)			Flies number	Sections number (%)			
Treatment	Total	Positive	Negative	*p*	Total	Positive	Negative	*p*	*p*
Control: BHI_g	9	162 (80)	41 (20)	*< 0.0001**	9	147 (68)	68 (32)	*< 0.0001**	*> 0.05***
Enzyme: DNase I	7	60 (48)	66 (52)		7	24 (14)	145 (86)		*< 0.0001***

p-values were calculated using the two-tailed Fisher's exact test. (*) Effect of enzyme treatment was compared to the control. (**) Effect of the duration was evaluated for each treatment.

D. melanogaster microbiota is important for larval growth and the fruit fly development [48]. We have noticed a minimal effect of *P. acnes* infection on life cycle of infected fruit flies. No lethality was observed. Infected fruit flies appeared smaller, with a few days delay in maturing. The fruit fly model has been previously used to study virulence and the immune response in *Pseudomonas aeruginosa* biofilm infection [49,50]. It is a well-studied relatable biological host system to investigate mono or polymicrobial biofilm, to identify microbial response factors or host immune response allowing the assessment of pathogens interactions with epithelial cells [27,49]. In this reported model, *Propionibacterium* spp. were able to attach to the gut epithelium and develop a mature biofilm in an oxygen-, nutrient-poor and lipid-rich environment.

3.3. Suitability of in vivo fruit fly model of Propionibacterium acnes biofilm for therapeutic intervention

The primary objective of this proof-of-concept study was to access if the fruit fly model can be suitable to evaluate dispersal agents against *P. acnes* biofilm. The role of eDNA in the development and the stability of biofilm has been well documented in several bacterial species [2,37,38] including *P. acnes* [46,47,51,52]. The use of biofilm dispersing enzyme targeting the eDNA is a strategy to change the matrix stability [2,3,37].

In order to demonstrate if *P. acnes* biofilm fruit fly model can be used to evaluate anti-biofilm therapeutic modalities, fruit flies were treated with DNase I. During the preliminary experiment the ability of the enzyme to degrade DNA at 25 °C, the maintenance temperature for the fruit fly, has been validated. We observed no lethality in response to a 3- or 5-day DNase I treatment. Our preliminary experiments showed that though all infected fruit flies harbored *P. acnes* biofilm after treatment, the biofilm was unevenly distributed in the gut. Therefore, the primary endpoint at evaluating the DNase I effect was to check the frequency of a positive section (Table 1). In total, 14 fruit flies were exposed to DNase I. Both the 3- and 5-day DNase I treatments were associated with a significantly lower presence of *P. acnes* biofilm. Moreover the 5-day treatment appeared more effective than the 3-day treatment (Table 1).

4. Conclusions

We developed a novel *in vivo* fruit fly model suitable to study *Propionibacterium* spp. biofilm. The model is applicable to *P. acnes*, *P. granulosum* and *P. avidum*. The developed fruit fly model has distinctive advantages to study the PSU as compared to previous models [12,13,24–26], namely anaerobic lipid-rich environment together with exposure to epithelial cells. This model can be used both for mechanistic studies of *Propionibacterium* spp. biofilm as well as biofilm targeting therapeutic modalities.

CRediT authorship contribution statement

Vicky Bronnec: conceived, designed, performed experiments and wrote the manuscript. **Oleg A. Alexeyev:** conceived, designed experiments and wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2021.102450.

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