Optimising synthetic cystic fibrosis sputum media for growth of non-typeable *Haemophilus influenzae*

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Abstract

Non-typeable *Haemophilus influenzae* (NTHi) is an early pathogen isolated from the lungs of children with cystic fibrosis (CF). However, its role in the progression of CF lung infection is poorly understood. Additionally, whether it forms biofilms in the lungs of people with CF is an open question. The development of synthetic cystic fibrosis sputum media has given key insights into the microbiology of later CF pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, through replicating the chemical composition of CF sputum. However, growth of NTHi in these media has not previously been reported. We show that NTHi grows poorly in three variants of synthetic cystic fibrosis sputum media commonly used to induce *in vivo*-like growth of *P. aeruginosa* and *S. aureus* (SCFM1, SCFM2 and SCFM3). The addition of NAD and hemin to SCFM1 and SCFM2 promoted the planktonic growth and biofilm formation of both laboratory and clinical NTHi isolates, and we were able to develop a modified variant of SCFM2 that allows culture of NTHis. We show that NTHi cannot be identified in an established *ex-vivo* model of CF infection, which uses SCFM and porcine bronchiolar tissue. This may in part be due to the presence of endogenous bacteria on the pig lung tissue which outcompete NTHi, but the lack of selective agar to isolate NTHi from endogenous bacteria, and the fact that NTHi is an exclusively human pathogen, make it hard to conclude that this is the case. Through spiking modified SCFM2 with filter sterilized lung homogenate, biofilm growth of clinical NTHi isolates was enhanced. Our results highlight that there are crucial components present in the lung tissue which NTHi require for growth, that are not present in any published variant of SCFM from the Palmer et al. 2007 lineage. Our results may inform future modifications to SCFM recipes to truly mimic the environment of CF lung sputum, and thus, to facilitate study of a wide range of CF pathogens.

Keywords: biofilms; Cystic fibrosis; infection models; non-typeable *Haemophilus Influenzae*; synthetic cystic fibrosis sputum media.

Data Summary

The authors confirm that all supporting data, code and protocols have been provided within the article or through supplementary data files. All raw data has been uploaded to FigShare (https://doi.org/10.6084/m9.figshare.28175300.v1).

Abbreviations: ANOVA, Analysis of Variance; BHI, brain heart infusion; CF, cystic fibrosis; CFTR; CFU, colony forming units; cystic fibrosis transmembrane conductance regulator, DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; DOPC, dioleoylphosphatidylcholine; eDNA, extracellular deoxyribonucleic acid; EVPL, *ex-vivo* pig lung; FBS, fetal bovine serum; GFP, green fluorescent protein; GlcNAc, N-acetyl glucosamine; Honestly Significant Difference Honestly significant difference, (HSD); NAD, nicotinamide adenine dinucleotide; NTHi, Non-typeable *Haemophilus influenzae*; PBS, phosphate buffered saline; SA, sialic acid; sBHI, supplemented brain heart infusion; SCFM, synthetic cystic fibrosis sputum media; UV, ultraviolet; VBC, vancomycin bacitracin clindamycin; XV, hemin and NAD.

Introduction

Cystic fibrosis (CF) is an autosomal genetic disorder, affecting over 70,000 individuals worldwide [1]. The accumulation of dehydrated mucus in the lungs harbors bacterial colonisation, resulting in chronic infection, a strong inflammatory response and lung damage [2]. The median life expectancy of a person with CF is currently 49 years in the United Kingdom [3], and many people with CF die prematurely from respiratory complications, despite treatment advances. The course of CF lung infection commonly begins with *Haemophilus influenzae* and *Staphylococcus aureus* in early childhood before progression to chronic infection with *Pseudomonas aeruginosa* during later childhood and adolescence [4].

There has been a dramatic increase in the survival of people with CF within the last decade thanks to a combination of antibiotic therapy, newborn screening and cystic fibrosis transmembrane conductance regulator (CFTR) modulators. CFTR modulators are now effective in correcting CFTR protein function in around 90% of people with CF [5][6][7]. There is increasing evidence that CFTR modulators reduce colonisation of later stage pathogens like *S. aureus* and *P. aeruginosa* [8]. However, whilst CFTR modulators decrease the prevalence of later stage pathogens, modulator therapy appears to result in an increase in prevalence of *H. influenzae* [9][10][11]. This means that investigating *H. influenzae* infection in CF lung mimicking conditions would be beneficial to better understand this changing landscape of CF lung infections. In this paper, we investigated the ability of *H. influenzae* to grow both planktonically and as a biofilm in a range of CF mimicking media to broaden the understanding of *H. influenzae* lung infection in people with CF.

Non-typeable *H. influenzae* (NTHi) are Gram-negative coccobacilli isolated from the respiratory tract in around 30% of children with CF [3][12]. Persistence of NTHi in the lower respiratory tract is commonly associated with periods of pulmonary exacerbation and chronic lung infection, contributing to the morbidity of people with CF [13][14] [15]. *H. influenzae* is defined by its requirement for Factors X (hemin) and V (nicotinamide adenine dinucleotide, NAD) to grow in laboratory conditions [16][17]. NTHi has emerged as the predominant group of *H. influenzae* strains in people with CF due to a lack of specific treatment, alongside a dramatic decline in invasive typeable *H. influenzae* disease due the development of a serotype b conjugate vaccine [18][19].

The existence of matrix-encased microbial communities known as biofilms is known to contribute to disease progression, pathogenesis and bacterial persistence in CF lung infections [20]. There is increasing evidence that *in vivo,* bacterial biofilms are increasingly tolerant to antibiotic therapy, surviving up to 100-1,000 fold higher antibiotic concentrations than planktonic cells [21]. Biofilms perpetuate chronic infection and recurrence of disease [22][23]. Mucin and extracellular deoxyribonucleic acid (eDNA) present in CF lungs have been demonstrated to provide a key bacterial niche for microcolony and biofilm formation *in vivo* [24][68]. Furthermore, hypoxic regions in the mucus have been demonstrated to increase transcription of

efflux pumps, promote anaerobic respiration, reduce bacterial metabolism and induce biofilm formation in the well-studied CF pathogen, *P. aeruginosa* [22][25][26].

There is evidence that NTHi can form aggregates that are consistent with the appearance of a biofilm, but whether NTHi truly form biofilms, or if NTHi biofilms contribute to disease, is under debate [27][28][29][30]. There is a wealth of research into NTHi biofilm formation in otitis media and chronic obstructive pulmonary disease, whilst research on biofilm formation in CF is limited [31][32][33]. Biofilms containing NTHi have been directly visualised within the ear canal of children with otitis media using scanning electron microscopy [34], and shown to produce mucosal biofilms in a chinchilla model of otitis media using scanning electron microscopy [33] and confocal laser scanning microscopy [35]. Furthermore, NTHi has been shown to grow as a biofilm within the chronic obstructive pulmonary disease ferret lung model by confocal fluorescent imaging [36]. Conversely, little is known about *H. influenzae* in the context of CF colonisation: although there is evidence that suggests NTHi can form biofilm aggregates in the lungs [23][37], this does not confirm whether NTHi biofilms are stable within a CF lung environment. As such, we strived to gain an understanding of if *H. influenzae* form biofilms in a CF lung environment.

The nutritional environment of CF sputum is poorly replicated in standard laboratory growth media. To better recapitulate the CF lung environment *in vitro,* synthetic cystic fibrosis sputum media (SCFM) was developed based on the concentrations of ions, free amino acids, lactate and glucose found in CF sputum [38]. This SCFM, later termed SCFM1, has been well documented in the literature to be capable of supporting high density bacterial growth and biofilm formation of *P. aeruginosa* and *S. aureus in vitro* and in an *ex vivo* model of lung biofilm using porcine tissue [39] [40] [41] [42]. However, SCFM1 lacks several polymers and molecules present in CF sputum such as mucin and eDNA, which contribute to the viscosity of CF mucus and biofilm structures of pathogens within the CF lung. The presence of mucin has been shown to be crucial for microcolony development and bacterial biofilm formation [43], whilst eDNA is known to be present in large amounts in in CF lung mucus, and high concentrations have been shown to be associated with increased biofilm viscoelasticity and more robust biofilms [44] [45]. SCFM1 was therefore adapted to form SCFM2, with additions of bovine maxillary mucin, salmon sperm DNA, dioleoylphosphatidylcholine (DOPC) and N-acetyl glucosamine (GlcNAc) to make up for previous shortcomings [46]. To create a further version, SCFM3, final additions of p-aminobenzoic acid, NAD+, adenine, guanine, xanthine, and hypoxanthine were added [47]. Although these SCFMs have been optimised for usage with *P. aeruginosa* and *S. aureus,* there is no published research investigating the growth of NTHi in any variant of artificial sputum media.

In this paper, we investigated the growth profiles of NTHi in several variants of SCFM. We hypothesized that the fastidious nature of NTHi may mean NTHi may require supplementation of additional factors to support growth within SCFM, such as NAD and hemin. Therefore, either fetal bovine serum (FBS) or purified hemin and NAD were added. We then investigated the growth of NTHi in an established model of CF lung infection [48]. This combines pig bronchiolar and alveolar tissue with SCFM to mimic the structural and chemical environment of a human CF lung, and has previously supported the studies of key CF pathogens *P. aeruginosa* and *S. aureus* [41][40][42][49]. This paper is the first publication that attempts to use the *ex-vivo* pig lung model (EVPL) to investigate the growth of NTHi. In summary, our results demonstrate that while some simple modifications of SCFM formulations can support growth and biofilm formation of NTHi, growth is relatively poor. Further, we found that the EVPL model in its current form is not suitable for culture of NTHi. Further investigation is needed if we are to optimise CF-mimicking media and models for research into the pathogenicity and management of NTHi in the CF lungs.

107 # Methodology

108 ## Bacterial strains

109 All *H. influenzae* were cultured on supplemented brain heart infusion (sBHI) agar (Millipore) at 37°C in 5% CO_2. Following
110 immersion in a water bath at 50°C, autoclaved agar was supplemented with NAD (2 μg/mL) (Merck) and hemin (10 μg/mL)
111 (Sigma), mixed, and 20 mL was added to 90 mm polystyrene petri dishes. sBHI plates were stored at 4°C.

112 The *H. influenzae* strains used in this study are listed in Supplementary Table 1. The reference strains NCTC 11315, NCTC
113 04560, NCTC 12699 and NCTC 12975 were obtained from the National Collection of Type Cultures
114 (www.culturecollections.org.uk). The streptomycin resistant NTHi375 and a GFP-tagged clinical otitis media isolate was
115 derived from a Finnish pneumococcal vaccine study on children undergoing tympanocentesis in 1994-1995 [50] [51]. When
116 cultured, NTHi-375 was plated on sBHI agar supplemented with 2μg/mL streptomycin. All *Haemophilus influenzae* clinical CF
117 isolates were obtained from patients of the SCILD cohort at the institute of infectious disease, University of Bern and are
118 listed in Supplementary table 2 [96][97].

119 The fluorescent *Pseudomonas aeruginosa* PAO1 was derived from the Holloway, 1955 study [52] and subcultured on Luria-
120 Bertani agar overnight at 37°C.

121 ## Synthetic Cystic Fibrosis Sputum Media

122 Synthetic Cystic Fibrosis Sputum Media 1 (SCFM1) was made following the recipe by Palmer *et al* [38], minus the addition of
123 glucose (following [41] (Supplementary Table 3)).

124 Synthetic Cystic Fibrosis Sputum Media 2 containing bovine maxillary mucin (SCFM2) was made following the recipe of
125 Turner *et al*. [46]. The recipe was modified slightly by removing the phenol extraction of salmon sperm deoxyribonucleic acid
126 (DNA) (Sigma). Instead, salmon sperm DNA was sterilised by ultraviolet (UV) light in 30 mg aliquots for 20 minutes. This was
127 done to simplify the recipe by removing the time-consuming and high-hazard step of phenol extraction (Supplementary Table
128 4), and followed advice from one of the original authors (K Turner, personal communication).

129 Modified Synthetic Cystic Fibrosis Sputum Media 2 with sialic acid replacing bovine maxillary mucin (SCFM2.SA) was
130 primarily prepared following the recipe of the Turner et al. [46]. Modifications included UV sterilisation of salmon sperm DNA
131 as per SCFM2, and replacing bovine maxillary mucin with sialic acid (n-acetylneuraminic acid (Neu5Ac)) (Millipore). A stock
132 concentration of 45 mg/mL Neu5Ac in dimethylsulfoxide (DMSO) was used. In 100 mL of SCFM2.SA, 1 mL of this stock was
133 added for a final concentration of 450 μg/ mL (Supplementary Table 4). The replacement of mucin with sialic acid is more
134 affordable than bovine maxillary mucin and also removes the need for a 4-hour UV sterilisation step and the potential
135 contamination of SCFM2 with impurities present in commercial mucin.

136 For experiments, each of SCFM1, SCFM2 or SCFM2.SA was used exactly as described above; or supplemented with 2% fetal
137 bovine serum (Gibco); or supplemented with NAD (2 μg/mL) and hemin (10 μg/mL).

138 Synthetic Cystic Fibrosis Sputum 3 was made using the recipe by Neve *et al.*, [47]. Modified SCFM2.SA was used as a base,
139 with the additions of p-aminobenzoate (200 μM) NAD (10 μg/ml), adenine, guanine, xanthine and hypoxanthine (10 μg/ml)
140 (Supplementary Table 4).

141 **Growth of bacteria**

142 All *H. influenzae* strains were maintained on sBHI plates at 37°C in 5% CO_2. A colony of *H. influenzae* was grown overnight in
143 BHI broth supplemented with NAD (2 μg/mL) and hemin (10 μg/mL) at 37°C in 5% CO_2. Bacteria were washed by centrifuging
144 1 mL of preculture in microfuge tubes for 1 minute at 10,500 rpm, removing the supernatant, adding 1 mL phosphate
145 buffered saline (PBS) and vortexing to resuspend cells. The centrifuge and PBS wash steps were then repeated. The desired
146 volume of overnight culture to achieve OD_{600} 0.1 was then added to fresh microfuge tubes and washed with PBS as before.
147 Cells were then resuspended in the SCFM media of choice.

148 **Growth curves**

149 All strains and isolates of *H. influenzae* were grown overnight as stated and adjusted to a desired optical density of OD_{600} 0.1
150 in SCFM media of choice. 200 μL of each suspension was added to a flat-bottom Corning 96 well polystyrene plate, with four
151 replica wells inoculated per condition. Media-only wells were used as a negative control. The plate was then incubated in a
152 Tecan Spark 10M multimode plate reader at 37°C, 5% CO_2 with shaking every 20 minutes. OD_{600} (nm) was read every 20
153 minutes for 24 hours. Carrying capacity (K) and growth rate (r) parameters were then extracted using the *growthcurver*
154 package in RStudio [53]. For plotting and analysis, OD_{600} values were blanked on the starting OD_{600} of each well.

155 To determine the colony forming units (CFU) per well, following 24 hours of growth, 100 μL from each well was removed and
156 serially diluted from 10^0 to 10^{-7}. 10 μL from each dilution were then plated on sBHI plates.

157 The colony forming units were then counted following static incubation for 24 hours at 37°C in 5% CO_2.

158 **Static biofilm assay**

159 *H. influenzae* were grown overnight, washed and re-suspended in media of choice to OD_{600} 0.1 as before. 100 μL of each
160 suspension was added to a flat-bottom Corning 96 well polystyrene plate (Sigma). Plates were incubated statically at 37°C
161 and 5% CO_2 for 48 hours. Media was then slowly aspirated from wells to remove planktonic cells and added to a fresh flat-
162 bottom 96 well plate, leaving behind the biofilm. 100 μL of PBS was added to each biofilm well, and the base of well was
163 scraped with a pipette tip. Plates were sealed with Parafilm and sonicated for 10 minutes in a sonicating water bath (Grant
164 XUBAL XUBA1, Grant Instruments (Cambridge Ltd.)). Following sonication, absorbance of both this plate and the aspirated
165 planktonic cell plate were measured at OD_{600} in a Tecan SPARK 10M multimode plate reader.

166 For crystal violet staining, following aspiration of media and planktonic cells, 100 μL of crystal violet (0.1% in PBS) (Sigma)
167 was added to each well and incubated at room temperature for 15 minutes. Crystal violet was aspirated and 100 μL of cell
168 culture water (HyClone) was added to each well, aspirated, and fresh cell culture water was added. 100 μL of 95% ethanol
169 was then added to each well and incubated at room temperature for 15 minutes. The bottom of the wells were scraped with
170 pipette tip and absorbance of each well measured at 590 nm in a Tecan SPARK 10M multimode plate reader.

171 Relative biofilm formation was calculated by taking this value, and dividing it by the OD_{600} of the aspirated planktonic cells.

172 **Biofilm assay using EBBA Biolight 680**

173 EBBA BioLight 680 (Ebba BioTech) was diluted 1:100 in HyClone water and then 1:10 in SCFM of choice. Bacteria of interest
174 (*H. influenzae* NCTC 11315, NCTC 4560, NCTC 12699 and NCTC 12975) were grown overnight, washed, and re-suspended in
175 SCFM of choice to OD_{600} 0.1 as before. 100 μL was added to black polystyrene transparent flat bottomed 96 well plates
176 (Greiner) and incubated for 48 hours in a Tecan SPARK 10M multimode plate reader at 37°C in 5% CO_2 with shaking, and
177 OD_{600} fluorescence (ex/em 540/670 nm) read every 20 minutes.

178 ***Ex vivo* pig lung model (EVPL) dissection and infection**

179 The EVPL model was prepared as previously described [54] [41][40]. Two independent lungs were used and provided by
180 Taylors Butchers (Coventry, UK) and Steve Quigley and Sons Butchers Ltd (Cubbington, UK). Following dissection, bronchiole
181 or alveolar tissue pieces were placed in 400 μL SCFM1 with ampicillin, or ampicilllin (50 μg/mL) + gentamicin (50 μg/mL) and
182 incubated at 37°C for 24 hours on a rocking platform. These antibiotics were chosen based on preliminary sequencing of
183 commensal bacteria present on the lung tissue (data not shown). Following incubation, the tissue was washed twice with
184 PBS and once with SCFM. Following the wash stages, the tissue was transferred to a fresh transparent 24-well plate with an
185 SCFM2.SA.XV agarose pad, and infected as previously described prior to addition of 500 µL of SCFM2.SA.XV and incubation
186 for 48 hours at 37°C and 5% CO_2.

187 **Bacterial recovery from EVPL**

188 Tissue was homogenised by bead beating as previously described [55][40]. To determine bacterial load, lung homogenate
189 was serially diluted in PBS, and plated on sBHI agar plates, which were then incubated for 24 hours at 37°C and 5% CO_2 to
190 determine CFU. Agar plates contained either sBHI, or sBHI containing vancomycin (5 μg/mL), bacitracin (300 μg/mL) and
191 clindamycin (1 μg/mL) (VBC).

192 **Visualisation of fluorescently-tagged bacteria in EVPL**

193 The fluorescence of uninfected tissue, NTHi-375 and PAO1 infected tissue was measured in an 8 x 8 grid at 0, 24 and 48 hours
194 post infection, at 484 ex / 505 em in a Tecan SPARK 10M multimode plate reader using a flat-bottom transparent Corning 24-
195 well plate (Costar).

196 **Synthetic CF sputum lung homogenate media**

197 Pig lung square sections were acquired through dissection and washed as per the EVPL model, above. Each tissue section
198 was homogenised in 1 mL of SCFM2.SA media in a FastPrep-24 machine (MP Biomedicals, UK) for 40 s at 4 m/s. Lung
199 homogenate was then transferred to a sterile 1.5 mL microfuge tube and centrifuged for 1 minute at 10,500 rpm to remove
200 tissue debris. The supernatant was removed and then diluted 1:4 in SCFM and filter sterilised through a 3 mm 0.2 μm syringe
201 filter (Fisher Scientific, China) with a 2 ml syringe (Becton Dickinson, Spain) into a sterile 50 mL tube. NAD (2 μg/mL) and
202 hemin (10 μg/mL) were then added. This was refrigerated for up to 1 month.

Statistical analysis

All data was analysed using RStudio (Mac OS Ventura 13.5.2 + Version 1.4.1717) (RStudio, https://www.rstudio.com) using general linear models followed by Analysis of Variance (ANOVA) and either post-hoc Dunnett, Estimated Marginal Means for pairwise comparisons or Tukey Honestly Significant Difference (HSD) tests as specified in the text.

Results

Adding NAD and hemin to SCFM2.SA significantly increases growth rate and carrying capacity of NTHi laboratory strains

The growth profiles of four laboratory strains, *H. influenzae* NCTC 12699, NCTC 12975, NCTC 4560 and NCTC 11315, were monitored in a total of nine different SCFM variations (SCFM1, SCFM2 and SCFM2.SA) alone or supplemented with either 2% FBS or NAD and hemin) over 24 hours to determine which SCFM variant was most suitable for NTHi growth. Growth curves are available in Supplementary Figure 1.

It was previously suggested that NAD is present in CF sputum, at concentrations up to 15 μM [47], whilst hemin has been determined as 'potentially available' [46]. Haemoptysis, which is blood streaking in sputum, is observed in 60% of CF patients, and often a marker of airway inflammation, pulmonary exacerbation and declining lung function, with up to 35 μM of hemin potentially found in CF sputum [56] [57]. Serum, containing NAD, hemin and additional factors which may influence bacteria growth, is also likely to be present in CF sputum due to vascular leakage following tissue damage from airway inflammation [57], and increased levels of serum proteins have been detected in the sputum of people with CF compared to healthy individuals [58]. We thus compared formulations of SCFM containing either purified NAD and hemin alone, or serum.

In SCFM2.SA, mucin in the original SCFM2 recipe was replaced with N-Acetylneuraminic acid at 450 μg/ml to increase affordability and ease of preparation. NTHi binds host mucin via sialic acid through its lipooligosaccharide to evade the immune response, and this sialic acid is the most common sialic acid found in human mucin [59] [60]. The concentration was determined based on reports of sialic acid plasma concentrations of 402-550 μg/ml in CF patients [61].

When growth of NTHi was investigated in these SCFM variations, there was a significant difference in carrying capacity and growth rate between media variations for all strains. Carrying capacity of all four NTHi laboratory strains when grown in SCFM2.SA supplemented with NAD + hemin (SCFM2.SA.XV) was significantly higher than when grown in unmodified SCFM1 **(Figure 1)**. There was some variation but, overall, SCFM2.SA resulted in a higher carrying capacity and growth rate compared to modified SCFM2 and unmodified SCFM1, and within SCFM2.SA, adding FBS or XV increased the carrying capacity. Meanwhile, the addition of FBS or XV to SCFM1 and SCFM2 did not enhance carrying capacity or growth media relative to these medias with no additions.

These results suggest that the modified SCFM has a more prominent effect on enhancing carrying capacity than growth rate, in particularly SCFM2.SA.XV. SCFM2.SA.XV was the only media variant to significantly promote carrying capacity over SCFM1 in all four strains, and growth rate in two strains (NCTC 11315 and NCTC 4560). SCFM2.SA and SCFM2.SA.FBS increased growth rate in the same two strains and carrying capacity in all but NCTC 4560. On the contrary, the original SCFM2 recipe produced highly variable results and did not significantly enhance either carrying capacity or growth rate except in one case,

237 with NCTC 12699 when supplemented with FBS. Supplementing SCFM1 with FBS enhanced carrying capacity in 3 strains (all
238 but NCTC 4560) and growth rate in one strain (NCTC 11315).

239 NTHi is known to reach an average bacterial load of 10^5 to 10^8 CFU/ml when recovered from broncoalveolar lavage [62] [63]
240 [64]. Therefore, it was investigated whether SCFM2.SA.XV could support a high bacterial load (CFU). Bacterial load of NTHi in
241 SCFM2.SA.XV averaged 10^8 CFU/ml for NCTC 12975, 10^7 CFU/ml for NCTC 12699 and NCTC 4560, and 10^6 CFU/ml for NCTC
242 11315 (Supplementary Figure 2). This was slightly higher than in SCFM1, in which bacterial load was 10^7 CFU/ml for NCTC
243 12975, NCTC 12699 and NCTC 4560, whilst NCTC 11315 reached 10^5 CFU/ml . This suggests both SCFM1 and SCFM2.SA.XV
244 can support NTHi growth to levels similar to that found in CF.

245 Biofilm forming ability of NTHi *in vitro* is poor in SCFM, and fetal bovine serum promotes planktonic 246 growth over biofilm growth

247 Several species of bacteria are known to form biofilms in the CF lung environment, and this often contributes to persistent
248 lung infection [65]. We investigated whether NTHi are able to form biofilms in SCFM media *in vitro,* and to what extent its
249 capability to form biofilm differs between the media variants. Biofilm formation was assessed by static growth in a flat-
250 bottomed 96-well plate and staining with crystal violet at 48 hours; this is a high-throughput method to determine biomass
251 based on optical density [66]. The relative biofilm allocation (amount of stain bound by biofilm / optical density of planktonic
252 culture) was then calculated.

253 At 48 hours, there was measurable NTHi biofilm formation (Supplementary Figure 3). The relative biofilm allocation of each
254 NTHi laboratory strain was determined in each SCFM variant to highlight whether any media variant promoted biofilm
255 growth specifically, or promoted both planktonic and biofilm growth indiscriminately. A significant increase in relative
256 biofilm, compared with unmodified SCFM1, was seen only seen in NCTC 12699 when grown in SCFM2.SA.XV. However, the
257 addition of FBS, both to SCFM1 and SCFM2.SA significantly reduced relative biofilm formation for all NTHi strains (**Figure 2)**.
258 SCFM2.SA also significantly reduced relative biofilm formation of NCTC 11315 and NCTC 12699. Overall, this suggests that
259 FBS promotes planktonic growth of bacteria over biofilm formation.

260 Overall, these results suggest that FBS may prevent biofilm formation of NTHi and may not be an ideal supplement for
261 SCFM. As such, despite FBS previously promoting the *in vitro* proliferation of NTHi, the presence of FBS hinders biofilm
262 growth. SCFM2.SA.XV was the only media variant to significantly promote biofilm formation, although in only one laboratory
263 strain.

264 Extracellular matrix production of *H. influenzae* is increased when grown in SCFM2.SA.XV

265 Crystal violet staining is a high-throughput and well-established assay used for quantifying biofilms *in vitro*. However, a
266 downside to crystal violet is that it can stain bacterial cells and biofilm matrix, and it stains cells regardless of whether they
267 are alive or dead [27]. As such, to specifically investigate biofilm matrix development, we utilised EBBA BioLight 680, a
268 chemically modified optotracer which binds to the extracellular matrix of Gram negative bacteria and fluoresces only when
269 bound [67]. NTHi was grown statically for 48 hours in SCFM2.SA and SCFM2.SA.XV with EBBA BioLight 680 to compare
270 biofilm formation in these media. FBS was also tested as supplement, but was found to bind to EBBA BioLight (data not
271 shown). Biofilm formation of NTHi was apparent when grown in SCFM2.SA and SCFM2.SA.XV. With all NTHi strains,
272 fluorescence intensity of EBBA Biolight 680 increased when grown in SCFM2.SA.XV over SCFM2.SA.

273 In SCFM2.SA, fluorescence peaked around 8 hours at 7,000 RFU for NCTC 4560, 10 hours and 3,000 RFU for NCTC 11315 and
274 less than 1,000 RFU for NCTC 12699 which did not appear to peak. On the other hand, biofilm formation in SCFM2.SA.XV was
275 most apparent with NCTC 4560 and NCTC 11315. With NCTC 4560, fluorescence peaking at around 22,000 RFU at 16 hours
276 before gradually decreasing up to 48 hours whilst NCTC 11315 peaked around 15,000 RFU at around 18 hours **(Figure 3)**.
277 NCTC 4560 and NCTC 11315 produced similar fluorescence profiles to each other, while fluorescence with NCTC 12699 and
278 NCTC 12975 were similar to each other but much lower, peaking around 10,000 RFU at 16 hours and 5,000 RFU at 20 hours
279 respectively. This suggested NCTC 12699 and NCTC 11315 do not produce as much extracellular matrix as NCTC 4560 or NCTC
280 11315, and may be weaker biofilm formers in SCFM. The time at which fluorescence peaked differed in all strains, but ranged
281 between 16 to 20 hours. For both NCTC 4560 and NCTC 11315, fluorescence peaked before dropping entirely in some
282 repeats. The peak of fluorescence appears later than when NTHi reached carrying capacity from the growth curves between
283 5 and 9 hours, consistent with the manufacturer's claim that EBBA BioLight does not bind to the bacterial cell directly, and is
284 indicative of biofilm formation by binding the extracellular matrix.

285 Overall, this data suggests all NTHi are capable of forming biofilms in SCFM2.SA and SCFM2.SA.XV, determined by
286 production of an extracellular matrix. However, more extracellular matrix was produced in in SCFM2.SA.XV, with biofilm
287 formation peaking between 16-20 hours. From this, SCFM2.SA.XV was determined to be the most suitable SCFM variant for
288 use with NTHi for further *in vitro* work.

289 Removing extracellular DNA from SCFM2.SA.XV significantly reduced growth of NTHi

290 Extracellular DNA is one of the major contributing factors to the progression of infection as well as biofilm formation in
291 people with CF, and can be derived from both bacterial and host origins. The majority of eDNA in the lungs has been
292 proposed to be due to neutrophil extracellular traps, but also comes from other sources such as dead host immune cells,
293 lysed bacteria and bacterial DNA released through quorum sensing regulation [98]. Large amounts of eDNA are known to be
294 present in CF lung mucus, and high concentrations have been shown to be associated with increased biofilm visco-elasticity
295 and more robust biofilms [44] [45]. To determine the importance of eDNA in NTHi growth, the growth profiles of NTHi were
296 investigated in SCFM2.SA.XV with and without eDNA. Growth curves are available in Supplementary Figure 4.

297 The removal of eDNA from SCFM2.SA.XV significantly reduced carrying capacity and growth rate in all laboratory strains
298 (**Figure 4**). This suggests that eDNA is a crucial component to promote NTHi growth. The importance of eDNA in growth and
299 biofilm has been previously suggested in the literature for other CF pathogens such as *P. aeruginosa* [68].

300 NAD is an essential growth factor of NTHi, and can reach up to 15 μM in CF sputum, which equates to around 10 μg/mL and
301 as a result is a component of the SCFM3 recipe [47]. Therefore, we also tested whether increasing NAD in SCFM2.SA.XV up to
302 10 μg/mL, alongside growth in modified SCFM3 which contains purines (adenine, guanine, xanthine and hypoxanthine),
303 NAD+ and p-aminobenzoic acid, would enhance growth of NTHi.

304 There was a significant increase in carrying capacity for NCTC 11315 when grown in SCFM3 but a decrease for NCTC 12975.
305 Also, 10 μg/mL NAD only enhanced growth of one strain, NCTC 11315, by raising carrying capacity. Increased NAD
306 significantly reduced the growth rate of NCTC 4560. These results suggest while the presence of NAD is important for NTHi
307 growth, it reaches saturating effects at low concentrations.

308 Overall, these results highlight that SCFM2.SA.XV remains as the most optimal SCFM variant for NTHi growth, and more so,
309 that the eDNA component of SCFM2.SA.XV plays a significant role in supporting the growth of NTHi *in vitro* in SCFM.
310 Although not investigated in this study, eDNA concentrations could be varied to further optimise SCFM2.SA.XV in biofilm
311 studies, as biofilm structure is thought to be highly sensitive to eDNA concentrations (seen in *P. aeruginosa*) [24], and
312 changes in concentration can be the difference between small, but well-developed biofilms, or gelatinous masses and
313 scattered cells. Furthermore, the source of eDNA may influence growth as a result of differences in DNA fragment length
314 incorporated into biofilms.

315 Growth of clinical *H. influenzae* isolates was enhanced when grown in SCFM2.SA.XV, demonstrated by 316 increased carrying capacity

317 The adequacy of laboratory strains to replicate real-world pathogenesis is debated. Many laboratory strains have been sub-
318 cultured over decades since their first isolation, and may have lost important characteristics [69]. We obtained twenty-four
319 clinical NTHi isolates from The Swiss Cystic Fibrosis Infant Lung Development (SCILD) cohort [96][97], and their growth
320 profiles were investigated over 24 hours in SCFM2.SA.XV. We hypothesized that if SCFM2.SA.XV replicated an *in vivo* CF lung
321 environment well, that the media should be able to support the growth of clinical isolates.

322 We found that all isolates grew in SCFM2.SA.XV. Four isolates (ID41, ID42, ID47 and ID55) had a significantly higher carrying
323 capacity than NCTC 4560, whilst three isolates (ID8, ID21 and ID37) isolates had a significantly lower carrying capacity (**Figure**
324 **5A**).

325 When investigating growth rate, three isolates (ID21, ID33 and ID48) had a significantly higher growth rate than NCTC 4560
326 in SCFM2.SA.XV (**Figure 5B**). These results suggest that only some NTHi clinical isolates are better adapted to grow in SCFM
327 than a laboratory strain. Growth curves are available in Supplementary Figure 5.

328 Only isolate ID21 demonstrated both a significantly higher growth rate and lower carrying capacity than NCTC 4560.
329 Potentially, there may be a trade-off such that ID21 is better adapted to utilise SCFM2.SA.XV substrates quickly, but as a
330 result, reaches a lower carrying capacity.

331 Sequencing of the clinical isolates is required to identify genetic differences between isolates which may result in enhanced
332 growth in SCFM2.SA.XV, and to clarify whether these NTHi isolates are the same strain persisting in one patient, as it is
333 common for individuals to remain colonised with an initial *H. influenzae* strain for up to 2 years without a strain turnover
334 [70].

335 Overall, it appears carrying capacity of clinical isolates in SCFM2.SA.XV is more variable than growth rate, with larger
336 standard deviation error bars for K than for r.

337 Growth of NTHi in *ex vivo* pig lung model with SCFM2.SA.XV

338 The presence of lung tissue has previously been shown to enhance growth of *S. aureus* and *P. aeruginosa* in an *ex-vivo* pig
339 lung model which utilises pig lungs and SCFM to mimic the CF lung environment [41][42]. However, there is no published
340 study investigating NTHi infection in an EVPL model. In our model, we do not completely sterilise the pig lung tissue prior to
341 use and so endogenous bacteria (pig commensals or environmental bacteria temporarily present in the lungs) can be

342 present. We found that *H. influenzae* is not morphologically very distinguishable from these bacteria by colony morphology.
343 Because there is a lack selective media for *H. influenzae*, a GFP-tagged, streptomycin resistant NTHi, NTHi-375 [51] was used
344 to facilitate identification of NHTi growth in the lung model. To provide a control for this method, a GFP-tagged strain of *P.*
345 *aeruginosa*, which is known to grow to high density and form biofilm in the EVPL model, was also assayed. An ampicillin +
346 gentamicin wash of the lung tissue was added to previous model preparation methods, to attempt to reduce the titre of
347 endogenous bacteria, before infecting with GFP-tagged NTHi or *P. aeruginosa*, incubating, and plating on VBC sBHI agar (+ 2
348 µg/mL streptomycin for NTHi). Preliminary work highlighted that this antibiotic wash and plating combination was the most
349 successful for limiting endogenous bacteria growth in the EVPL and on plates used to enumerate target bacteria,
350 respectively. As NTHi is able to invade both bronchioles [71] and alveolar regions of the lung, [15], both bronchiolar and
351 alveolar tissue was dissected from the pig lung and infected to determine whether infection site affected the ability of NTHi
352 to grow.

353 Our washing step did not completely remove endogenous bacteria, as shown by the high bacterial titre in uninfected tissues
354 (Supplementary Figure 6). Furthermore, endogenous bacteria grew on the selective agar plates, so again we could not
355 reliably distinguish NTHi.

356 The fluorescence profiles of each tissue section were measured at 0 hours, 24 hours and 48 hours post infection to attempt
357 to visualize NTHi colonization of the lung tissue compared to un-infected tissue (**Figure 6**). Uninfected tissue presented some
358 background fluorescence due to the auto-fluorescence of collagen. However, fluorescence of *NHTi375*-infected tissue did not
359 exceed fluorescence of uninfected tissue at any time point, unlike *P. aeruginosa* for which growth was clearly visible. This
360 suggests that NTHi is unable to establish itself in the EVPL model, despite the presence of optimised SCFM. We hypothesise
361 that this is due to an inability to out-compete endogenous bacteria, but maybe also be because NTHi is an exclusively human
362 pathogen. Further model optimisation is needed to determine whether the EVPL model can be used with NTHi.

363 Lung homogenate media enhances biofilm growth of some NTHi CF isolates over SCFM2.SA.XV alone

364 Since the presence of lung tissue has shown to be beneficial to the growth of other CF pathogens [42], but the presence of
365 endogenous bacteria seemingly prevented NTHi establishing in the model, lung homogenate media was created by
366 homogenising fresh, uninfected lung tissue in SCFM2.SA.XV and filter sterilising. This retains the presence of host factors,
367 whilst removing potential competitors. We investigated whether lung homogenate media enhanced *in vitro* biofilm
368 formation of both NTHi laboratory isolates and CF clinical isolates through crystal violet staining after 48 hours of static
369 growth. Nine clinical isolates which had previously demonstrated enhanced growth rate or carrying capacity in SCFM2.SA.XV
370 were used for this experiment.

371 We found that lung homogenate significantly increased biofilm formation in four of the clinical isolates (ID21, ID42, ID55
372 and ID58), alongside laboratory strain NCTC 4560, when compared to SCFM2.SA.XV alone. Meanwhile, a significant decrease
373 in biofilm formation was seen with ID33. The other isolates were unaffected by the presence of lung homogenate **(Figure 7)**.

374 Lung homogenate media also significantly enhanced planktonic growth of five clinical isolates (ID21, ID42, ID47, ID55 and
375 ID58) and the laboratory strain NCTC 11315. Overall, four of the nine clinical isolates tested (ID21, ID42, ID55 and ID58) had
376 a significant increase in both biofilm and planktonic growth when grown in lung homogenate media compared to
377 SCFM2.SA.XV alone. These results suggest that the presence of lung components is capable of enhancing biofilm formation
378 and planktonic growth of some clinical isolates. However, the growth of replica cultures when in lung homogenate media

379 was much more variable than in SCFM2.SA.XV alone, suggesting the usage of lung homogenate media results in less
380 reproducible growth.
381

382 ## Discussion

383 Cystic fibrosis imposes a large economic burden on healthcare systems, individuals with CF and their caregivers. The burden
384 is thought to be underestimated, with many studies only reporting healthcare costs [72]. The increasing prevalence of NTHi
385 [73][74], alongside the changing landscape of CF lung infection with the availability of CFTR modulators, may place a greater
386 importance on handling and treating NTHi infections in people with CF [8][9][10][11] [75].

387 There are increasing concerns about the relevance of standard nutrient-rich laboratory media to replicate *in vivo* conditions
388 [76] [77] [78]. Host-mimicking media such as SCFM have been demonstrated to better recapitulate the growth dynamics of
389 pathogens such as *P. aeruginosa* and *S. aureus* in the CF lung [38][47] [46]. However, to our knowledge, the growth of the
390 early CF pathogen *H. influenzae* has not previously been studied in any form of CF mimicking media *in vitro*. In this study, we
391 present data on the planktonic growth profiles and biofilm formation of NTHi laboratory and clinical CF isolates in different
392 synthetic sputum media *in vitro*, and present a partially optimised SCFM for usage with NTHi *in vitro*: SCFM2 with mucin
393 replaced by N-acetylneuraminic acid, supplemented with NAD and hemin.

394 We found that all NTHi grew poorly planktonically in SCFM1. This is the simplest form of SCFM we used, and lacks common
395 CF sputum components such as mucin and eDNA. The carrying capacity and growth rate of NTHi was significantly improved
396 when grown in modified SCFM2 (SCFM2.SA), which contained additional components including sialic acid, GlcNAc and eDNA.
397 We also investigated SCFM2.SA with a source of NAD and hemin either directly, or in the form of FBS. We found that FBS as a
398 source of NAD and hemin hindered biofilm formation, and instead promoted planktonic growth, whilst NAD and hemin as a
399 supplement directly appeared to increase extracellular matrix production, particularly in the laboratory strains NTHi NCTC
400 4560 and NCTC 11315. Potentially, different ratios of NAD and hemin could be investigated to further optimise SCFM. The
401 negative effect of FBS on biofilms has previously been documented with *P. aeruginosa* [79] as well as *S. aureus* alone and in a
402 polymicrobial biofilm with *Candida albicans* [80]. Components of FBS such as the serum proteins lactoferrin, apo-transferrin,
403 and albumin may have contributed the inhibition of biofilm formation [81] [79] [82].

404 We found that the presence of eDNA in SCFM2.SA.XV was crucial for the enhanced the growth of NTHi. We found that both
405 the growth rate and carrying capacity of NTHi were significantly reduced when eDNA was omitted from SCFM2.SA.XV.
406 Extracellular DNA has been shown to be present within the matrix of biofilms and is essential for biofilm maintenance of
407 *Haemophilus influenzae* in an otitis media model, and the structure of an otitis media isolate [83][84] [85]. Our results
408 support the idea that eDNA is crucial for NTHi growth and biofilm formation, and the presence of eDNA is also important in
409 the cystic fibrosis lung. The eDNA used as part of SCFM is of low molecular weight (100-200b), but eDNA fragments *in vivo*
410 can vary widely in size: DNA released from cell lysis can be up to several kilobases, while secreted DNA is often smaller.
411 Therefore, we suggest exploring whether alternate sources of eDNA, with varying lengths, also influence the growth of NTHi
412 [99][100].

413 We also found that replacing bovine maxillary mucin specified in the original SCFM2 recipe with the sialic acid N-
414 acetylneuraminic acid increased the growth rate and carrying capacity of NTHi laboratory strains when compared to SCFM1.
415 NTHi is unable to synthesise its own sialic acid, and therefore depends on uptake of sialic acid from the host [86]. Sialic acid

416 has been shown to be important in the matrix formation of NTHi biofilms through a backbone of polysaccharide and 2,6
417 sialic acid [87], and NTHi deficient in the ability to sialylate its lipooligosaccharide have been shown to exhibit an impaired
418 ability to form biofilms *in vitro* [88] [87] [37]. Our results align with the idea that biofilm formation of NTHi is enhanced in
419 media containing sialic acid, and that sialic acid is an important component for NTHi biofilm formation [87][29][89][90]. As
420 such, we deemed SCFM2.SA.XV the most optimal media for usage with NTHi as it supports biofilm formation, and
421 *Haemophilus influenzae* isolated from people with CF have been shown to be strong biofilm producers *in vitro* in the
422 literature [91].

423 We then went on to investigate the ability of NTHi to form biofilms *ex vivo*. We attempted to grow a GFP-tagged isolate,
424 NTHi-375 in an EVPL model, to replicate both the structural and biochemical environment in the CF lung [48] [54]. However,
425 we were unable to successfully grow or identify the presence of NTHi on either bronchiole or alveolar tissue by fluorimetry,
426 and we could not culture NTHi despite the usage of selective agar plates. NTHi is an obligate human pathogen, and does not
427 naturally infect pigs [92]. As such, it is likely this may have affected the ability of NTHi to colonise the lung tissue. We also
428 found that the specificity of NTHi isolation agar was poor. In the literature, haemin-bacitracin chocolate agar is only able to
429 isolate *H. influenzae* from CF sputa at a rate of 13.7%, and cefsulodin-chocolate agar at 17.8% [93]. We found chocolate agar
430 supplemented with vancomycin, bacitracin and clindamycin (VBC) was the most successful at inhibiting endogenous bacteria
431 present on the lung tissue, which aligns with literature results reporting an isolation rate of 59.9% [94]. However, when
432 compared to the specificity of mannitol salt agar for *S. aureus*, which is as high as 97.6%, it is clear there is a lack of selective
433 media to isolate *H. influenzae* from respiratory cultures [95]. We also found that even washing the lung tissue with high
434 concentrations of ampicillin and gentamicin prior to infection did not remove a sufficient level of endogenous bacteria, which
435 may have interfered with NTHi colonisation. Other pathogens including *P. aeruginosa* and *S. aureus* have been successfully
436 grown in the EVPL model [41] [42]. *P. aeruginosa* has been shown to easily out-compete endogenous bacteria present on the
437 bronchiole tissue sections [41], and can be readily isolated using selective agar, whilst *S. aureus* could successfully be
438 distinguished from endogenous bacteria using through plating on highly selective mannitol salt agar [42]. As such, further
439 work is required to better isolate NTHi in the presence of endogenous lung bacteria, and we recommend using an NTHi
440 isolate with an antibiotic resistance marker to improve isolation. However, it is important to note that this may interfere with
441 antibiotic susceptibility studies due to antagonism or synergy between antibiotics used for selection and those under
442 investigation, and resistant mutants may have altered fitness over their wild-type variant.

443 As the presence of lung tissue has been shown to be important in enhancing the growth of other CF pathogens [42] [41],
444 we added lung homogenate into SCFM2.SA.XV, to keep important components of the lung tissue *in vitro,* whilst removing
445 endogenous bacteria. We found that lung homogenate media enhanced biofilm formation of some clinical NTHi isolates,
446 suggesting that there are components in the lung tissue that contribute to the growth of NTHi and these are not captured in
447 any published SCFM recipe.

448 We have optimised SCFM in the form of SCFM2.SA.XV for the usage with NTHi, and have highlighted the importance of the
449 presence of eDNA in supporting NTHi growth and biofilm formation. However, further work is required to take NTHi into the
450 EVPL model, including higher specificity isolation agar, and removal of endogenous bacteria. We suggest that
451 proteomic/metabolomic analysis of the lung homogenate media would help suggest a new formulation of SCFM for better
452 replicating the CF lung environment to facilitate further research into NTHi biology.

Conclusion

An array of synthetic CF sputum media has been developed to attempt to capture the complex nutritional composition of CF sputum [38][46][47]. We found that additions of eDNA, sialic acid and NAD and hemin to SCFM were crucial for NTHi growth and biofilm formation, and that presence of pig lung homogenate enhanced the growth of some clinical NTHi CF isolates even further. Although SCFM2.SA.XV and lung homogenate media improved the growth of NTHi *in vitro,* more work is required to develop a representative CF sputum mimicking media that allows suitable growth of NTHi to better replicate what occurs *in vivo*. We suggest that chemical analysis of the components found in lung homogenate could help to develop an SCFM better suited for studying NTHi microbiology. Our work highlights the need for a representative CF sputum medium that is optimised to support NTHi growth, and we have performed preliminary development of such a medium. Through this development, we can start to better understand the infection dynamics of NTHi, its interactions with other CF pathogens, and its antibiotic susceptibility profile in the CF lung.

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Bibliography

[1] Endres TM, Konstan MW. What Is Cystic Fibrosis? Jama. 2022;327(2):191-1.

[2] Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. American journal of respiratory and critical care medicine. 2003;168(8):918-51.

[3] UK Cystic Fibrosis Registry; 2020.

[4] Abman SH, Ogle JW, Harbeck RJ, Butler-Simon N, Hammond KB, Accurso FJ. Early bacteriologic, immunologic, and clinical courses of young infants with cystic fibrosis identified by neonatal screening. The Journal of pediatrics. 1991;119(2):211-7.

[5] Goetz DM, Savant AP. Review of CFTR modulators 2020. Pediatric pulmonology. 2021;56(12):3595-606.

[6] Ramsey BW, Davies J, McElvaney NG, Tullis E, Bell SC, Dˇrev´ınek P, et al. A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. New England Journal of Medicine. 2011;365(18):1663-72.

[7] Heijerman HG, McKone EF, Downey DG, Van Braeckel E, Rowe SM, Tullis E, et al. Efficacy and safety of the elexacaftor plus tezacaftor plus ivacaftor combination regimen in people with cystic fibrosis homozygous for the F508del mutation: a double-blind, randomised, phase 3 trial. The Lancet. 2019;394(10212):1940-8.

[8] Watts SC, Judd LM, Carzino R, Ranganathan S, Holt KE. Genomic Diversity and Antimicrobial Resistance of Haemophilus Colonizing the Airways of Young Children with Cystic Fibrosis. Msystems. 2021;6(4):101128.

[9] Heltshe SL, Mayer-Hamblett N, Burns JL, Khan U, Baines A, Ramsey BW, et al. Pseudomonas aeruginosa in cystic fibrosis patients with G551D-CFTR treated with ivacaftor. Clinical Infectious Diseases. 2015;60(5):703-12.

[10] Bessonova L, Volkova N, Higgins M, Bengtsson L, Tian S, Simard C, et al. Data from the US and UK cystic fibrosis registries support disease modification by CFTR modulation with ivacaftor. Thorax. 2018;73(8):731-40.

[11] Ronan NJ, Einarsson GG, Twomey M, Mooney D, Mullane D, NiChroinin M, et al. CORK study in cystic fibrosis: sustained improvements in ultra-low-dose chest CT scores after CFTR modulation with ivacaftor. Chest. 2018;153(2):395-403.

[12] Bilton D, Pye A, Johnson M, Mitchell J, Dodd M, Webb A, et al. The isolation and characterization of nontypeable Haemophilus influenzae from the sputum of adult cystic fibrosis patients. European Respiratory Journal. 1995;8(6):948-53.

[13] Rayner R, Hiller E, Ispahani P, Baker M. Haemophilus infection in cystic fibrosis. Archives of Disease in Childhood. 1990;65(3):255-8.

[14] Bhatt JM. Treatment of pulmonary exacerbations in cystic fibrosis. European Respiratory Review. 2013;22(129):205-16.

[15] Moller LV, Timens W, Van der Bij W, Kooi K, de Wever B, Dankert J, et al. Haemophilus influenzae in lung explants of patients with end-stage pulmonary disease. American journal of respiratory and critical care medicine. 1998;157(3):950-6.

[16] King P. Haemophilus influenzae and the lung (Haemophilus and the lung). Clinical and translational medicine. 2012;1(1):1-9.

[17] Erwin AL, Smith AL. Nontypeable Haemophilus influenzae: understanding virulence and commensal behavior. Trends in microbiology. 2007;15(8):355-62.

[18] Peltola H. Worldwide Haemophilus influenzae type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. Clinical microbiology reviews. 2000;13(2):302-17.

[19] Oliver SE, Rubis AB, Soeters HM, Reingold A, Barnes M, Petit S, et al. Epidemiology of Invasive Nontypeable Haemophilus influenzae Disease—United States, 2008–2019. Clinical Infectious Diseases. 2023;76(11):1889-95.

[20] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. science. 1999;284(5418):1318-22.

[21] Høiby N. Understanding bacterial biofilms in patients with cystic fibrosis: current and innovative approaches to potential therapies. Journal of Cystic Fibrosis. 2002;1(4):249-54.

[22] Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. Trends in microbiology. 2005;13(1):34-40.

[23] Swords WE. Nontypeable Haemophilus influenzae biofilms: role in chronic airway infections. Frontiers in cellular and infection microbiology. 2012;2:97.

[24] Haley CL, Colmer-Hamood JA, Hamood AN. Characterization of biofilm-like structures formed by Pseudomonas aeruginosa in a synthetic mucus medium. BMC microbiology. 2012;12(1):1-20.

[25] Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, et al. Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Developmental cell. 2002;3(4):593-603.

[26] Schaible B, Taylor CT, Schaffer K. Hypoxia increases antibiotic resistance in Pseudomonas aeruginosa through altering the composition of multidrug efflux pumps. Antimicrobial agents and chemotherapy. 2012;56(4):2114-8.

[27] Moxon ER, Sweetman WA, Deadman ME, Ferguson DJ, Hood DW. Haemophilus influenzae biofilms: hypothesis or fact? Trends in microbiology. 2008;16(3):95-100.

[28] Dohar JE. Evidence that otitis media is not a biofilm disease. Ear, Nose & Throat Journal. 2007;86(11 suppl):8-12.

[29] Langereis JD, Hermans PW. Novel concepts in nontypeable Haemophilus influenzae biofilm formation. FEMS microbiology letters. 2013;346(2):81-9.

[30] Ng J, Kidd SP. The concentration of intracellular nickel in Haemophilus influenzae is linked to its surface properties and cell–cell aggregation and biofilm formation. International Journal of Medical Microbiology. 2013;303(3):150-7.

[31] Gallaher TK, Wu S, Webster P, Aguilera R. Identification of biofilm proteins in non-typeable Haemophilus influenzae. BMC microbiology. 2006;6(1):1-9.

[32] Weeks JR, Staples KJ, Spalluto CM, Watson A, Wilkinson TM. The role of non-typeable Haemophilus influenzae biofilms in chronic obstructive pulmonary disease. Frontiers in Cellular and Infection Microbiology. 2021;11:720742.

[33] Hong W, Juneau RA, Pang B, Swords WE. Survival of bacterial biofilms within neutrophil extracellular traps promotes nontypeable Haemophilus influenzae persistence in the chinchilla model for otitis media. Journal of innate immunity. 2009;1(3):215-24.

[34] Post JC. Direct evidence of bacterial biofilms in otitis media. 2001. The Laryngoscope. 2015;125(9):2003-14.

[35] Ehrlich GD, Veeh R, Wang X, Costerton JW, Hayes JD, Hu FZ, et al. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. Jama. 2002;287(13):1710-5.

[36] Hunt BC, Stanford D, Xu X, Li J, Gaggar A, Rowe SM, et al. Haemophilus influenzae persists in biofilm communities in a smoke-exposed ferret model of COPD. ERJ open research. 2020;6(3).

[37] Starner TD, Zhang N, Kim G, Apicella MA, McCray Jr PB. Haemophilus influenzae forms biofilms on airway epithelia: implications in cystic fibrosis. American journal of respiratory and critical care medicine. 2006;174(2):213-20.

[38] Palmer KL, Aye LM, Whiteley M. Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis sputum. Journal of bacteriology. 2007;189(22):8079-87.

[39] La Rosa R, Johansen HK, Molin S. Adapting to the airways: metabolic requirements of Pseudomonas aeruginosa during the infection of cystic fibrosis patients. Metabolites. 2019;9(10):234.

[40] Harrington NE, Sweeney E, Alav I, Allen F, Moat J, Harrison F. Antibiotic efficacy testing in an ex vivo model of Pseudomonas aeruginosa and Staphylococcus aureus biofilms in the cystic fibrosis lung. JoVE (Journal of Visualized Experiments). 2021;(167):e62187.

[41] Harrington NE, Sweeney E, Harrison F. Building a better biofilm-formation of in vivo-like biofilm structures by Pseudomonas aeruginosa in a porcine model of cystic fibrosis lung infection. Biofilm. 2020;2:100024.

[42] Sweeney E, Hassan M, Harrington N, Smyth A, Hurley M, Tormo-Mas M, et al. An ex vivo cystic fibrosis model recapitulates key clinical aspects of chronic. Staphylococcus aureus. 2019.

[43] Aiyer A, Manos J. The Use of Artificial Sputum Media to Enhance Investigation and Subsequent Treatment of Cystic Fibrosis Bacterial Infections. Microorganisms. 2022;10(7):1269.

[44] Peterson BW, van der Mei HC, Sjollema J, Busscher HJ, Sharma PK. A distinguishable role of eDNA in the viscoelastic relaxation of biofilms. MBio. 2013;4(5):10-1128.

[45] Lethem M, James S, Marriott C, Burke J. The origin of DNA associated with mucus glycoproteins in cystic fibrosis sputum. European Respiratory Journal. 1990;3(1):19-23.

[46] Turner KH, Wessel AK, Palmer GC, Murray JL, Whiteley M. Essential genome of Pseudomonas aeruginosa in cystic fibrosis sputum. Proceedings of the National Academy of Sciences. 2015;112(13):4110-5.

[47] Neve RL, Carrillo BD, Phelan VV. Impact of artificial sputum medium formulation on Pseudomonas aeruginosa secondary metabolite production. Journal of Bacteriology. 2021;203(21):10-1128.

[48] Harrison F, Diggle SP. An ex vivo lung model to study bronchioles infected with Pseudomonas aeruginosa biofilms. Microbiology. 2016;162(10):1755-60.

[49] Sweeney E, Sabnis A, Edwards AM, Harrison F. Effect of host-mimicking medium and biofilm growth on the ability of colistin to kill Pseudomonas aeruginosa. Microbiology. 2020;166(12):1171.

[50] Cody AJ, Field D, Feil EJ, Stringer S, Deadman ME, Tsolaki AG, et al. High rates of recombination in otitis media isolates of non-typeable Haemophilus influenzae. Infection, Genetics and Evolution. 2003;3(1):57-66.

[51] Mulay A, Akram KM, Williams D, Armes H, Russell C, Hood D, et al. An in vitro model of murine middle ear epithelium. Disease Models & Mechanisms. 2016;9(11):1405-17.

[52] Holloway B. Genetic recombination in Pseudomonas aeruginosa. Microbiology. 1955;13(3):572-81.

[53] Sprouffske K, Wagner A. Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. BMC bioinformatics. 2016;17:1-4.

[54] Harrison F, Muruli A, Higgins S, Diggle SP. Development of an ex vivo porcine lung model for studying growth, virulence, and signaling of Pseudomonas aeruginosa. Infection and immunity. 2014;82(8):3312-23.

[55] Harrington NE, Littler JL, Harrison F. Transcriptome analysis of Pseudomonas aeruginosa biofilm infection in an ex vivo pig model of the cystic fibrosis lung. Applied and Environmental Microbiology. 2022;88(3):e01789-21.

[56] Glasser NR, Hunter RC, Liou TG, Newman DK, Investigators MWCC. Refinement of metabolite detection in cystic fibrosis sputum reveals heme correlates with lung function decline. PLoS One. 2019;14(12):e0226578.

[57] Barben JU, Ditchfield M, Carlin JB, Robertson CF, Robinson PJ, Olinsky A. Major haemoptysis in children with cystic fibrosis: a 20-year retrospective study. Journal of Cystic Fibrosis. 2003;2(3):105-11.

[58] Sagel SD, Kapsner R, Osberg I, Sontag MK, Accurso FJ. Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction. American journal of respiratory and critical care medicine. 2001;164(8):1425-31.

[59] Lewis AL, Chen X, Schnaar RL, Varki A. Sialic acids and other nonulosonic acids. Essentials of Glycobiology [Internet] 4th edition. 2022.

[60] Jackson MD, Wong SM, Akerley BJ. Sialic Acid Protects Nontypeable Haemophilus influenzae from Natural IgM and Promotes Survival in Murine Respiratory Tract. Infection and Immunity. 2021;89(6).

[61] Cantin AM, Bilodeau G, Lariv´ee P, Richter MV. Plasma biomarkers and cystic fibrosis lung disease. Clinical and Investigative Medicine. 2012:E173-81.

[62] Rom´an F, Cant´on R, P´erez-V´azquez M, Baquero F, Campos J. Dynamics of long-term colonization of respiratory tract by Haemophilus influenzae in cystic fibrosis patients shows a marked increase in hypermutable strains. Journal of clinical microbiology. 2004;42(4):1450-9.

[63] Begum N, Byrnes CA, Cheney J, Cooper PJ, Fantino E, Gailer N, et al. Factors in childhood associated with lung function decline to adolescence in cystic fibrosis. Journal of Cystic Fibrosis. 2022;21(6):977-83.

[64] Rosenfeld M, Emerson J, Accurso F, Armstrong D, Castile R, Grimwood K, et al. Diagnostic accuracy of oropharyngeal cultures in infants and young children with cystic fibrosis. Pediatric pulmonology. 1999;28(5):321-8.

[65] Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sørensen SR, Moser C, Kühl M, et al. The in vivo biofilm. Trends in microbiology. 2013;21(9):466-74.

[66] Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically feasible biofilm susceptibility assay for isolates of Pseudomonas aeruginosa from patients with cystic fibrosis. Journal of clinical microbiology. 2004;42(5):1915-22.

[67] Choong FX, Bäck M, Fahlén S, Johansson LB, Melican K, Rhen M, et al. Real-time optotracing of curli and cellulose in live Salmonella biofilms using luminescent oligothiophenes. npj Biofilms and Microbiomes. 2016;2(1):1-11.

[68] Ferguson DL, Gloag ES, Parsek MR, Wozniak DJ. Extracellular DNA enhances biofilm integrity and mechanical properties of mucoid Pseudomonas aeruginosa. Journal of Bacteriology. 2023;205(10):e0023823.

[69] Fux C, Shirtliff M, Stoodley P, Costerton JW. Can laboratory reference strains mirror 'realworld'pathogenesis? Trends in microbiology. 2005;13(2):58-63.

[70] M¨oller LV, Regelink AG, Grasselier H, Dankert-Roelse JE, Dankert J, van Alphen L. Multiple Haemophilus influenzae strains and strain variants coexist in the respiratory tract of patients with cystic fibrosis. Journal of Infectious Diseases. 1995;172(5):1388-92.

[71] Morey P, Cano V, Mart´ı-Lliteras P, Lopez-Gomez A, Regueiro V, Saus C, et al. Evidence for a nonreplicative intracellular stage of nontypable Haemophilus influenzae in epithelial cells. Microbiology. 2011;157(1):234-50.

[72] Cheema ZM, Gomez LC, Johnson N, Laflamme OD, Rabin HR, Steele K, et al. Measuring the burden of cystic fibrosis: A scoping review. Journal of Cystic Fibrosis. 2023.

[73] Ebbing R, Robertson C, Robinson P. Haemophilus influenzae and Haemophilus parainfluenza in cystic fibrosis: 15 years experience. J Med Microbiol Diagn. 2015;5:2161-0703.

[74] Razvi S, Quittell L, Sewall A, Quinton H, Marshall B, Saiman L. Respiratory microbiology of patients with cystic fibrosis in the United States, 1995 to 2005. Chest. 2009;136(6):1554-60.

[75] Abavisani M, Keikha M, Karbalaei M. First global report about the prevalence of multi-drug resistant Haemophilus influenzae: a systematic review and meta-analysis. BMC Infectious Diseases. 2024;24(1):90.

[76] Garcia Maset R, Hapeshi A, Hall S, Dalgliesh RM, Harrison F, Perrier S. Evaluation of the antimicrobial activity in host-mimicking media and in vivo toxicity of antimicrobial polymers as functional mimics of AMPs. ACS Applied Materials & Interfaces. 2022;14(29):32855-68.

[77] Barton TE, Frost F, Fothergill JL, Neill DR. Challenges and opportunities in the development of novel antimicrobial therapeutics for cystic fibrosis. Journal of Medical Microbiology. 2022;71(12):001643.

[78] Ersoy SC, Heithoff DM, Barnes L, Tripp GK, House JK, Marth JD, et al. Correcting a fundamental flaw in the paradigm for antimicrobial susceptibility testing. EBioMedicine. 2017;20:173-81.

[79] Hammond A, Dertien J, Colmer-Hamood JA, Griswold JA, Hamood AN. Serum inhibits P. aeruginosa biofilm formation on plastic surfaces and intravenous catheters. Journal of surgical research. 2010;159(2):735-46.

[80] Hernandez-Cuellar E, Guerrero-Barrera AL, Avelar-Gonzalez FJ, Díaz JM, de Santiago AS, Chávez-Reyes J, et al. Characterization of Candida albicans and Staphylococcus aureus polymicrobial biofilm on different surfaces. Revista Iberoamericana de Micolog´ıa. 2022;39(2):36-43.

[81] Ardehali R, Shi L, Janatova J, Mohammad SF, Burns GL. The inhibitory activity of serum to prevent bacterial adhesion is mainly due to apo-transferrin. Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials. 2003;66(1):21-8.

[82] Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. Nature. 2002;417(6888):552-5.

[83] Domenech M, Pedrero-Vega E, Prieto A, García E. Evidence of the presence of nucleic acids and β-glucan in the matrix of non-typeable Haemophilus influenzae in vitro biofilms. Scientific Reports. 2016;6(1):36424.

[84] Jurcisek JA, Bakaletz LO. Biofilms formed by nontypeable Haemophilus influenzae in vivo contain both double-stranded DNA and type IV pilin protein. Journal of bacteriology. 2007;189(10):3868-75.

[85] Das J, Mokrzan E, Lakhani V, Rosas L, Jurcisek JA, Ray WC, et al. Extracellular DNA and type IV pilus expression regulate the structure and kinetics of biofilm formation by nontypeable Haemophilus influenzae. MBio. 2017;8(6):10-1128.

[86] Apicella MA. Nontypeable Haemophilus influenzae: the role of N-acetyl-5-neuraminic acid in biology. Frontiers in cellular and infection microbiology. 2012;2:19.

[87] Greiner L, Watanabe H, Phillips N, Shao J, Morgan A, Zaleski A, et al. Nontypeable Haemophilus influenzae strain 2019 produces a biofilm containing N-acetylneuraminic acid that may mimic sialylated O-linked glycans. Infection and immunity. 2004;72(7):4249-60.

[88] Swords WE, Moore ML, Godzicki L, Bukofzer G, Mitten MJ, VonCannon J. Sialylation of lipooligosaccharides promotes biofilm formation by nontypeable Haemophilus influenzae. Infection and immunity. 2004;72(1):106-13.

[89] Hong W, Mason K, Jurcisek J, Novotny L, Bakaletz LO, Swords WE. Phosphorylcholine decreases early inflammation and promotes the establishment of stable biofilm communities of nontypeable Haemophilus influenzae strain 86-028NP in a chinchilla model of otitis media. Infection and immunity. 2007;75(2):958-65.

[90] Jurcisek J, Greiner L, Watanabe H, Zaleski A, Apicella MA, Bakaletz LO. Role of sialic acid and complex carbohydrate biosynthesis in biofilm formation by nontypeable Haemophilus influenzae in the chinchilla middle ear. Infection and immunity. 2005;73(6):3210-8.

[91] Cardines R, Giufrè M, Pompilio A, Fiscarelli E, Ricciotti G, Di Bonaventura G, et al. Haemophilus influenzae in children with cystic fibrosis: antimicrobial susceptibility, molecular epidemiology, distribution of adhesins and biofilm formation. International Journal of Medical Microbiology. 2012;302(1):45-52.

[92] Dickerman A, Bandara AB, Inzana TJ. Phylogenomic analysis of Haemophilus parasuis and proposed reclassification to Glaesserella parasuis, gen. nov., comb. nov. International Journal of Systematic and Evolutionary Microbiology. 2020;70(1):180-6.

[93] Smith A, Baker M. Cefsulodin chocolate blood agar: a selective medium for the recovery of Haemophilus influenzae from the respiratory secretions of patients with cystic fibrosis. Journal of medical microbiology. 1997;46(10):883-5.

[94] Chapin K, Doern G. Selective media for recovery of Haemophilus influenzae from specimens contaminated with upper respiratory tract microbial flora. Journal of Clinical Microbiology. 1983;17(6):1163-5.

[95] Kampf G, Lecke C, Cimbal AK, Weist K, Ruden H. Evaluation of mannitol salt agar for detection of oxacillin resistance in Staphylococcus aureus by disk diffusion and agar screening. Journal of clinical microbiology. 1998;36(8):2254-7.

[96] Korten I, Kieninger E, Yammine S, Regamey N, Nyilas S, Ramsey K, et al. The swiss cystic fibrosis infant lung development (SCILD) cohort. Swiss medical weekly. 2018;148(1718):w14618-8.

[97] Mostacci N, Wüthrich TM, Siegwald L, Kieser S, Steinberg R, Sakwinska O, et al. Informed interpretation of metagenomic data by StrainPhlAn enables strain retention analyses of the upper airway microbiome. Msystems. 2023;8(6):e00724-3.

[98] Dwyer M, Shan Q, D'ortona S, Maurer R, Mitchell R, Olesen H, et al. Cystic fibrosis sputum DNA has NETosis characteristics and neutrophil extracellular trap release is regulated by macrophage migration-inhibitory factor. Journal of innate immunity. 2014;6(6):765-79.

[99] Zafra O, Lamprecht-Grandío M, de Figueras CG, González-Pastor JE. Extracellular DNA release by undomesticated Bacillus subtilis is regulated by early competence. PloS one. 2012;7(11):e48716.

[100] Sharma DK, Rajpurohit YS. Multitasking functions of bacterial extracellular DNA in biofilms. Journal of Bacteriology. 2024;206(4):e00006-24.

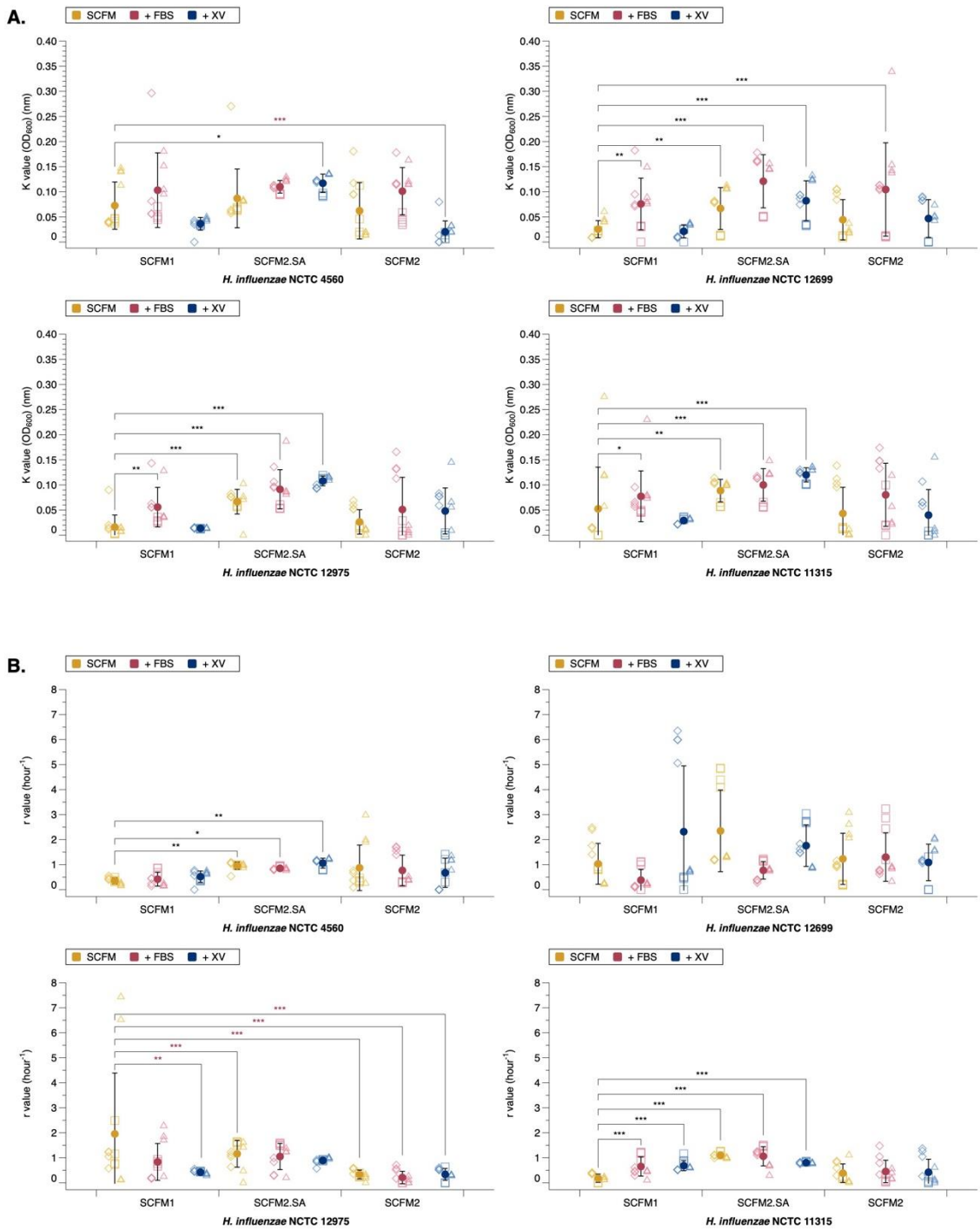

Figure 1: The influence of different SCFM media on the growth of four laboratory *H. influenzae* strains. Laboratory *H. influenzae* strains were grown in either SCFM1, SCFM2 or SCFM2.SA alone or supplemented with either fetal bovine serum or NAD and hemin. Carrying capacity and growth rate were calculated from 3 independent experiments (demonstrated by different shapes), with the mean and standard deviation shown by the solid circle. Significance was defined as $p \leq 0.05$. An ANOVA was run to test the effect of strain type on carrying capacity (NCTC 12975 $F_{8,97} = 13.17$, $p = < 0.001$, NCTC 12699 $F_{8,97} = 13.31$, $p = < 0.001$, NCTC 11315 $F_{8,97} = 8.70$, $p = < 0.001$, NCTC 4560 $F_{8,97} = 12.84$, $p = < 0.001$). Post-hoc Dunnett analysis identified which media variations resulted in a significant change in K compared to SCFM1. A significant increase in K for all strains was identified when grown in SCFM2.SA.XV (NCTC 12975 $p = < 0.001$, NCTC 12699 $p = < 0.001$, NCTC 13315 $p = < 0.001$, NCTC 4560 $p = 0.03$). K was also significantly increased in all strains except NCTC 4560 when grown in SCFM2.SA (NCTC 12975 $p = < 0.001$, NCTC 12699 $p = 0.007$, NCTC 11315 $p = 0.003$), SCFM2.SA.FBS (all except NCTC 4560 $p < 0.001$) and SCFM1.FBS (NCTC 12975 $p = 0.002$, NCTC 12699 $p = 0.002$, NCTC 11315 $p = 0.023$). A significant increase in K was seen with NCTC 12699 when grown in SCFM2.FBS ($p < 0.001$) and a significant decrease in K was seen with NCTC 4560 when grown in SCFM2.XV) ($p < 0.001$).

For growth rate, an ANOVA was run to test the effect of strain type on growth rate (NCTC 12975 $F_{8,97} = 7.05$, $p = < 0.001$, NCTC 12699 $F_{8,97} = 3.87$, $p = < 0.001$, NCTC 11315 $F_{8,96} = 12.44$, $p = < 0.001$, NCTC 4560 $F_{8,97} = 4.14$, $p = < 0.001$). Dunnett analysis identified which media variations resulted in a significant change in r compared to SCFM1. Growth rate was significantly increased when grown in SCFM2.SA (NCTC 4560 $p < 0.001$, NCTC 11315 $p = 0.006$), SCFM2.SA.FBS (NCTC 4560 $p < 0.001$, NCTC 11315 $p = 0.022$), SCFM2.SA.XV (NCTC 4560 $p < 0.001$, NCTC 11315 $p = 0.001$) SCFM1.FBS (NCTC 11315 $p < 0.001$) and SCFM1.XV (NCTC 11315 $p < 0.001$). Growth rate of NCTC 12975 was significantly reduced in all variants of SCFM2 (all $p < 0.001$) as well as SCFM1.XV ($p = 0.003$). There was no change in r for NCTC 12699 in any media variant.

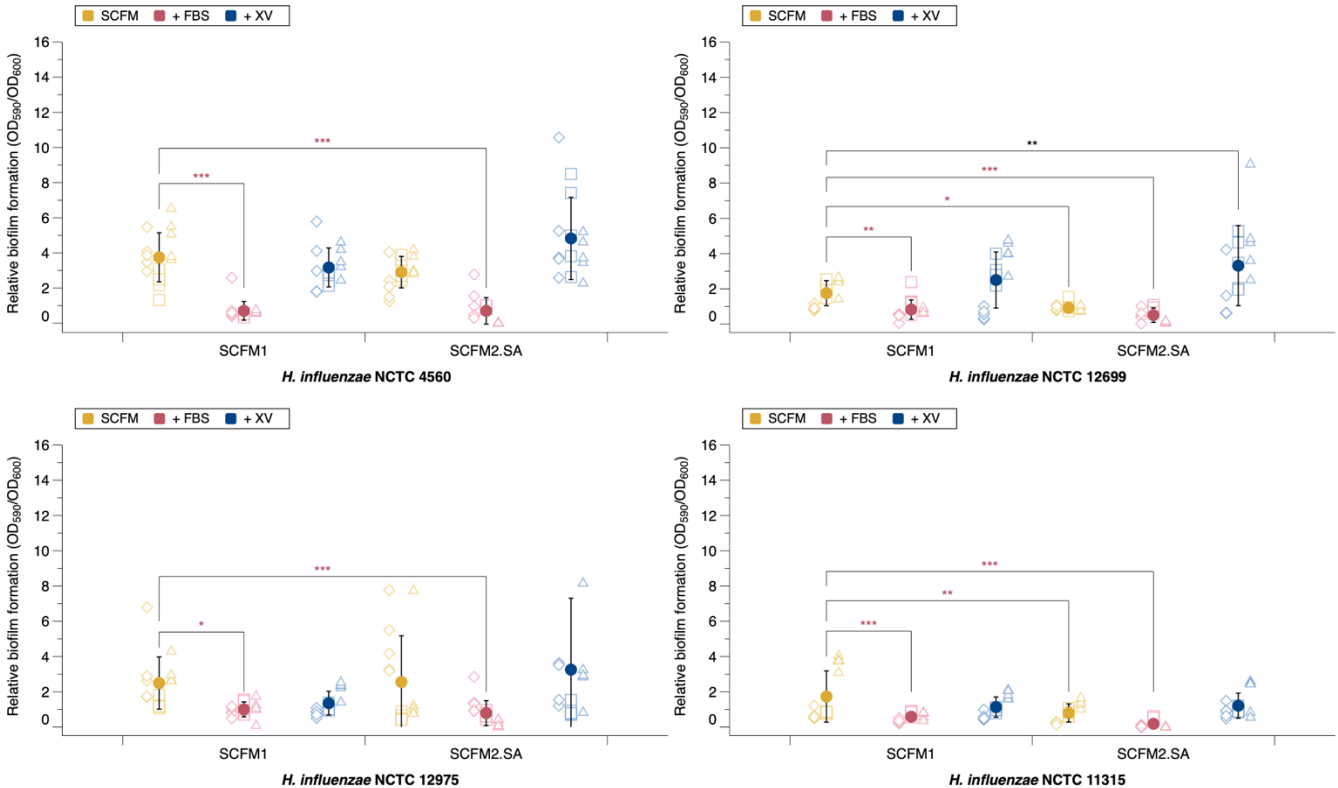

Figure 2: Relative biofilm allocation of four laboratory *H. influenzae* strains in varying SCFM conditions at 48 hours. Absorbance of biofilm was measured at 590 nm and planktonic at 600 nm. Mean and standard deviation was calculated from 3 independent experiments (demonstrated by different shapes), and shown by the solid circle. Significance was defined as $p \leq 0.05$. An ANOVA was run to test the effect of media variation on relative biofilm formation for each strain (NCTC 12975 $F_{5,82} = 5.93$, $p = <0.001$, NCTC 12699 $F_{5,82} = 20.21$, $p = <0.001$, NCTC 11315 $F_{5,82} = 21.41$, $p = <0.001$, NCTC 4560 $F_{5,82} = 37.51$, $p = <0.001$). Post-hoc Dunnett analysis identified which media and strain comparisons resulted in a significant change in relative biofilm formation compared to SCFM1. There was a significant reduction in relative biofilm formation with SCFM1.FBS (NCTC 12975 $p < 0.016$, NCTC 12699 $p < 0.005$, NCTC 11315 $p < 0.001$, NCTC 4560 $p < 0.001$) and SCFM2.SA (all $p < 0.001$). SCFM2.SA.XV significantly promoted relative biofilm formation in NCTC 12699 ($p = 0.007$). SCFM2.SA significantly reduced relative biofilm formation of NCTC 11315 ($p = 0.034$) and NCTC 12699 ($p = 0.002$).

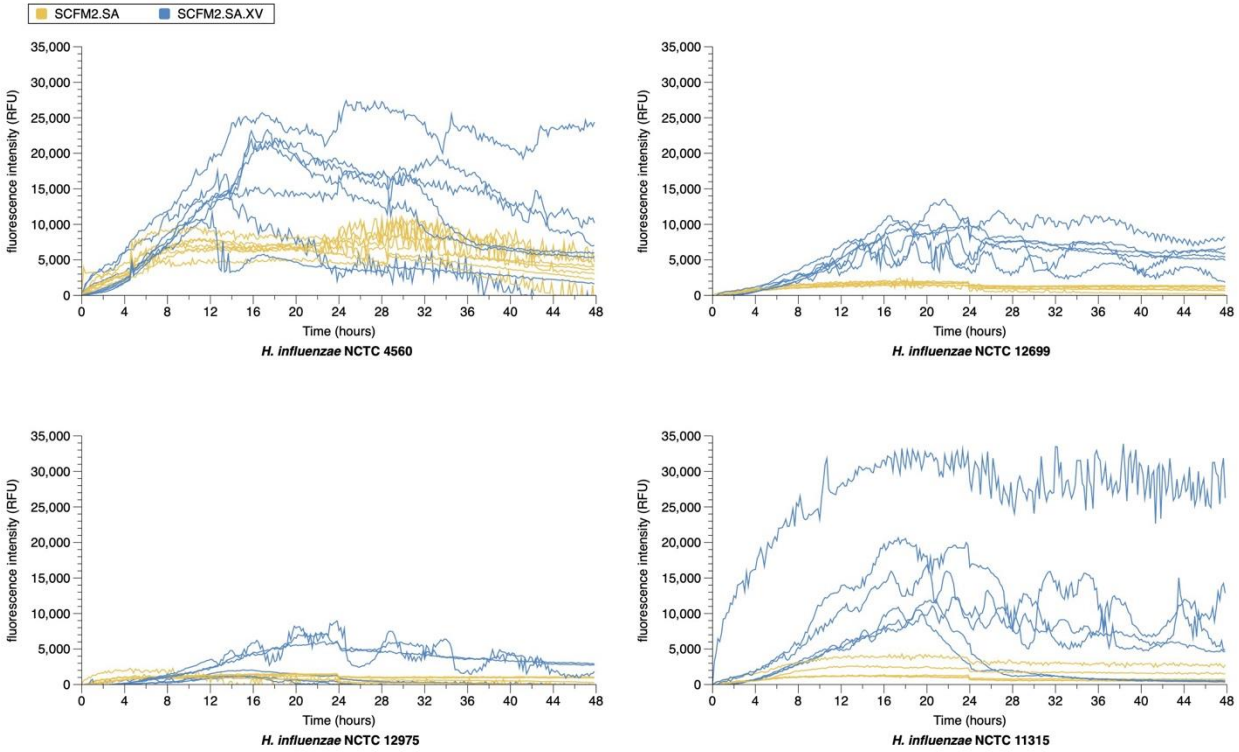

Figure 3: 48-hour growth of *H. influenzae* with extracellular matrix marker EBBA BioLight. NTHi laboratory strains NCTC 4560, NCTC 12699, NCTC 12975 and NCTC 11315 were grown statically for 48 hours in SCFM2.SA and SCFM2.SA.XV. Fluorescence was measured at 540/570 ex/em (nm) over two independent experiments.

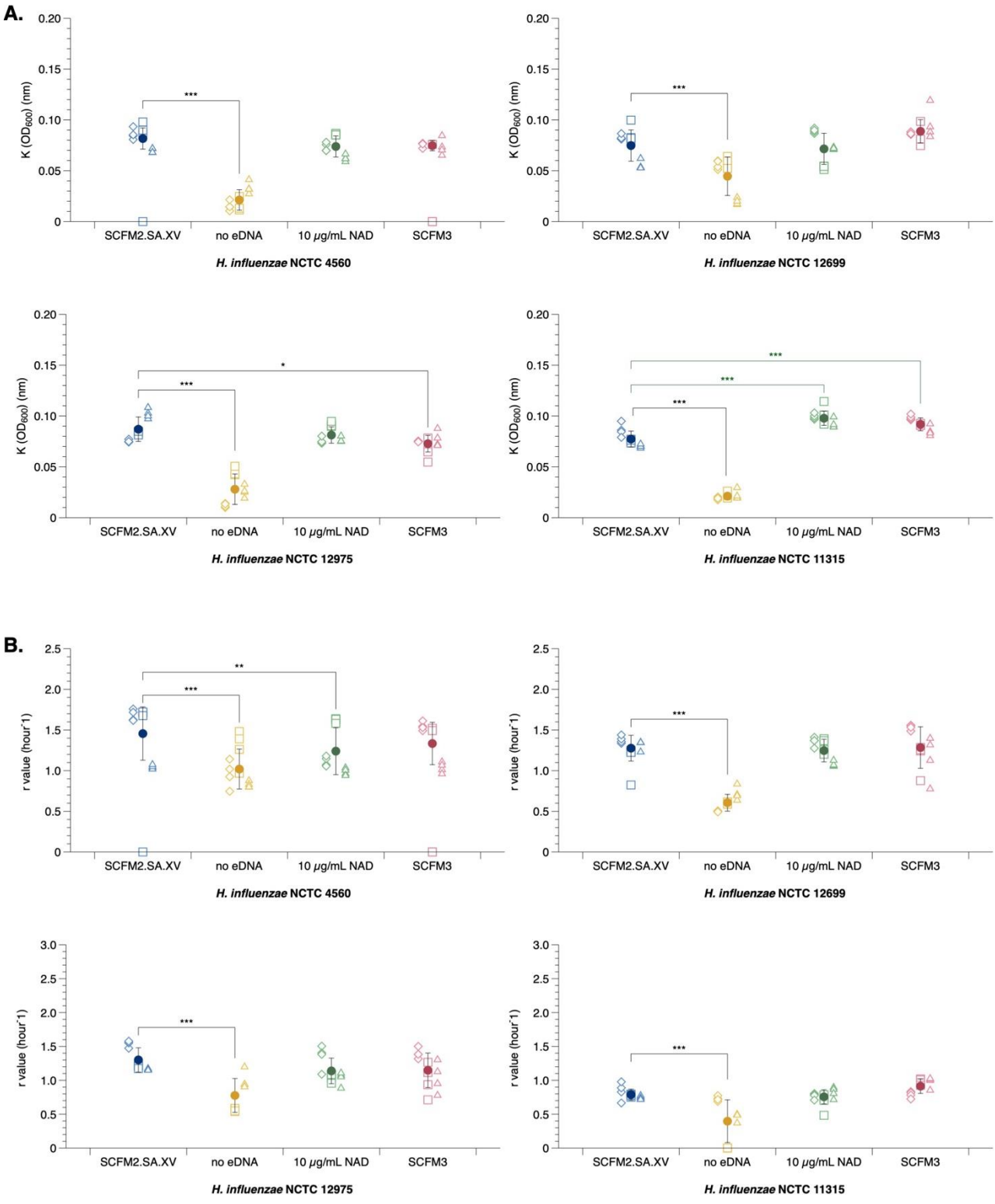

Figure 4: Carrying capacity and growth rate of *H. influenzae* in different SCFM medias. NTHi laboratory strains NCTC 4560, NCTC 12699, NCTC 12975 and NCTC 11315 were grown statically for 24 hours in either SCFM2.SA.XV, SCFM2.SA.XV minus eDNA, SCFM2.SA.XV with NAD at 10 µg/mL or SCFM3. Optical density was measured at 600 nm, and the mean and standard deviation are represented by the solid circle and were calculated over three independent experiments. An ANOVA was run to test the effect of media variation on carrying capacity and growth rate. There was a significant difference in carrying capacity between media types (NCTC 4560 $F_{3,42} = 8.789$, $p = 1.218e^{-04}$, NCTC 12975 $F_{3,42} = 82.3191$, $p = < 0.001$, NCTC 12699 $F_{3,42} = 21.4965$, $p = <0.001$) and NCTC 11315 $F_{3,42} = 650.81$, $p = < 0.001$) as well as growth rate (NCTC 4560 $F_{3,39} = 22.494$, $p = < 0.001$, NCTC 12975 $F_{3,38} = 11.578$, $p = < 0.001$, NCTC 12699 $F_{3,42} = 49.1127$, $p = < 0.001$ and NCTC 11315 $F_{3,42} = 15.797$, $p = < 0.001$). Post-hoc Dunnett analysis identified which media variations resulted in a significant change in K compared to SCFM2.SA.XV. SCFM2.SA.XV without eDNA significantly reduced K in all strains (all $p < 0.001$). There was an increase in K for NCTC 11315 when grown in SCFM3 ($p < 0.001$) and SCFM2.SA.XV with 10 μg/mL NAD ($p < 0.001$). In SCFM3, there was a decrease in K of NCTC 12975 ($p = 0.034$). For r, SCFM2.SA.XV without eDNA significantly reduced r in all strains (all $p < 0.001$). SCFM2.SA.XV with 10 μg/mL NAD reduced the r of NCTC 4560 ($p = 0.00137$).

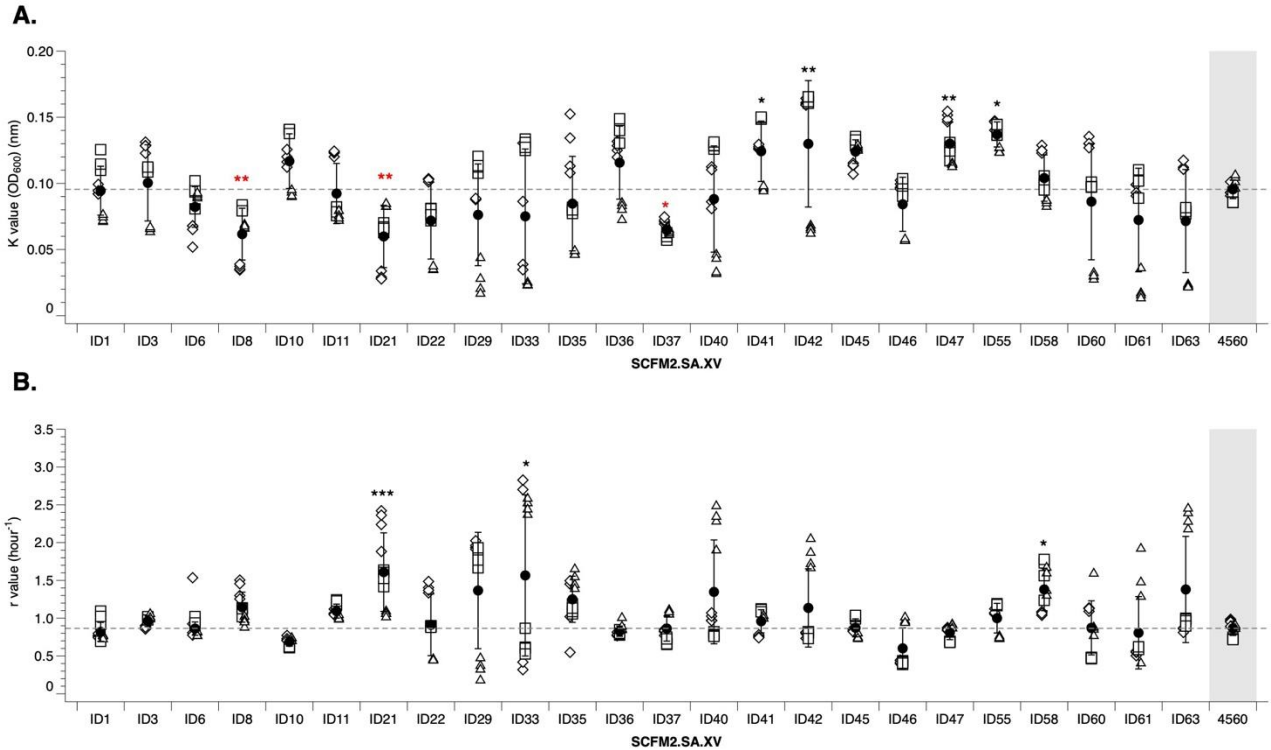

Figure 5: The influence of SCFM2.SA.XV on the growth profiles of clinical *H. influenzae* isolates. NTHi CF clinical isolates were grown statically for 24 hours in SCFM2.SA.XV and both the **A.** carrying capacity and **B.** growth rate were calculated and compared to that of laboratory strain NCTC 4560. Optical density was measured at 600 nm, and the mean and standard deviation are represented by the solid circle and were calculated over three independent experiments. An ANOVA was run to test the effect of isolate on carrying capacity and growth rate (K $F_{24,271}$ = 13.376 p = < 0.001, r $F_{24,271}$ = 5.0137 p = < 0.001). Post-hoc Dunnett analysis identified which clinical isolates had a significant change in K or r compared to NCTC 4560 laboratory strain. 4 clinical isolates had significantly higher carrying capacity than NCTC 4560 which were ID41 (p = 0.04597), ID42 (p = 0.00692), ID47 (p = 0.00724) and ID55 (p = <0.001), whilst 3 isolates had a significantly lower carrying capacity, which were ID8 (p = 0.00321), ID21 (p = 0.00692) and ID37 (p = 0.0104). 3 isolates had a significantly higher growth rate than NCTC 4560 which were ID21 (p = <0.001), ID33 (p = 0.0311), and ID58 (p = 0.0357).

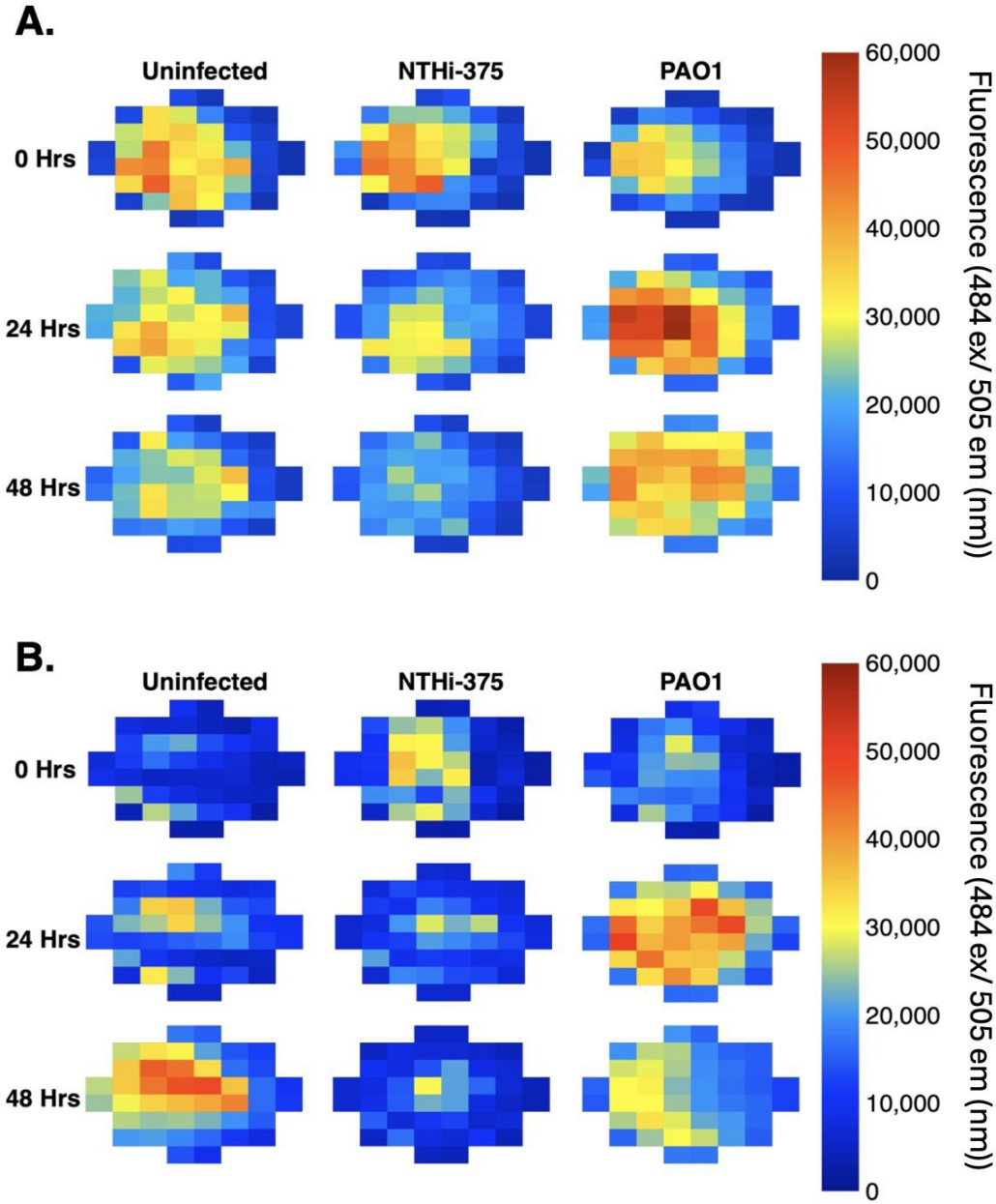

Figure 6: Fluorescence profiles of NTHi-375 in an EVPL model. Bacterial growth was tested on bronchiole lung tissue and alveolar tissue in SCFM2.SA.XV and plating on selective VBC-sBHI agar. Growth of a GFP-tagged PAO1 was used as a positive control. The **A.** fluorescence on bronchiole tissue and **B.** alveolar tissue at 0, 24 hours and 48 hours was monitored. Fluorescence was measured at 484 (nm) ex / 505 (nm) em.

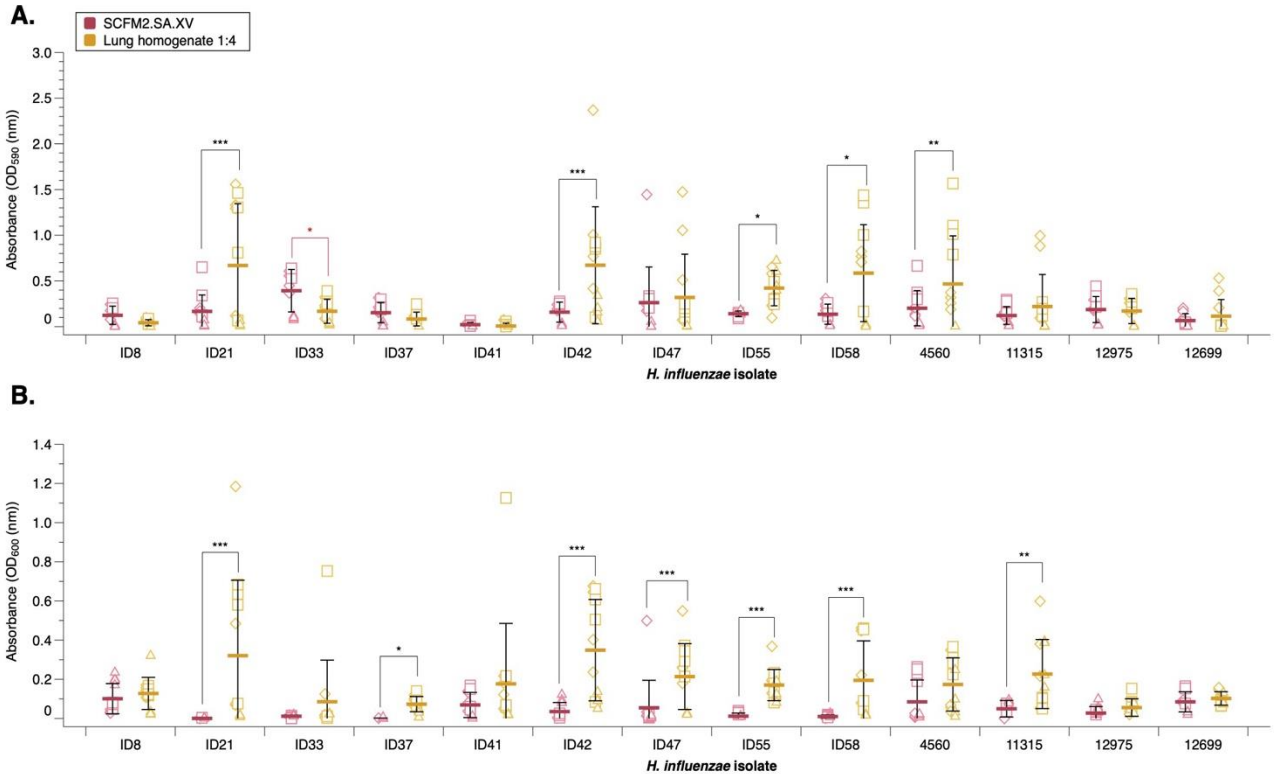

Figure 7: 48-hour biofilm and planktonic growth of NTHi laboratory and clinical CF isolates when grown in lung homogenate media. A. Biofilm growth and **B**. Planktonic growth were measured by crystal violet staining and OD_{600} respectively. The mean and standard deviation were calculated from 3 independent experiments and represented by the solid line. Significance was defined as $p \leq 0.05$. An ANOVA was run to test the effect of media type and isolate/strain on biofilm and planktonic growth. There was a significant effect on biofilm formation by media ($F_{1,249}$ = 12.0637 p = < 0.001), strains ($F_{12,249}$ = 4.9871 p = < 0.001) and an interaction strain*media ($F_{12,249}$ = 3.0237 p = < 0.001). There was also a significant change in planktonic growth between media (ANOVA: $F_{1,251}$ = 76.1778 p = < 0.001), strains (ANOVA: $F_{12,251}$ = 3.4339 p = < 0.001) and an interaction strain*media (ANOVA: $F_{12,251}$ = 2.9556 p = < 0.001). Post-hoc analysis using estimated marginal means for pairwise comparisons highlighted which media*strain interactions were significant. There was a significant increase in biofilm formation when grown in lung homogenate media with ID21 (p = 0.0007), ID42 (p = 0.0009), ID55 (p = 0.0262), ID58 (p = 0.0158) and NCTC 4560 (p = 0.0056). A significant decrease was noted with ID33 (p = 0.0348). Planktonic growth was significantly increased in ID21 (p = ≤0.0001), ID42 (p = ≤0.0001), ID47 (p = 0.0002), ID55 (p = 0.0005), ID58 (p = 0.0001) and NCTC 11315 (p = 0.0077).