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Cutibacterium acnes biofilm forming clinical isolates modify the formation and structure of *Staphylococcus aureus* biofilms, increasing their susceptibility to antibiotics

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ABSTRACT

Cutibacterium acnes (formally *Propionibacterium acnes*) is frequently identified within surgical device related infections. It is often co-isolated from infection sites with other opportunistic pathogens. Recent studies have demonstrated that *C. acnes* is able to form biofilms and when co-cultured with *Staphylococcus* spp. both inhibitory and stimulatory effects have been reported across several studies. Here, we investigated the biofilm-forming ability of 100 clinical *C. acnes* isolates from various infection sites in human patients, both deep tissue and superficial, followed by an investigation of how the supernatants of *C. acnes* cultures influenced the attachment and maturation of *Staphylococcus aureus* NCTC 6571 biofilms. All of the *C. acnes* isolates were able to form biofilms *in vitro*, although biofilm biomass varied between isolates. Nineteen isolates were weakly adherent, 33 were moderately adherent and the majority (48) showed strong adherence. The presence of *C. acnes* sterile supernatants reduced the biomass of *S. aureus* cultures, with a > 90% reduction observed in the presence of several of the *C. acnes* isolates. We observed that this decrease was not due to *C. acnes* affecting *S. aureus* viability, nor due to the presence of propionic acid. Biofilm maturation was however delayed over a 24-h period as was biofilm surface structure, although initial (up to 8 h) surface attachment was not affected. We hypothesis that this defective biofilm maturation is the cause of the observed biomass decrease. In turn, these altered biofilms showed a greater susceptibility to antibiotic treatments. In contrast the presence of *C. acnes* supernatant in planktonic (defined as a free moving, non-surface attached population within the liquid column) *S. aureus* cultures increased antibiotic tolerance, via a currently undefined mechanism. This study suggests that complex interactions between *C. acnes* and other opportunistic pathogens are likely to exist during colonisation and infection events. Further investigation of these interactions may lead to increased treatment options and a better prognosis for patients.

1. Introduction

C. acnes is a Gram-positive member of the skin microbiota and also an opportunistic pathogen able to cause deep tissue, implant-related, infections [1]. These infections are a significant cause of morbidity and mortality and have a significant financial burden [2]. The persistence of *C. acnes* within implant infections is not yet fully understood, but it is likely that biofilm formation, alongside an ability to modulate the biofilm formation of other species, plays a significant role [3,4]. Biofilms are defined as single or multi-species microbial populations surrounded by an extracellular matrix (ECM) which is self-produced [5,6]. *C. acnes* has been identified in multi-species biofilm communities on the skin and within sebaceous glands [7]. Biofilm forming ability appears to be variable across the species. Recent studies show that *C. acnes* isolates belonging to clonal complex (CC) 36 were less able to be internalised by osteoblasts and osteoclasts than isolates from either CC 18 or 28 [8].

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Interestingly however, CC36 and 53 are reported by Aubin et al. [9] to be more frequently associated with prosthetic joint infections than CC18 or 28. It has also been reported that *C. acnes* isolated from deep tissue sites show increased biofilm formation potential [10,11]. Biofilm formation is linked with increased tolerance to antibiotics and increased virulence factor production [4]. Taken together, these findings suggest that biofilm formation is an important persistence and immune evasion factor for *C. acnes*.

Within the sebaceous gland, *C. acnes* is the dominant member of a complex microbial community [12] and it is likely that *C. acnes* is able to both communicate with, and influence, the community structure. Within infection sites *C. acnes* is frequently co-isolated with *Staphylococcus aureus* and *Staphylococcus epidermidis* [13]. A recent study demonstrated that combining *C. acnes* and *S. aureus* cultures led to increased biofilm biomass and better survival of *C. acnes* in aerobic conditions [14]. In contrast, others have shown that *S. epidermidis* is able to inhibit growth of *C. acnes* clinical isolates, and vice versa [15]. Taken together these data suggest that *C. acnes* interactions with other opportunistic pathogens, particularly *Staphylococcus* sp., are multifactorial and require significant further investigation to understand their complexity. Recently interest of these interactions has risen, with reference to the role *C. acnes* and *S. epidermidis* play in modulating the skin microbiome, stimulating inflammation and subsequently driving conditions such as acne [16,17].

The aim of the study presented here was to gain a better understanding of biofilm formation capability of *C. acnes* and understand its interactions with *S. aureus*. To do this, we first carried out a screen of biofilm formation by 100 clinical *C. acnes* isolates from multiple infection sites. Following the initial screen, the influence of *C. acnes* supernatant on *S. aureus* biofilm formation and maturation was determined. The results presented within this study highlight that community interactions are highly influential in biofilm structure and progression and understanding these interactions in more detail may allow the development of new treatment strategies for surgical implant infections.

2. Methods

2.1. Bacterial isolates and routine maintenance

C. acnes isolates were obtained from the Anaerobe Reference Unit (ARU), Cardiff, UK. A total of 100 isolates were obtained from stocks and stored at −80 °C. With the exception of the two isolates from the National collection of Tissue Culture (NCTC) and the four isolates obtained from animals, all isolates were clinical in nature, isolated as part of investigations to determine the source of infections. It should however be noted that it was impossible to determine from the ARU database if isolates were the primary cause of the infections investigated, or an incidental organism found at the site of infection. Isolates selected represented a wide range of isolation sites, full details of the isolates provided by the ARU, their site of origin and the site groupings assigned by the authors can be found in Supplementary Table 1. A total of nine site groupings (animal, brain cavity, bone, cardiac, head (including all sites from head, neck, eye, outer ear and oral cavity), spinal, tissue, NCTC strain and urogenital) were used to allow isolates to be grouped in relation to either the infection type or physical location of the site. Long term storage was at −80 °C in fastidious anaerobe broth (FAB) (Lab M) containing 10% v/v sterile glycerol (Sigma). Isolates were revived and routinely maintained on fastidious anaerobe agar (FAA) (Lab M), containing 5% v/v horse blood (Sigma), incubated at 37 °C in anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2) for 48 h. *S. aureus* NCTC 6571 was stored at −80 °C in tryptic soy broth (TSB) (Oxoid) supplemented with 10% v/v sterile glycerol. The isolate was revived and maintained on tryptic soy agar (TSA) (Oxoid) at 34 °C in aerobic conditions. Following revival, *S. aureus* isolates were stored for up to 30 days at 2−8 °C, allowing repeated use.

The five *C. acnes* isolates used throughout the work were sequenced to allow MLST typing. Genomic DNA was extracted following the manufacturers instructions and using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich). DNA was suspended in nuclease-free water and shipped to MicrobesNG for whole genome analysis. Sequencing and alignment was carried out using the standard providers protocol. Briefly, DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: 2 ng of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 s. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on an Illumina instrument using a 250bp paired-end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [18]. *De novo* assembly was performed on samples using SPAdes version 3.7 [19]. Sequence types (ST) were determined using The Center for Genomic Epidemiology MLST 2.0 tool [20] with the *Propionibacterium acnes* MLST configuration selected. Further analysis of the identity of isolate 37298 was carried out using standard nucleotide BLAST [21]. Assemblies were submitted to Genbank and have the following accession numbers: isolate 1026 ID JAJNRK000000000, isolate 37298 ID JAJNRJ000000000, isolate 38833 ID JAJNRI000000000, isolate 41121 ID JAJNRH000000000 and isolate 44487 ID JAJNRG000000000.

2.2. Preparation of sterile supernatants

Unless otherwise stated supernatants were collected from 48 h *C. acnes* broth cultures incubated with no shaking at 37 °C in anaerobic conditions in polypropylene tubes (broth cultures are hereafter referred to as planktonic cultures). The cultures were harvested by pelleting the cells at 3000 g for 10 min at ambient temperature and passing the collected supernatant through a 0.2 μM PES filter. Supernatant from triplicate cultures of the same isolate were pooled prior to experimental use and stored at 2−8 °C for up to two weeks to allow repeated use. *C. acnes* were grown in BHI, except for use in antibiotic susceptibility assays where isolates were cultured in Mueller Hinton Broth (MHB).

2.3. Routine biofilm formation by C. acnes isolates

Prior to biofilm assay a streak of colonies from the plate was transferred into FAB and incubated at 37 °C in anaerobic conditions for a further 48 h to allow culture expansion. Following incubation the cells were diluted to an OD_{600} of between 0.08 and 0.1 (equivalent to approximately 1×10^7 CFU/ml) and then further diluted 1 in 100 before addition of 200 μl of cell suspension to a 96 well tissue culture treated microtiter plate (Corning). Unless otherwise stated all plates were incubated statically for 72 h at 37 °C in anaerobic conditions to allow attachment of bacterial isolates to the plates and subsequent biofilm formation. All dilutions were carried out using BHI, and BHI was used as a negative control. Biofilms were quantified by crystal violet staining, described below.

2.4. Crystal violet staining of biofilms

Following static incubation the cell suspension was removed from the plates and all wells were rinsed gently using 200 μl of sterile PBS. Any biofilm present was fixed with 200 μl of methanol for 15 min, before removal of the methanol suspension and addition of 200 μl 1% w/v crystal violet solution. A further 15 min incubation was carried out to allow staining of any biofilm biomass present followed by washing with copious water to remove unbound dye. The dye was resolubilised by the addition of 200 μl 30% v/v acetic acid and measured using an absorbance wavelength of 590 nm (FLOUstar Omega, BMG Labtechs).

2.5. Classification of biofilm biomass

The ability of each *C acnes* isolate to form biofilm was quantified following the method used by Stepanovic et al. [22] to quantify *S. aureus* biofilm formation. Briefly, for each isolate the mean optical density (OD) and upper limit of the BHI only control (mean + 3 x SD, referred to as OD_c) was calculated. The relationship between the two was used to determine the biofilm forming ability of each isolate. Using this relationship, *C. acnes* isolates were classified as being non-adherent or able to form a biofilm which was weakly/moderately/strongly adherent to the plate surface. It is considered that as the level of staining increases relative to the BHI only control, so does the ability of the bacterium to adhere to a surface and from a robust biofilm. The classification boundaries of biofilm formation used within the work is shown below.

Grouping	Definition
$OD \leq OD_c$	Non-adherent (0)
$OD_c < OD \leq 2$ x OD_c	Weakly adherent (+)
2 x $OD_c < OD \leq 4$ x OD_c	Moderately adherent (++)
4x $OD_c < OD$	Strongly adherent (+++)

2.6. Biofilm formation by S. aureus NCTC 6571

Suspensions for biofilm cultures were prepared by adding a sweep of colonies from TSA plates to TSB and incubated overnight at 37 °C, before dilution to between OD_{600} 0.08 and 0.1 (equivalent to approximately 1×10^7 CFU/ml). Suspensions were further diluted 1 in 100 before carrying out a second 1 in 50 dilution. All dilutions were carried out in TSB + 0.5% (w/v) glucose. An equal volume of the final suspension was mixed with either sterile TSB + 0.5% (w/v) glucose, BHI, PBS or filtered *C. acnes* supernatant. Cultures were then incubated statically to allow biofilm formation. Unless otherwise stated all incubations to allow biofilm formation were carried out without shaking for 24 h at 37 °C in aerobic conditions supplemented with 5% CO_2.

Where mature biofilms were required: biofilm cultures were prepared as described above in TSB broth + 0.5% (w/v) glucose (no supplementation with other medium or *C. acnes* supernatant) and incubated for 24 h at 37 °C, in aerobic conditions supplemented with 5% CO_2. Following incubation, the supernatant was removed carefully, and the wells washed once with sterile PBS. The medium in the wells was then replaced with either sterile TSB + 0.5% (w/v) glucose, BHI or *C. acnes* suspensions diluted as described previously. Microtiter plates were then incubated for a further 24 h as described previously before crystal violet staining.

2.7. Live/dead staining of surface-attached S. aureus NCTC 6571 quantification of surface coverage

S. aureus biofilm suspensions were diluted as described in the previous section and added to the wells of 12 well microtiter plate, each containing a sterile glass microscopy coverslip. Following incubation, the coverslip was removed and washed once with PBS to remove non-adhered cells. The coverslips were then stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) following manufacturer's instructions. Attached cells were visualised using an Olympus IX50 fluorescent microscope at 200× magnification at emission/excitation wavelengths of 485/530–630 nm respectively. Five random images of the disc surface were taken and bacterial coverage of the surface was quantified using a macro written in ImageJ which calculated the percentage of each image occupied by fluorescence.

2.8. Scanning electron microscope analysis

S. aureus NCTC 6571 biofilms were allowed to form on sterile cover slips as described in the previous section. Following a 24 h incubation the coverslips were removed and washed once with sterile PBS. Samples were then fixed by adding 1 ml of 2.5% v/v glutaraldehyde (Sigma) diluted in sterile distilled H_2O and dehydrated by incubation in increasing concentrations of ethanol (50, 70, 90, 100% v/v respectively), followed by serial incubation in hexamethyldisilazane (HMDS) diluted in ethanol. The HMDS to ethanol ratios were as follows: 1:2, 2:1, HMDS only. After addition of the final volume of HDMS the excess liquid was left to evaporate overnight before sputter coating using a K650x sputter coater (Quorum Technologies) and imaging with a Tescan Vega 3 (Tescan Ltd). For each cover slip images at low and high magnification were taken at each of the cardinal compass points and in the centre of the disc.

2.9. Antibiotic susceptibility testing

For both minimum inhibitory concentration (MIC) and Minimum biofilm eradication concentration (MBEC) testing an adapted EUCAST microbroth dilution assay was used [23]. Gentamicin, ciprofloxacin, vancomycin, linezolid and rifampicin (all Sigma), were diluted to a stock concentration 5.12 mg/ml following manufacturer's instructions and aliquoted into single use volumes. On the day of use the antibiotic stocks were removed from storage and diluted to a working concentration then diluted two-fold 11 times in sterile MHB, or *C. acnes* MHB supernatant. Any excess stocks were discarded after use and not re-used.

For MIC testing *S. aureus* NCTC 6571 cultures were prepared by adding a sweep of colonies from TSA plates to TSB and incubated overnight at 37 °C, before dilution to between OD_{600} 0.08 and 0.1 (equivalent to approximately 1×10^7 CFU/ml). Suspensions were further diluted 1 in 100 in MHB or sterile filtered MHB in which had been conditioned by allowing growth of *C. acnes* for 48 h. Diluted cell cultures were added to the antibiotic dilutions in a 1:1 ratio before incubating for 16–20 h at 37 °C, aerobic conditions. The OD_{600} of the suspensions we measured at the start and end of the incubation period and the MIC was the lowest concentration of antibiotic at which there was no increase in OD_{600} recorded.

For MBEC testing *S. aureus* NCTC 6571 cultures were diluted in TSB supplemented with 0.5% w/v glucose and incubated for 24 h as previously described to allow biofilm formation. Following incubation of the biofilm plate, the supernatant was removed and plates washed carefully once with sterile PBS to avoid disrupting biofilms. Doubling dilutions of the antibiotic solutions were added to the attached biofilms within the microtiter plate (200 μl) before re-

incubation for 16–20 h at 37 °C, aerobic conditions. Cell viability was measured using the viability dye resazurin adapted from the method described previously by Elshikh et al. [24]. Briefly, resazurin powder (Sigma) was diluted to a stock concentration of 0.015% (w/v) in sterile water and stored at 2–8 °C in the dark until use. This stock solution was added to each well of the plate (30 μl) and incubated at 37 °C, aerobic conditions for approx. 60 min to allow to allow colour development. Resazurin changes from a deep blue to bright pink colour in the presence of metabolically active cells. This is due to resazurin being reduced to resorufin by oxidoreductases within viable cells [25]. The MBEC was the lowest concentration of antibiotic at which no colour change was visible. The presented MIC values represent the final concentration the bacterial inoculum was exposed to following all dilutions in broth and *C. acnes* supernatant.

2.10. Statistical analysis

For all biofilm quantification analysis three biological replicates, each containing three technical replicates were completed. Microscopic analysis of biofilms used three biological replicates, each with 5 technical replicate images. Mean and standard error of mean (SEM) values were calculated and are displayed throughout, unless otherwise stated. Analysis of data was carried out using GraphPad Prism software. Statistical analysis was performed within GraphPad Prism V9 using the one way or two way ANOVA analysis packages (as appropriate) followed by Dunnetts posthoc testing to compare values.

3. Results

3.1. Assessment of biofilm forming ability of one hundred C. acnes clinical isolates

The ability of all 100 *C. acnes* isolates within our collection to form biofilms was assessed. As can be seen in Table 1 all the isolates within our collection were able to attach to the polystyrene microtitre plate surface and produce quantifiable biomass. None of the isolates were classified as non-adherent, with the majority (48 isolates) showing strong adherence according to the classification system developed by Stepanovic et al. [22]. A further 33 isolates were moderately adherent and 19 isolates were weakly adherent. There was a high level of inter-isolate variation in biofilm forming ability and no statistical relationship was observed between clinical origin and quantity of biofilm biomass produced. The full details of each isolates biofilm biomass, the standard deviation and biofilm classification are detailed in Supplementary Table 1.

Table 1
Quantification of *C. acnes* biofilm biomass indicates that isolates display a variable ability to form biofilms. *C. acnes* biofilm biomass was quantified and the relationship between the BHI only control (OD_c) and staining mass of the samples was used to determine the biofilm forming capability of each isolate. Within the table each column shows the number of isolates from each infection area.

Isolate origin	Number of isolates				Total number
	+	++	+++		
Animal	3		1		4
Brain cavity	3	7	9		19
Bone	5	5	10		20
Cardiac	1	1	4		6
Head	4	7	5		16
Spinal	2	3	4		9
Tissue	3	9	6		18
Urogenital		2	6		8
				Total	**100**

Following screening of the isolates, five isolates, 1026 (ST 4, phylogroup IA_1), 37298 (Unknown *Cutibacterium* sp. with no discernible *C. acnes* phylogroup classification), 38833 (ST 22, phylogroup IA_2), 41121 (ST 72, phylogroup II) and 44487 (ST 57, phylogroup IA_2) were selected for further analysis. These isolates were chosen as they were able to reproducibly produce a high biofilm biomass and were all from different infection sites and so represented a wide range of potential infections types. MLST analysis also indicated that the selected isolates came from multiple ST types and three major phylogroups, with one isolate not being identified as *C. acnes* upon further MLST analysis. Phenotypically this isolate was identified as *C. acnes* by ARU, however genomic analysis and BLAST analysis of the assemble contigs showed it was most likely a closely related, but previously unidentified *Cutibacterium* sp.. BLAST analysis indicated that it was closely related to *Propionibacterium* sp. oral taxon 193 strain F0672. Unless specified, all further investigations were carried out using these five isolates.

3.2. The supernatant of C. acnes planktonic cultures can inhibit the biofilm formation of S. aureus

Previous studies report that *C. acnes* and *S. aureus* can be co-isolated from infection sites, in particular from post-surgical deep tissue infections [26]. It was therefore of interest to us to see what impact *C. acnes* supernatants and cultures would have on the structure and biomass of *S. aureus* biofilms. *S. aureus* NCTC 6571 was selected as it is a widely used and well characterised type strain of *S. aureus* [27]. Supplementation of BHI with the sterile supernatant of the five *C. acnes* planktonic cultures (50:50 (v/v) dilution) uniformly decreased the surface coverage (Fig. 1A) and biofilm biomass (Fig. 1B) of *S. aureus* NCTC 6571. This effect was not due to the impact of decreased nutrient availability within the biofilm cultures since static incubation in a 50:50 PBS:TSB + 0.5% glucose mix did not significantly alter biofilm formation compared to biofilms formed in the presence of BHI.

We speculated that the decreased biofilm formation may be due to the supernatant inhibiting growth of *S. aureus* NCTC 6571 and therefore also affecting biofilm formation. A comparison of *S. aureus* NCTC 6571 growth in BHI medium, and medium supplemented with the five *C. acnes* supernatants or PBS was carried out (Fig. 1C). As expected, supplementation of medium with PBS inhibited growth of *S. aureus* NCTC 6571 ($P \leq 0.001$), likely because of limited access to nutrients. Except for *C. acnes* 41121 there was no statistically significant difference between *S. aureus* NCTC 6571 growth in BHI medium alone and in cultures where BHI medium was supplemented with *C. acnes* supernatant. This implies that the observed effects in reducing biofilm biomass are not due to a significant reduction in growth. Supplementation with *C. acnes* isolate 41121 did decrease *S. aureus* NCTC 6571 growth ($P \leq 0.0001$), and so in this specific instance the inhibition of biofilm formation may partly be due to the ability of *C. acnes* 41121 supernatant to limit the growth of *S. aureus* NCTC 6571.

Since all five isolates tested were all able to decrease *S. aureus* NCTC 6571 biofilm we investigated how widespread the biomass reducing phenomenon was within our collection of 100 *C. acnes* clinical isolates. Supernatant from all 100 isolates were able to reduce the biofilm biomass of *S. aureus* NCTC 6571 (Fig. 1D and Supplementary Table 1). Only six of the *C. acnes* isolates (Isolates 481, 17678, 17680, 26854, 26948 and 40696) did not produce a statistically significant decrease in biofilm biomass ($P > 0.05$) however, a reduction in biofilm biomass was still present. The biomass reduction ranged from 9.54% to 97.83% with a mean reduction of 39.3% (±21.05 standard deviation) (Fig. 1D). This highlights that the observed effect was both widespread within our collection and the impact on the biofilm biomass of *S. aureus* NCTC

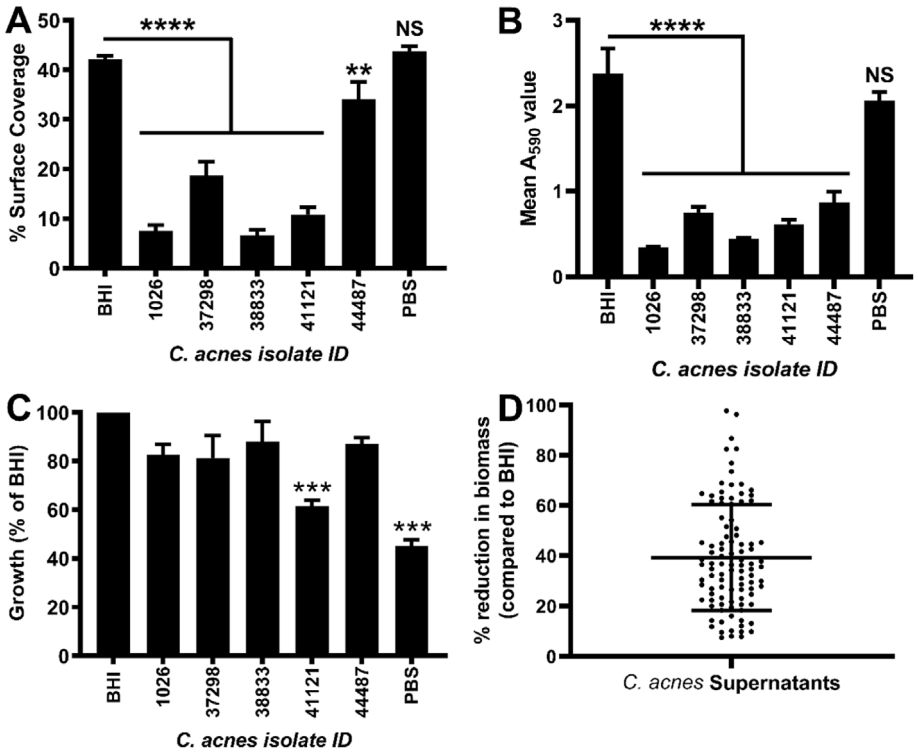

Fig. 1. Sterile supernatant from *C. acnes* planktonic cultures reduces *S. aureus* NCTC 6571 biofilm biomass. *S. aureus* NCTC 6571% surface coverage (A), total biomass of biofilms (B) and bacterial growth, displayed as a percent of the growth achieved in BHI medium, (C) of *S. aureus* NCTC 6571 was assessed in the presence of BHI, PBS and *C. acnes* supernatants. (D) shows mean % reduction in S. *aureus* NCTC 6571 biofilm biomass in the presence of all 100 of the *C. acnes* isolates. Bars show mean values and error bars show SEM (A to C) or standard deviation (D). Significance was calculated using post hoc tests following ANOVA analysis (****$P \leq 0.0001$, **$P \leq 0.01$).

6571 cultures was substantial for most of the *C. acnes* isolates.

3.3. Co-culture of C. acnes isolates with S. aureus NCTC 6571 also leads to decreased biofilm biomass, but only in aerobic conditions

The results shown in Fig. 1 highlight that an unidentified component within the *C. acnes* stationary phase supernatant can elicit an alteration in *S. aureus* NCTC 6571 biofilm biomass. Next, we assessed if this reduction would also occur in the presence of actively growing *C. acnes* cultures, despite the growth and biofilm formation rate of *C. acnes* being slower than that of *S. aureus* NCTC 6571, potentially providing a competitive advantage to *S. aureus* NCTC 6571. This allowed us to determine if the active component(s) were able to elicit a response early in the *C. acnes* growth cycle or if accumulation were required before a biomass reduction could be observed. Since both bacterial species are likely to be inoculated within wound sites at low concentrations and at the same time we hoped that this would provide information as to the likelihood that the effect on biomass may also be observed *in vivo*. It was also of interest to see if the effect could be observed in both aerobic and anaerobic conditions, since *S. aureus* and *C. acnes* are known to be able to survive in both. In aerobic conditions there was a statistically significant decrease ($P \leq 0.001$) in the biomass formed by *S. aureus* NCTC 6571 when mixed with the five *C. acnes* isolates (Fig. 2A). Indeed, the biofilm biomass was very similar in quantity to the single isolate *C. acnes* cultures. No statistical differences were observed between single species *C. acnes* cultures and the respective combined cultures. These observations were not matched in anaerobic conditions (Fig. 2B). In anaerobic conditions the biomass quantity formed by *S. aureus* NCTC 6571 was lower, and as such there was no statistical difference between the biomass of single or mixed species cultures. Interestingly the biomass of *C. acnes* monospecies biofilms was increased in aerobic conditions.

3.4. The inhibitory effect on S. aureus biofilm formation is not due to the C. acnes isolates lowering medium pH

C. acnes is also known to produce propionic acid, which has antimicrobial properties [28] and pH also influences *S. aureus* biofilm formation [29]. We speculated that a decrease in the pH of the medium, or the presence of propionic acid specifically, may be responsible for the effects observed in Figs. 1 and 2. The pH of the five *C. acnes* supernatants and TSB + 0.5% glucose were assessed and found to each be approximately pH 6, with standard BHI having a pH of 7 (Fig. 3A). This difference, although small was significantly significant ($P \leq 0.0001$). To determine if any observed effects were due to simply a change in pH or the presence of propionic acid, the pH of the medium was adjusted using both HCl and propionic acid. Contrary to the effect observed in the presence of *C. acnes* supernatants, reduction of the pH of BHI with either propionic acid or HCl increased the biofilm biomass of *S. aureus* NCTC 6571 cultures (Fig. 3B). Although an increase was observed following adjustment with both acids, this increase was only statistically significant ($P \leq 0.001$) when the pH was adjusted with HCl.

3.5. Scanning electron microscopy of S. aureus NCTC 6571 biofilms supplemented with C. acnes supernatants highlighted biofilm structure alterations and inhibition of biofilm maturation

To determine the effect of biofilm biomass on biofilm structure, microscopic analysis of the biofilms was carried out. The gross architecture of *S. aureus* NCTC 6571 biofilms supplemented with supernatant from the five *C. acnes* isolates was analysed by SEM. Biofilm development and maturation was also imaged via live/dead

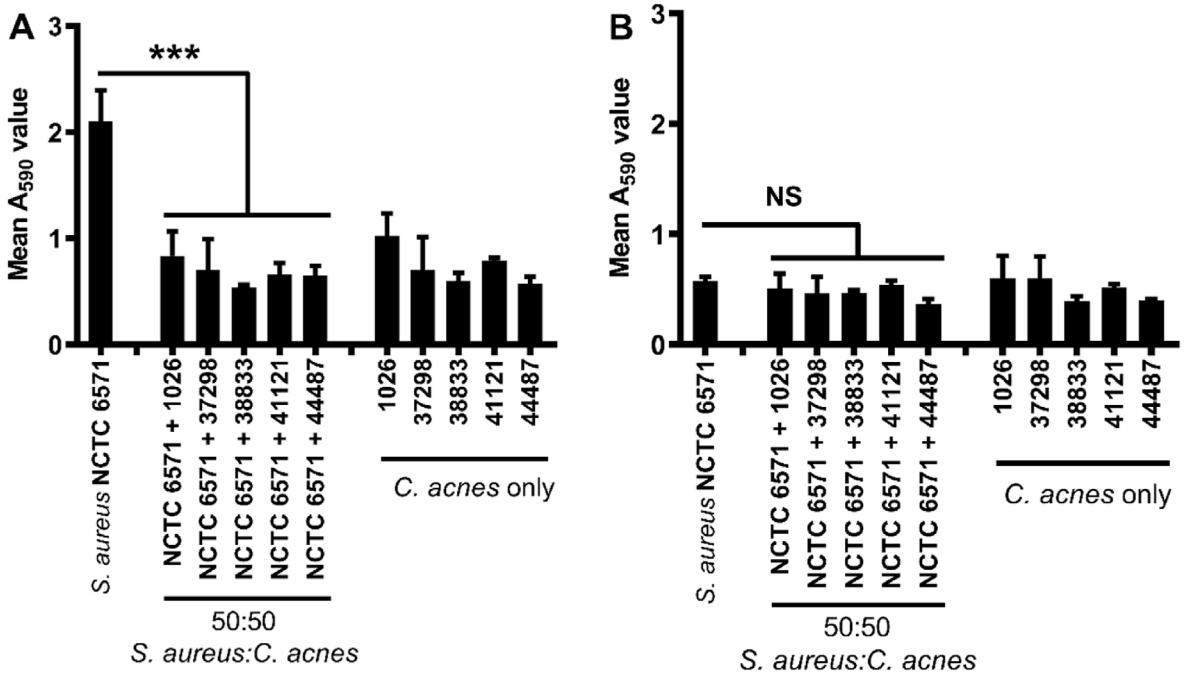

Fig. 2. Co-culture of *C. acnes* and *S. aureus* NCTC 6571 indicates that *S. aureus* takes on a biomass similar to the *C. acnes* biofilms when incubated aerobically. Single and dual *C. acnes* -*S. aureus* NCTC 6571 biofilms were formed either aerobic (A) or anaerobic (B) conditions. Bars show mean values and error bars show SEM. Significance was calculated using post hoc tests following ANOVA analysis (***P ≤ 0.001).

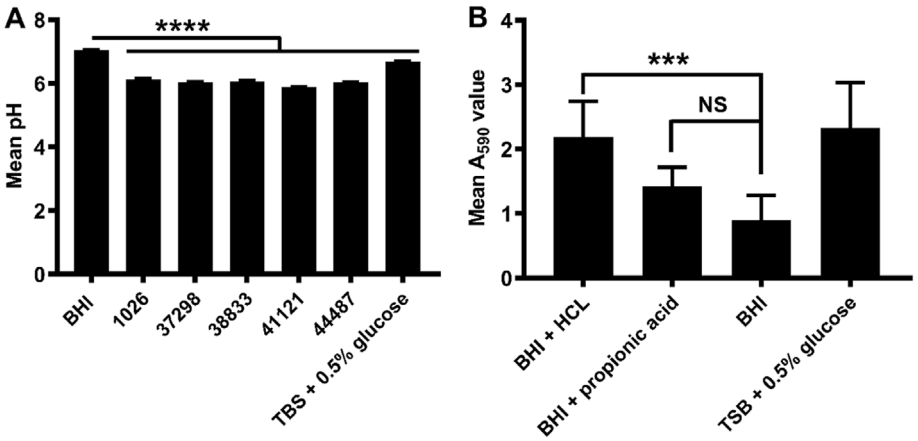

Fig. 3. The pH **reduction observed within *C. acnes* supernatants is not responsible for the observed biomass decrease.** Measurement of pH of *C. acnes* supernatant, glucose supplemented TSB and BHI was measured (A). Adjustment of BHI to pH 6.0 increased *S. aureus* NCTC 6571 biofilm biomass (B). Bars show mean values and error bars show SEM. Significance was calculated using post hoc tests following ANOVA analysis (****P ≤ 0.0001, ***P ≤ 0.001).

staining combined with fluorescent microscopy. SEM imaging showed that *S. aureus* NCTC 6571 biofilms formed in the presence of BHI medium were complex structures containing many bacterial cells (Fig. 4A). In contrast, the biofilms supplemented with *C. acnes* supernatant show limited three-dimensional structure compared to biofilms formed in BHI only (Fig. 4B–F). In the presence of *C. acnes* supernatant biofilms did not appear to have progressed beyond the early stages of biofilm formation, showing only single-cell attachment (Fig. 4E), or the formation of very small micro colonies on the coverslip surface (Fig. 4B, C and D) and limited 3D architecture. Although biofilm formed in the presence of *C. acnes* 44487 supernatant (Fig. 4E) appeared to have a more mature structure, with ECM apparently present, the architecture was still morphologically altered compared to the BHI medium control sample.

Biofilm formation occurs over a series of four phases: initial attachment, microcolony formation, maturation and dispersal [30]. A delay at any of the first three of these stages could lead to the structural changes shown in Fig. 4. In order to determine if initial attachment was affected by the ability of *S. aureus* NCTC 6571 to attach to a coverslip after 1, 2, 4, 8 or 24 h in the presence of *C. acnes* supernatant, PBS or BHI during static incubation was measured (Fig. 5A and B). At 1, 2, 4 and 8 h there was no statistically significant difference in surface coverages of *S. aureus* NCTC 6571 incubated in the presence of 50% *C. acnes* or BHI medium (Fig. 5A). In contrast, cultures incubated in 50% PBS showed significantly higher levels of surface coverage ($P \leq 0.01$), and by extension bacterial attachment, at these early time points. Representative images for each time

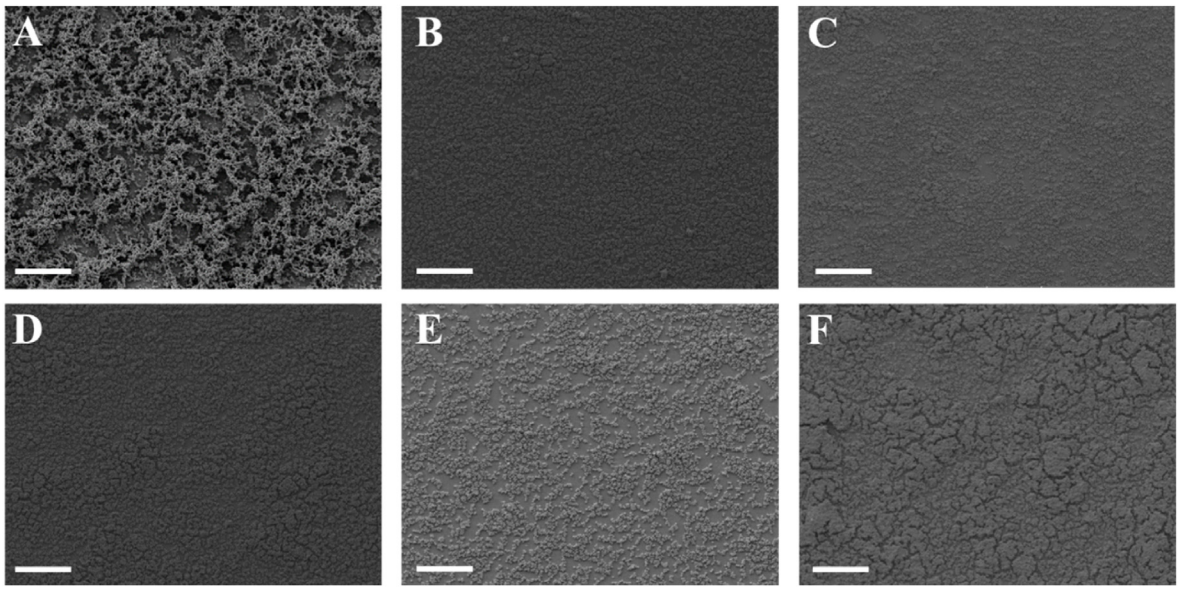

Fig. 4. *S. aureus* NCTC 6571 biofilms supplemented with *C. acnes* supernatant show reduced complexity and 3D structure. *S. aureus* NCTC 6571 cultures were incubated in either BHI medium (A) or BHI medium supplemented 1:1 with the sterile filtered supernatant of either *C. acnes* isolate 1026 (B), 37298 (C), 38833 (D), 41121 (E) or 44487 (F) before fixing and SEM imaging. Images shown are representative of all the replicates and the scale bar in the bottom left corner of each image represents 20 μm.

point are shown in Fig. 5B. Only images for *S. aureus* NCTC 6571 biofilms grown in the presence of *C. acnes* isolate 1026 supernatant are shown, however these images were representative of images obtained for the biofilms grown with the other *C. acnes* supernatants. Where cultures are incubated in the presence of PBS a clear progression from attachment to microcolony formation and maturation can be observed (Fig. 5B bottom panels). In contrast those cultures incubated in the presence of either *C. acnes* supernatant or BHI do not show such a continuous development. Little alteration in images is seen between 1 and 8 h, with only microcolonies and single cell attachments observed (Fig. 5B top and middle panels). By 24 h of incubation this trend had altered, with no statistically significant difference in attachment of *S. aureus* NCTC 6571 culture incubated in BHI medium or PBS, whereas as the *C. acnes* supernatant containing cultures showed a statistically significant reduction in surface coverage. Taken together Fig. 5A and B suggest that progression from microcolony formation to maturation is delayed or inhibited by the presence of *C. acnes* supernatants. The data also confirms that the initial stages of attachment and microcolony formation are not affected by the presence of *C. acnes* supernatants, with this progressing in a similar fashion to cultures incubated in standard BHI medium. Taken together the results presented in Figs. 4 and 5 suggest that it is biofilm maturation, rather than attachment which is affected by the presence of *C. acnes* supernatant.

To assess if biofilm maturation was delayed or unable to take place at all, biofilm cultures were incubated for an extended period of up to 96 h. It was expected that if maturation were delayed then biomass would begin to increase with longer incubation. No obvious trend of consistently increasing biomass was observed in the presence of *C. acnes* supernatants over the time period of the experiment, suggesting the possibly of defective rather than delayed biofilm maturation (Fig. 5C.).

Since maturation, rather than attachment, appeared to be the biofilm formation stage affected by the supernatants we assessed if an effect was also present in biofilms which had already undergone maturation. The medium of 24 h *S. aureus* NCTC 6571 biofilms was supplemented with 50% *C. acnes* supernatants or BHI before incubation for a further 24 h, (Fig. 5D). Although a slight reduction in biofilm biomass was observed in the presence of *C. acnes* supernatants, it was not statistically significant. In contrast, biofilms formed for 48 h in the presence of *C. acnes* supernatant showed clear, statistically significant, reduction; as was repeatedly observed across multiple experiments (Fig. 1B, D, 2A, 5A and 5C).

3.6. *C. acnes* conditioned medium also alters the antibiotic susceptibility of *S. aureus* NCTC 6571 planktonic and biofilm cultures

Biofilm formation is known to be a significant factor in the reticence to antibiotic treatment. Since the presence of *C. acnes* supernatant has been shown to lead to reduction and modification in the *S. aureus* NCTC 6571 biofilm biomass we speculated that these modifications may also impact antibiotic susceptibility.

The susceptibility of *S. aureus* NCTC 6571 planktonic and biofilm cultures to five, clinically relevant, antibiotics were tested both in MHB only and filtered MHB in which *C. acnes* isolates had been cultured as described in the materials and methods. When compared to the MHB only MIC values, there was an overall trend in decreased susceptibility of planktonic cultures (see Table 2). As was expected the antibiotic concentrations needed to inactivate biofilms were much higher than that needed to eradicate planktonic cultures. However, it was interesting that a trend of increased susceptibility of biofilms to antibiotics was observed when cultures were supplemented with 50% v/v *C. acnes* supernatant. With the exception of Vancomycin this was observed for all the antibiotics tested. Two to eight fold increases in susceptibility were common, although in the case of Rifampicin the increase in susceptibility was much higher, with susceptibility increasing 128 fold. The observed phenomenon appeared to be linked to both the antibiotic class and *C. acnes* strain present, with no overall trend being discernibly able to be linked to either a particular *C. acnes* strain or antibiotic. Only in the case of rifampicin did *C. acnes* supernatants increased the susceptibility of both *S. aureus* NCTC 6571 planktonic and biofilm cultures, although only a slight MIC reduction was observed in planktonic cultures, compared to the large susceptibility increases

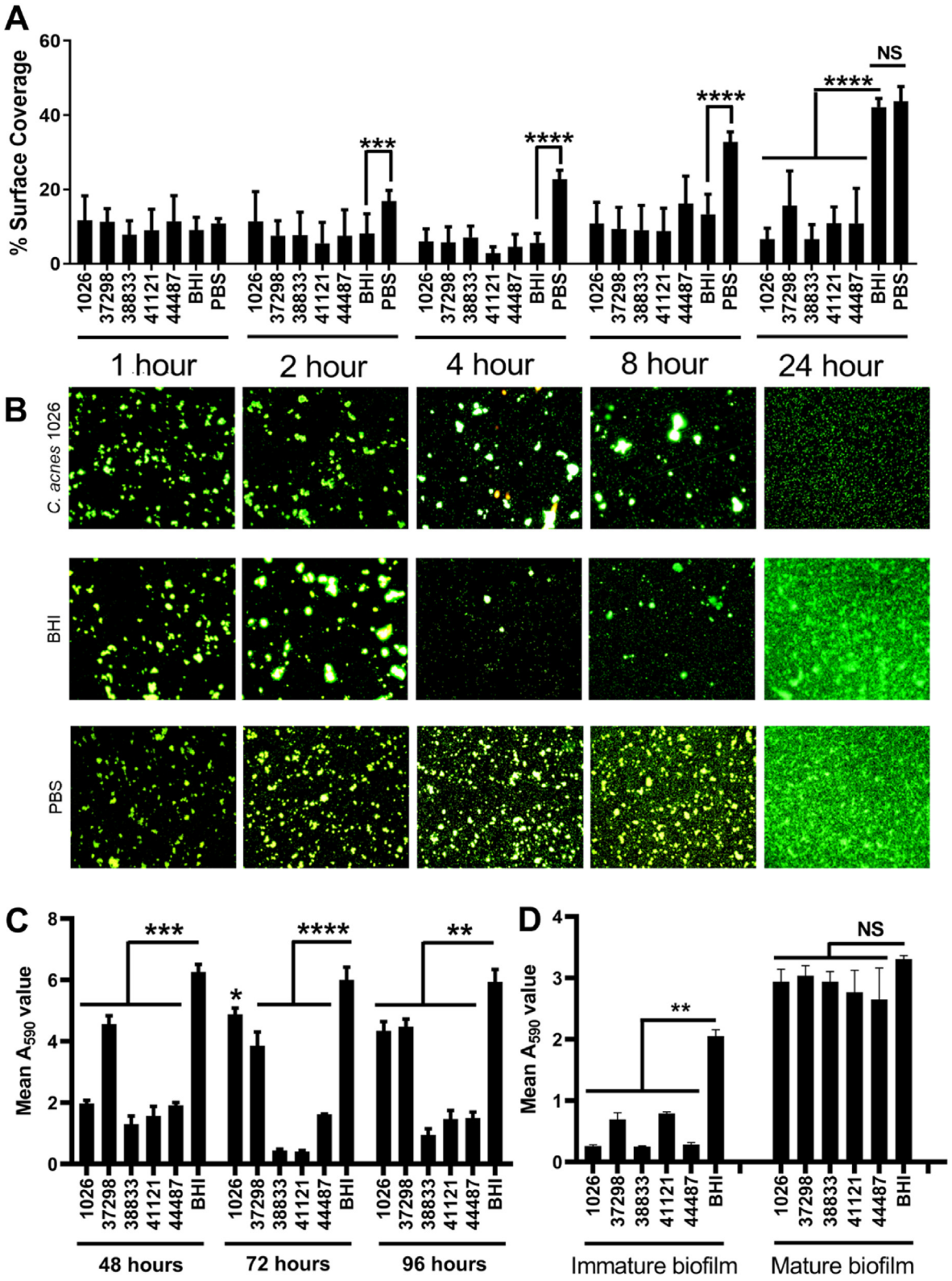

Fig. 5. *C. acnes* spent supernatants do not inhibit early attachment of *S. aureus* NCTC 6571 to surfaces but do inhibit progression to biofilm maturation. *S. aureus* NCTC 6571 surface attachment and biofilm maturation was assessed at 1, 2, 4, 8 (immature biofilms) or 24 h (mature biofilm), data is represented graphically in (A) with representative images in the presence of *C. acnes* supernatant (top panel), BHI (middle panel) or PBS (bottom panel) shown in (B). Biofilm biomass of *S. aureus* NCTC 6571 biofilms grown in either 50% *C. acnes* supernatant or BHI for 48, 72 or 96 h are shown in (C). Finally, the impact of *C. acnes* supernatants on the biomass of mature biofilms (24 h) was assessed (D). Bars show mean values and error bars show SEM. Statistical significance was calculated by comparing the BHI only control. Post hoc tests following ANOVA analysis were used (*P $\leq$ 0.05, **P $\leq$ 0.01***P $\leq$ 0.001, ****P $\leq$ 0.0001).

Table 2
Minimum inhibitory antibiotic concentrations (g/L) for planktonic and biofilm cultures. Where susceptibility is decreased in the presence of *C. acnes* supernatant, compared to MHB, values are highlighted in bold. Where susceptibility is not affected values are underlined.

			Antibiotic				
			Gentamicin	Cipro-floxacin	Vanco-mycin	Linezolid	Rifampicin
MIC (g/L)	*S. aureus* only		0.5	1	0.5	0.25	0.004
	With *C. acnes* superna-tant	1026	4	2	2	2	**0.002**
		37298	8	**0.5**	1	2	**0.002**
		38833	4	1	1	2	0.004
		41121	8	1	1	2	**0.002**
		44487	8	1	1	2	**0.002**
MBEC (g/L)	*S. aureus* only		>512	>512	8	128	64
	With *C. acnes* superna-tant	1026	**32**	**512**	64	**64**	64
		37298	**16**	**256**	16	**32**	**0.5**
		38833	**128**	**128**	8	**8**	**0.032**
		41121	>512	**64**	8	**8**	64
		44487	**256**	**128**	32	**16**	**0.5**

observed within the biofilm cultures.

4. Discussion

This study provides an analysis of the biofilm formation by 100 *C. acnes* isolates of clinical origin and the effect their supernatants have on the biofilm formation of *S. aureus* NCTC 6571. Most importantly we show that *C. acnes* can influence the biofilm formation of *S. aureus* NCTC 6571 and increase the susceptibility of these biofilms to several antibiotics. The results presented here indicate how relationships between two important opportunistic pathogens may influence biofilm maturation, and by extension infection progression within deep tissue infections.

The biofilm forming ability of the clinical *C. acnes* isolates used within this study has not previously been assessed, although biofilm formation within other isolates has been quantified. The finding of widespread biofilm-forming ability is consistent with the results presented by previous authors [10,31,32]. Taken together previous studies, combined with the results presented here, suggest that the ability to form biofilms is widespread within *C. acnes* although the exact amount of biomass produced is variable and related to the genetic makeup and phylotype of isolates. Although extensive genomic analysis of the isolates used within the study was outside the scope of this current work, MLST analysis of the five isolates used identified that all belonged to different MLST types and covered phylogroups IA_1, IA_2 and II.

The ability of *C. acnes* to form *de novo* monospecies biofilms is important in allowing it to establish biofilms *in vivo*, particularly at surgical sites and upon implanted abiotic surfaces. It is well recognised however, that infections related to implants and at surgical sites many contain multiple bacterial species within a single biofilm [33,34]. As such as ability to interact and compete with other species is also critical in allowing opportunistic pathogens to successfully establish a population. Importantly, we also observed that in our hands *C. acnes*, and its supernatants, interacted with *S. aureus* NCTC 6571, lowering the latter's ability to form mature biofilms. Investigation of *C. acnes* pathogenicity is still in its infancy and so the findings reported here are of importance in understanding how interactions between common opportunistic pathogens may affect the progression and persistence of infections. To the best of the authors knowledge this is the first study of how *C. acnes* and its soluble factors might influence *S. aureus* biofilm formation. This work is limited to only investigating the effect of *C. acnes* supernatants on a single *S. aureus* strain. It would be of significant future interest to determine of the affects observed here also occur in other strains of *S. aureus* and *S. epidermidis*. *C. acnes* isolates from the I2 phylogroup were reported to have inhibitory activity against *S. epidermidis* isolates [15] hinting that their influence is not just limited to *S. aureus* NCTC 6571.

We speculate that, at least in part, the observed antibiofilm activity could be due to the production of soluble products by *C. acnes*. The broad range of reductions in *S. aureus* NCTC 6571 biofilm biomass which the different *C. acnes* isolates could elicit, suggests that production of these products is likely to have some strain specificity. As such, further proteomic and genomic investigation of the *C. acnes* isolates presented here and their secreted components would be of significant interest. To the best of the authors knowledge there have been no description of antibiofilm components produced by *C. acnes* to date, however the species is known to produce several antimicrobial components. *C. acnes* 2.3.A1 and 27.1.A1 were both shown in previous studies to be able to inhibit growth of multiple *S. epidermidis* isolates using an agar overlay assay, whereas other isolates tested in the same study did not inhibit growth [15]. Similarly recent investigation has identified cutimycin, a *C. acnes* peptide with biocidal activity against *Staphylococcus* sp. but not *Corynebacterium* sp. [35]. It is important to note that in our hands *S. aureus* NCTC 6571 growth was not significantly reduced by the presence of four of the five tested *C. acnes* supernatants (Fig. 1C), suggesting that either compounds were not antimicrobial, or possibly any antimicrobial components were at too low a concentration to have an antimicrobial effect. Alongside the direct production of antimicrobial compounds, *C. acnes* can produce antimicrobial metabolites such as the short-chain fatty acid (SCFA) propionic acid which reduces growth of *S. aureus* USA 300 at a concentration of 25 mM [36]. Similarly, *C. acnes* ATCC 6919 was shown to have an inhibitory effect on *S. aureus* USA 300 [37] due to propionic acid production. Propionic acid is also reported to have antibiofilm activity. Yoneda et al. [38] reported the antibiofilm propionic acid effects against *Actinomyces naeslundii* but not *Streptococcus gordonii*. This partially supports our findings, where supplementation of medium with propionic acid alone did not decrease biofilm formation. Taken together these studies indicated that further investigation of active compounds within our *C. acnes* collection would be of interest in future investigations.

It should be noted that the results presented here do differ from those published by Gannesen et al. [39] and Tyner and Patel [14]. Both studies showed that *C. acnes* presence within a biofilm increased *S. aureus* biofilm biomass. Various factors could be responsible for these differences. To the best of our knowledge the *C. acnes* isolates used within this study have not previously been reported since they are clinical isolates collected and stored by ARU as part of their diagnostic activities. It is likely that these isolates will differ in the exact cocktail of secreted products produced compared to other *C. acnes* isolates. Neither of the studies above

attempted to quantify or identify the secreted products which might have accounted for their observations. Here we determined that the biomass reduction of *S. aureus* NCTC 6571 biofilms was not due to the ability of the *C. acnes* isolates to reduce the pH of their growth medium or due to the presence of propionic acid itself, however a further and more comprehensive analysis of the *C. acnes* secretome was outside the scope of the current investigation. Secondly, both the studies mentioned used methodology, bacterial isolates and medium different to each other and the work presented here. All of these variables may contribute to the observed differences between the studies. Wijesinghe et al. [40] highlighted the effect of different mediums on biofilm formation by *S. aureus* and *Pseudomonas aeruginosa* mixed-species biofilms. Similar findings were reported by Haney et al. [41] indicating that both temperature and medium influenced biofilm biomass, determined by crystal violet staining, of both *P. aeruginosa* PA01 and PA14. It is also important to note that medium may influence the secreted components produced by *C. acnes*, again possibly account for the difference in our findings and those of two other studies. Yuan et al. [42] noted that protein secretion via the type 3 secretion system within *Escherichia coli* could be influenced by the growth medium selected. Finally, it is also likely that the genetic background of the *S. aureus* may influence its response to the presence of *C. acnes* supernatant. Here *S. aureus* NCTC 6571 was chosen as it is a well characterised *S. aureus* isolate and extensively used in *in vitro* studies. Christensen et al. [15] showed variability in the biocidal activity of 20 *S. epidermidis* isolates against *C. acnes*, with a similar variability observed in the biocidal activity of the 77 *C. acnes* isolates they tested. Due to these factors it is difficult to make comparisons across studies, since not only the technique used to quantify biofilms but also the growth medium, environmental conditions and choice of attachment surface are all likely to in some way effect the experimental outcome. Standardisation of biofilm techniques is an ongoing issue within the field, however we hope that the very recent publication of minimal information guidelines for biofilm assays [43] will go some way towards resolving these issues and allowing easier comparison of future studies, more in line with other areas of biological research such as MIC testing [44], exosome research [45] and qPCR [46].

The incidence of AMR within *S. aureus* isolates is well reported and reports of *C. acnes* antibiotic resistance are increasing [47]. As such susceptibility of organisms to antibiotics is of great interest, as AMR leads to significant patient morbidity and mortality [48]. Our data indicated that in our hands *S. aureus* NCTC 6571 biofilms had greater susceptibility to antibiotics when in the presence of *C. acnes* supernatants. It is perhaps unsurprising that this would occur as *S. aureus* NCTC 6571 biofilm biomass was significantly reduced in the presence of *C. acnes* and its supernatants. It is likely that the observed delays in maturation, and subsequent biomass reduction played a significant role in increasing susceptibility. Ito et al. [49] showed that *Escherichia coli* mature biofilms were less susceptible to antibiotics than immature biofilms. Similarly, Shih and Huang [50] showed that reduction in ECM production by *P. aeruginosa* increased antibiotic susceptibility. It is more difficult to explain the increase in antibiotic resistance observed in planktonic cultures when *C. acnes* supernatant was present. Work by other groups has highlighted that the presence of two or more species alters susceptibility of individual bacterial species [51], with various mechanisms of activity hypothesised to be responsible for the observed effects. Thickening of *Streptococcus anginosus* cell walls in the presence of *S. aureus* supernatants decreased its susceptibility to vancomycin, but not ciprofloxacin [52]. *S. aureus* supernatants were also shown to have a protective effect on clinically adapted *P. aeruginosa* isolates grown in the presence of tobramycin [53]. It is possible that either physiological or transcriptional alterations, or a combination of both, are responsible for the decrease in *S. aureus* antibiotic susceptibility observed in the presence of *C. acnes* supernatants. As with other areas of this research further investigation of the components present in the *C. acnes* supernatants and the response *S. aureus* isolates have to their presence will further elucidate the mechanism of action underpinning the observations presented here.

5. Conclusions

In conclusion, this study has demonstrated that 100 clinical isolates of *C. acnes* are able to form biofilms. Furthermore, *C. acnes* supernatants inhibited the maturation of *S. aureus* NCTC 6571 biofilms and altered its antimicrobial susceptibility. This study highlights that in both the *in vitro* and *in vivo* environment it is likely that interactions between microbial species modulate both bacterial accumulation, biofilm formation and resistance to antimicrobial treatments. As the supernatant of all the *C. acnes* isolates tested inhibited biofilm maturation in *S. aureus* NCTC 6571, and for the vast majority this reduction was statistically significant, we conclude that the inhibitory effects of *C. acnes* supernatants are likely to be widespread across the species. Future work is planned to investigate further the components responsible for these modifications and to establish the molecular basis for the effects shown within this study. Further consideration should also be given as to how *C. acnes* can influence more complex microbial communities containing multiple other opportunistic and commensal species.

It is possible that the modifications to architecture and susceptibility of the *S. aureus* biofilm which occur in the presence of *C. acnes* and its supernatants are also present during clinical infections. This highlights the possibility that *S. aureus:C. acnes* co-infections may respond differently to treatment strategies, potentially being more easily resolved than mono-species *S. aureus* infections. Further work is needed to establish if this is the case and to elucidate the mechanism of action and *C. acnes* components possibly responsible for the observed effects. Further research must also be supported by more thorough testing of suspected deep tissue infections, with particularly emphasis on detecting the anaerobic species present and their phylogroup.

Author contributions

HLB carried out experimental work, drafted manuscript and designed study. CA carried out optimisation of biofilms formation and assessment of biofilm formation. EG performed MIC and MBEC testing. TEM provided isolates, technical advice and manuscript review.

Declaration of competing interest

The authors declare no competing conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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