ORIGINAL ARTICLE

An *in vitro* biofilm model system to facilitate study of microbial communities of the human oral cavity

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Significance and Impact of the Study: The CDC biofilm reactor is a continuous-flow culture model that has been deployed to study complex interactions between members of human microbiotas. In this study, we used the CDC biofilm reactor to efficiently grow and maintain diverse oral microbiota. This robust model could be used to both study the safety of antimicrobial-containing oral care products and examine novel approaches to modify plaque composition using pre- or probiotics.

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Abstract

The human oral cavity is host to a diverse microbiota. Much of what is known about the behaviour of oral microbes derives from studies of individual or several cultivated species, situations which do not totally reflect the function of organisms within more complex microbiota or multispecies biofilms. The number of validated models that allow examination of the role that biofilms play during oral cavity colonization is also limited. The CDC biofilm reactor is a standard method that has been deployed to study interactions between members of human microbiotas allowing studies to be completed during an extended period under conditions where nutrient availability, and washout of waste products are controlled. The objective of this work was to develop a robust *in vitro* biofilm-model system from a pooled saliva inoculum to study the development, reproducibility and stability of the oral microbiota. By employing deep sequencing of the variable regions of the 16S rRNA gene, we found that the CDC biofilm reactor could be used to efficiently cultivate microbiota containing all six major phyla previously identified as the core saliva microbiota. After an acclimatisation period, communities in each reactor stabilised. Replicate reactors were predominately populated by a shared core microbiota; variation between replicate reactors was primarily driven by shifts in abundance of shared operational taxonomic units. We conclude that the CDC biofilm reactor can be used to cultivate communities that replicate key features of the human oral cavity and is a useful tool to facilitate studies of the dynamics of these communities.

Introduction

The human oral cavity allows the colonization of a diverse and unique microbiota. After the gut, the oral cavity is the second largest microbial community in humans (Deo and Deshmukh 2019), and undergoes continuous environmental changes in parameters such as carbohydrate source and availability, pH, oxygen tension and redox potential (Kilian *et al.* 2016; Deo and Deshmukh 2019). The microbial community associated with the oral

cavity has been highly studied in part due to its significance to health but also to provide insight into the functions of multi-species communities and underlying interspecies interactions (Edlund *et al.* 2013; Kilian *et al.* 2016; Deo and Deshmukh 2019). Difficulties in understanding this complex and assorted environment are multidimensional, and include issues related to microbiota, the variation in human subjects, lack of continuous access to samples, lack of robust sample sizes and ethical consent issues. Both artificial consortia biofilm and *in vitro* model systems using human oral microbial isolates have been studied by using growth systems that have included chemostats (Bradshaw *et al.* 1989), saliva-conditioned flow cells (Foster and Kolenbrander 2004), constant-depth film fermenters (Kinniment *et al.*1996) and synthetic mouths (Sissons *et al.* 1991). These studies demonstrated that the biofilms containing a collection of bacterial species are functionally reproducible and have some consistencies with those of natural plaque (Bradshaw *et al.* 1989; Sissons *et al.* 1991; Kinniment *et al.* 1996; Foster and Kolenbrander 2004; Edlund *et al.* 2013).

A hurdle to understanding the structure and function of the oral microbiota is the undetermined role of the unculturable fraction of the bacteria present in the community. Additionally, it is difficult to track species and strains temporally and spatially in these communities due in part to the microbiota taxonomic variability between study subjects, (Campbell *et al.* 2013; Zhao *et al.* 2020). Moreover, the small sample sizes and costs limit the statistical power needed to detect and understand small but likely significant differences between these communities.

To gather a more complete ecological understanding of the mechanisms that are involved in the succession of healthy to disease-associated oral microbiotas, it is important to develop oral microbial model systems which allow for experiments to be conducted in a controlled environment. There are many advantages to such systems given they provide novel openings to examine the microbial community ecology with systems biology perspectives using omics experimental tools including metagenomics, metabolomics and metatranscriptomics. Such a model system would also allow for the generation of biological replicates and contribute to the analyses of large numbers of samples that are needed to obtain reliable data (Campbell *et al.* 2013).

In this study, we aimed to develop a multispecies biofilm model system with high bacterial diversity, representative of the resident saliva-derived microbiota responsible for biofilm formation in the oral cavity. We used the CDC biofilm reactor that has previously been shown to support growth of multispecies biofilms (Rudney *et al.* 2012; Touzel *et al.* 2016). We have evaluated the reproducibility of this model system by analysis of the 16S rRNA data.

Results and Discussion

Cultivating oral microbiota using the CDC biofilm reactor

The goal of this study was to examine the reproducibility of a complex oral biofilm generated in the CDC biofilm reactor (Fig. 1). Biofilms were grown on hydroxyapatite coupons to mimic the tooth surface where plaque develops. We employed supplemented Brain Heart Infusion (BHIS) medium in the CDC biofilm reactor to obtain the high species diversity to be representative of the human oral cavity microbiota, with high reproducibility and stability. An inoculum of pooled saliva and plague samples from six subjects was used to overcome individual microbiota variability and three reactors were used in the study. The pH of the start culture was approximately 7·0, which was similar to the pH value of the culturing medium used in the study. To monitor the development of the biofilm, samples from Days 1, 3, 5 and 8 were taken for analysis.

After 24 h growth in BHIS, the pH value decreased to ~5·5. At Day 3, the pH increased to ~6·8 and stayed between 6·5 and 7·0 over the remaining course of the experiment (Fig. 2a). This was consistent across all three bioreactors. In parallel, colony forming units (CFUs) of the samples were quantified by growth on BHIS agar plates (see Materials and Methods). As shown on Fig. 2b, Day 1–Day 8, the CFU counts from planktonic samples were about 10 times higher than those of the samples taken from the biofilms. Interestingly, there was no significant change in CFU counts from Day 1 to 8 on BHIS agar plates for both sample types, suggesting stable growth over time. To visualize the biofilm structure over the 8-day period, samples were also harvested for confocal microscopy analysis. The results of the acquired microscopy data are presented in Fig. 2c. Visualization of 3D reconstructed biofilms revealed distinct outcomes between samples. Biofilms obtained from Day 5 and Day 8 exhibited completely developed 3D structured masses. In contrast, biofilms obtained from Day 1 and Day 3 were characterized as aggregates that gradually colonized the surface. The loss of some less adherent aggregates during the processing of the younger biofilm samples for confocal microscopy could account for the apparent increase in coverage of the coupon with time while the colony counts were relatively stable.

Bacterial community and relative abundance analysis by 16S rRNA sequencing

16S rRNA metagenomic analysis was deployed to examine the bacterial community diversity and abundance of samples from the CDC biofilm reactor. This entailed
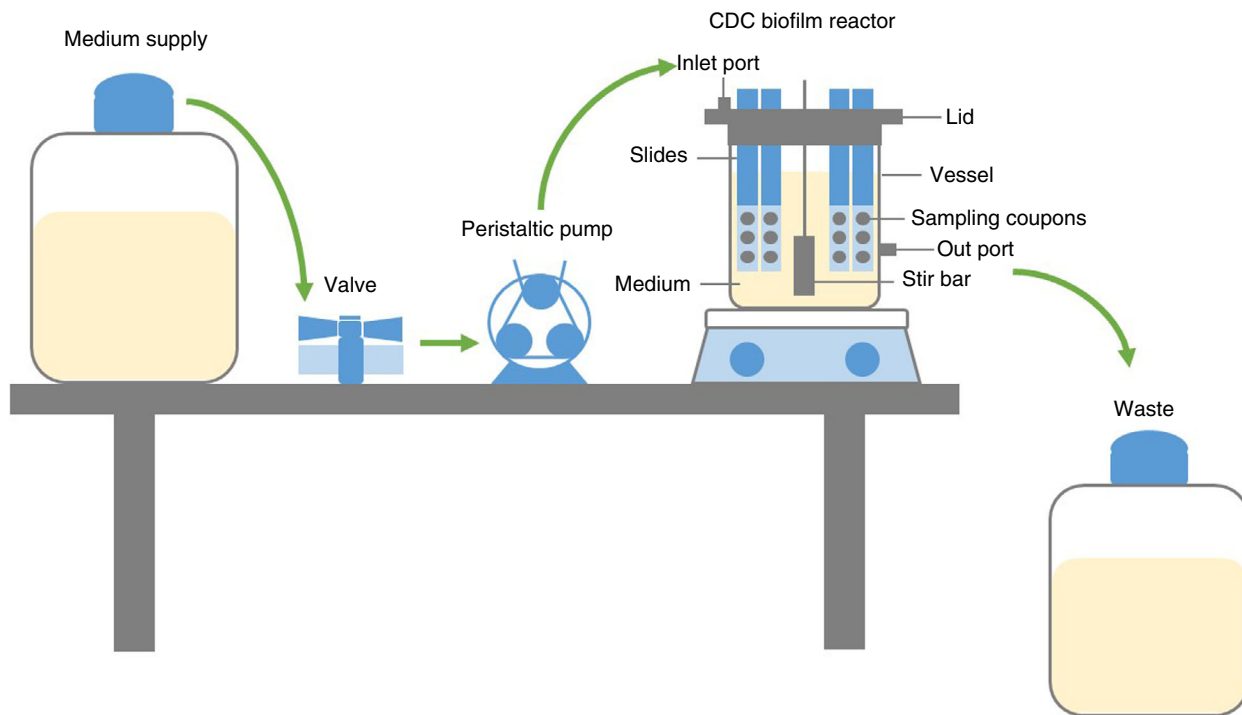
Figure 1 A schematic of a CDC biofilm reactor used in this study for biofilm growth and development.
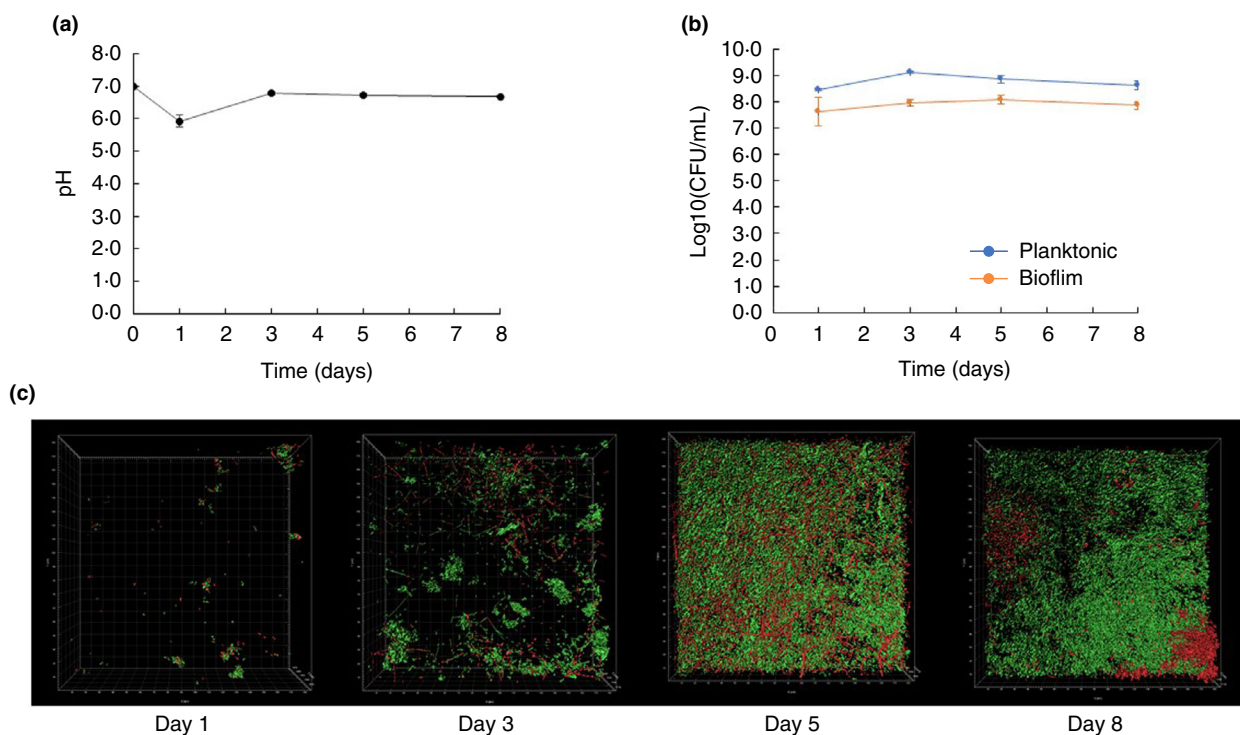

Figure 2 Growth characteristics of oral biofilms developed in the CDC biofilm reactor experiments. (a) pH variation throughout the operation of the CDC biofilm bioreactor. Data are presented as mean values $\pm$ SD of triplicate experiments. (b) Changes in the microbial population measured by colony forming units (CFU) obtained on supplemented brain heart infusion (BHIS) agar. Data are presented as mean values $\pm$ SD of triplicate experiments. (c) Live/Dead staining confocal microscopy images of biofilms obtained from the CDC biofilm reactor at Days 1, 3, 5, 8.

amplifying 16S rRNA genes from 25 samples (three biofilm and three planktonic samples from Days 1, 3, 5 and 8; water was used as control) using specific primers (see Materials and Methods) that spanned variable regions V3–V4 and incorporating specific bar code tags for identification. Amplicon libraries were prepared and sequenced using Illumina MiSeq, as described in the Materials and Methods.

The number of high-quality reads per bioreactor sample was between 73 and 190 K, with a total of high-quality reads from all conditions of 2 441 554 (Tables S1 and S2). The resulting OTU table contained 500 OTUs which were assigned based on the SILVA 16S (clustered at 97% similarity) (see Table S1). As expected, there was no bacterial DNA signal identified in the control sample (water) (Table S2). The relative bacterial abundance in each sample is presented in Fig. S1. The rarefaction analysis suggested that there were enough sequences to characterize the majority of bacteria present in these communities Fig. S2.

A total of 22 orders belonging to nine phyla of bacteria in the cultivated oral microbiota samples were found. The most abundant OTUs at the phylum level were: Firmicutes (44%), Bacteroidetes (21%), Actinobacteria (16%), Fusobacteria (9%) and Proteobacteria (3%), which is consistent with previous reported predominant phyla of the human oral microbiota (Dewhirst *et al.* 2010; Deo and Deshmukh 2019). In addition, 0·002% of the sequences could not be classified at the phylum level. The relative abundance of each individual sample is shown in Fig. S1. The total relative abundance of the predominant 12 orders (arbitrarily defined as >1% abundance) represented 97·30% of the OTUs, and Lactobacillales (25·00%), Bacteroidales (21·00%) and Selenomonadales (13·89%) were the top three. Meanwhile, 0·45% of the sequences were not classified at the order level (Fig. S1). These 12 high abundance orders were distributed among multiple bacterial phyla, four of them were Actinobacteria, three were Firmicutes, and one each were Bacteroidetes, Fusobacteria, Proteobacteria, Epsilonbacteraeota and Patescibacteria. Therefore, the cultivated human oral microbiota also has a high complexity in order composition. These observations are consistent with previous reported from samples taken from the oral cavity and propagated in biofilm bioreactor (Edlund *et al.* 2013).

Comparison of bacterial biofilm communities from different biofilm reactors

PERMANOVA principal coordinate analysis (PCoA) was used to compare samples from different biofilm reactors. This analysis showed that the overall profile of bacterial communities was largely similar between the samples taken from different bioreactors (Fig. 3). There were no statistically significant differences between the samples taken from across different reactors ($Padj = 1·0$) (Table S3), which suggested the reproducibility of the model was good.

Comparison of samples taken at different time points showed that the community at Day 1 was distinct from those at Days 3, 5 and 8 ($Padj = 0·01$), whereas bacterial communities in samples from Days 3, 5 and 8 clustered together ($Padj > 0·01$) (Fig. 4 and Table S4). The predominant order at Day 1 was Lactobacillales (40·60%) which reduced to 21·28% at Day 8. However, Fusobacteriales and Coriobacteriales increased from 1·31% (Day 1) to 16·80% (Day 8), and 0·18% (Day 1) to 6·44% (Day 8) respectively. There was no significant difference between biofilm and planktonic composition (Fig. 5 and Table S5).

The oral bacterial community is dominated by the six major phyla, Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes and Fusobacteria. We found that the CDC biofilm reactor could be used to cultivate microbiota containing all six major phyla previously identified as the core saliva microbiota although Spirochaetes were less abundant. The analysis also allowed us to identify OTUs that are not included in the reference database. Seven of them were identified in our study with reads >60 (Table S6). Nucleotide BLAST analysis suggests they are likely to be uncultured bacteria.

The use of such a model system can aid in the understanding of the interactions in microbial communities of the oral cavity. It will facilitate discovery and functional characterization of known, as well as uncultivated bacteria, within a multispecies system that is approaching the diversity of *in vivo* conditions. It could be used to generate data for novel oral care antimicrobials to understand their efficacy and help ensure they are safe for consumers.

Materials and Methods

Saliva-plaque collection

Saliva-plaque was collected from six healthy subjects as described by Hall *et al.* (2017). Study subjects were recruited through the Southampton Research Biorepository volunteer database. Consents from study subjects, including consent to participate in the study and consent to publish findings from saliva/plaque samples, were obtained. Ethical approval of all protocols related to saliva collection and experimental research was confirmed by University of Southampton recognised Research Ethics Committees (RECs) (approval reference: 17/NW/0632). Subjects were asked to refrain from any food or drink 9 h before samples were taken. Approximately 5 ml sample
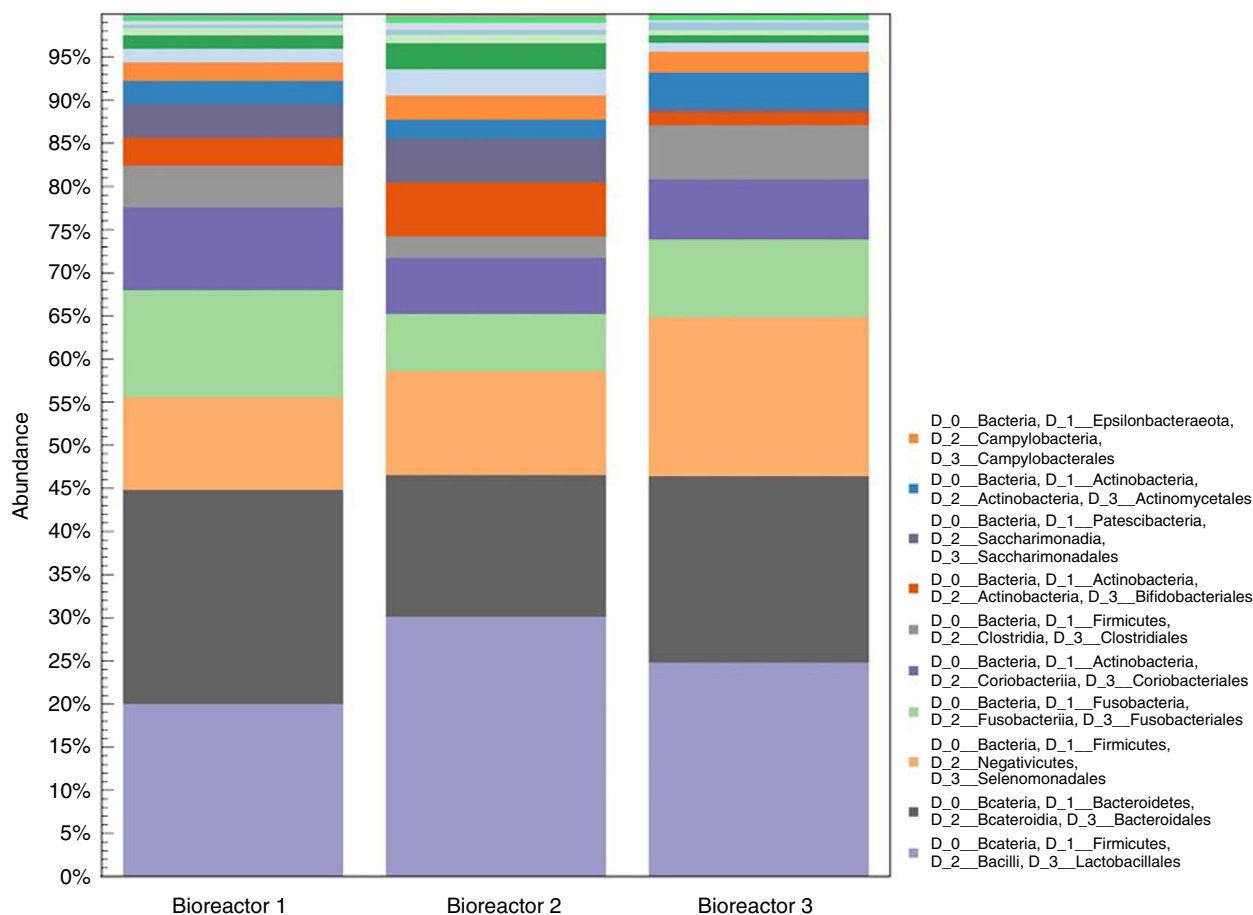
Figure 3 Relative abundances of bacterial orders identified as operational taxonomic units (OTUs) from the sequence reads generated from biofilm samples (CDC biofilm reactors 1, 2, 3).

was collected from each person and pooled together for further analysis.

Culturing and growth of saliva-derived biofilm using the CDC biofilm reactor

A CDC biofilm reactor (BioSurface Technologies, Bozeman, MT, USA; Fig. 1) was used to culture and grow the saliva-derived biofilms. Based on the American Society for Testing and Materials standard E2562-07 (https://infostore.saiglobal.com/en-gb/standards/astm-e2562-07-156230_SAIG_ASTM_ASTM_379236/), the reactor was run using the following parameters to mimic in part the oral environment of the mouth: 500 μl saliva sample was inoculated into 350 ml of Brain Heart Infusion broth (BHI) supplemented with hog gastric mucin (1 g l^{-1}), haemin (10 mg l^{-1}) and vitamin K (0·5 mg l^{-1}) (brain heart infusion-supplemented, BHIS) in the biofilm reactor (Kistler *et al*. 2015). The rotator was stirred at

100 rev min^{-1}, and the unit was run at 37°C which was monitored by a temperature probe. BHIS broth flowed through the reactor at 0·5 ml min^{-1} for the course of the experiment. These experiments examined the biofilm and planktonic samples taken at Days 1, 3, 5 and 8. Biofilm samples were obtained from hydroxyapatite sampling coupons (BioSurface Technologies); planktonic samples were taken from the reactor chamber.

CFU counts

At Days 1, 3, 5 and 8 biofilm and planktonic samples were taken as discussed above. The biofilm was removed from the coupons using cell scrapers and sonication (150 W for 10 min). Serial dilutions were made in phosphate buffered saline and spread-plated in triplicates on BHIS agar plates. Plates were incubated at 37°C for 24 h, colonies were manually counted, and results were recorded and expressed in log CFU per ml.
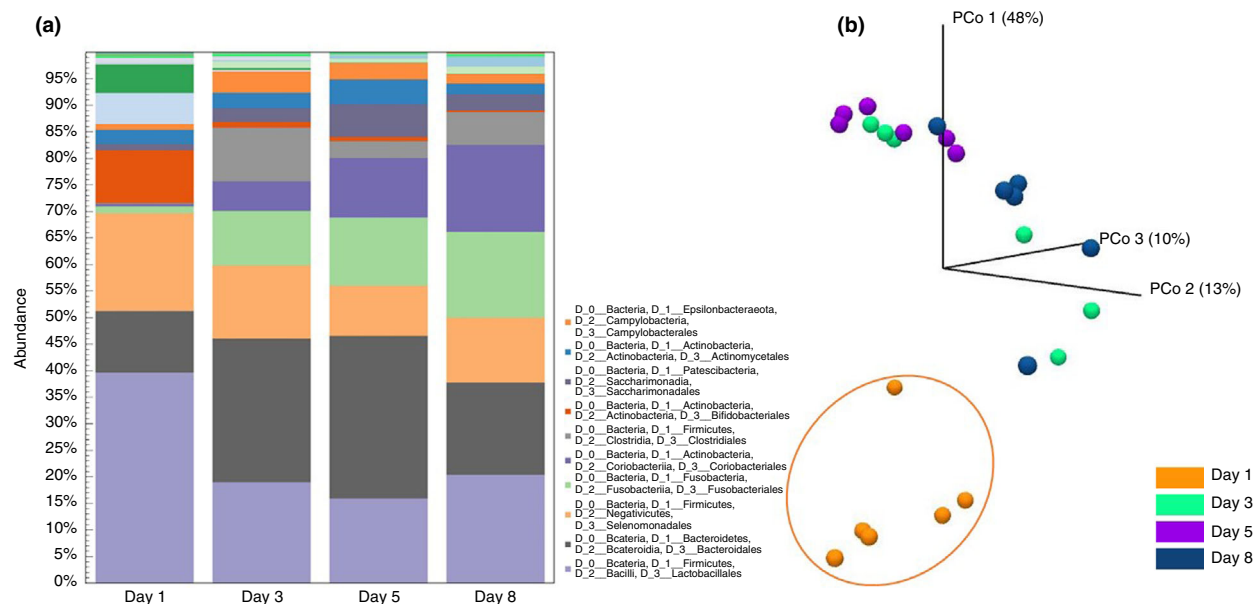
Figure 4 Comparison of bacterial communities identified from biofilm samples taken at different time points. (a) Relative abundances of bacterial order identified as operational taxonomic units (OTUs) from the sequence reads generated from samples taken from different time points (Days 1, 3, 5, 8). (b) Diversity analysis demonstrating differences in the bacterial order community between samples. Principal coordinate (PCo) analysis of all samples based on D_0.5 UniFrac distance. Colour denotes different sample groups (Days 1, 3, 5, 8).

Confocal laser scanning microscopy analysis

The biofilms on sampling coupons were fixed in 4% paraformaldehyde for 10 min. and staining with LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen, Thermo Fisher Scientific, Loughborough, UK) was performed according to the instructions provided by the manufacturer. Stained biofilms were examined with a Confocal laser scanning microscopy (CLSM) (Leica model TCS SP8, Imaging and Microscopy Centre, University of Southampton) using a 63× oil objective. A 488 nm laser line was used to excite SYTO® 9, while the fluorescent emission was detected from 500 to 540 nm. Propidium iodide (PI) was excited with 561 nm laser line and its fluorescent emission was detected from 600 to 695 nm. For each sample, sequential optical sections were collected along the z-axis over the complete thickness of the sample. Images were subsequently rendered into 3D mode by IMARIS 9 software.

DNA extraction and sequencing

DNA extraction was performed using the MasterPure Complete DNA and RNA Purification kit according to the manufacturer's instructions. Briefly, bacterial cell pellets were resuspended in 150 μl TE buffer (10 mmol l^{-1} Tris-HCl [pH 7.5], 1 mmol l^{-1} EDTA) containing 1250 U of Ready-Lyse Lysozyme Solution (Lucigen, Middleton,

WI) and then incubated at 37°C overnight. Subsequently, 150 μl of Tissue and Cell Lysis Solution containing 1 μl of Proteinase K (50 μg μl^{-1}) were added. The mixture was incubated for 30 min at 65°C, and vortexed every 5 min. After placing the samples on ice for 7 min, 175 μl of MPC Protein Precipitation Reagent was added. Cell debris was pelleted by centrifugation at 4°C for 10 min at 13 100 *g* in a microcentrifuge. The supernatant was transferred to a fresh microcentrifuge tube and the pellet was discarded. A volume of 500 μl isopropanol was added to the recovered supernatant and then carefully mixed by inverting the tube several times. The precipitated DNA was pelleted by centrifugation at 4°C for 10 min at 13 100 *g* in a microcentrifuge. After washing DNA pellets twice with 500 μl of 75% ethanol, the DNA was resuspended in 25 μl TE buffer and then stored at −80°C until further use.

16S gene amplification and sequencing

16S Metagenomic Sequencing Library Preparation Guide (Illumina, Inc.), was followed to prepare sequencing libraries targeting the variableV3 and V4 regions of the 16S rRNA gene (Forward Primer: TCGTCGGCAGCGTCA GATGTGTATAAGAGACAGCCTACGGGAGGCAGCAG; Reverse Primer: GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACAAGGGTATCTAATCC) and paired-end sequencing was performed on the MiSeq
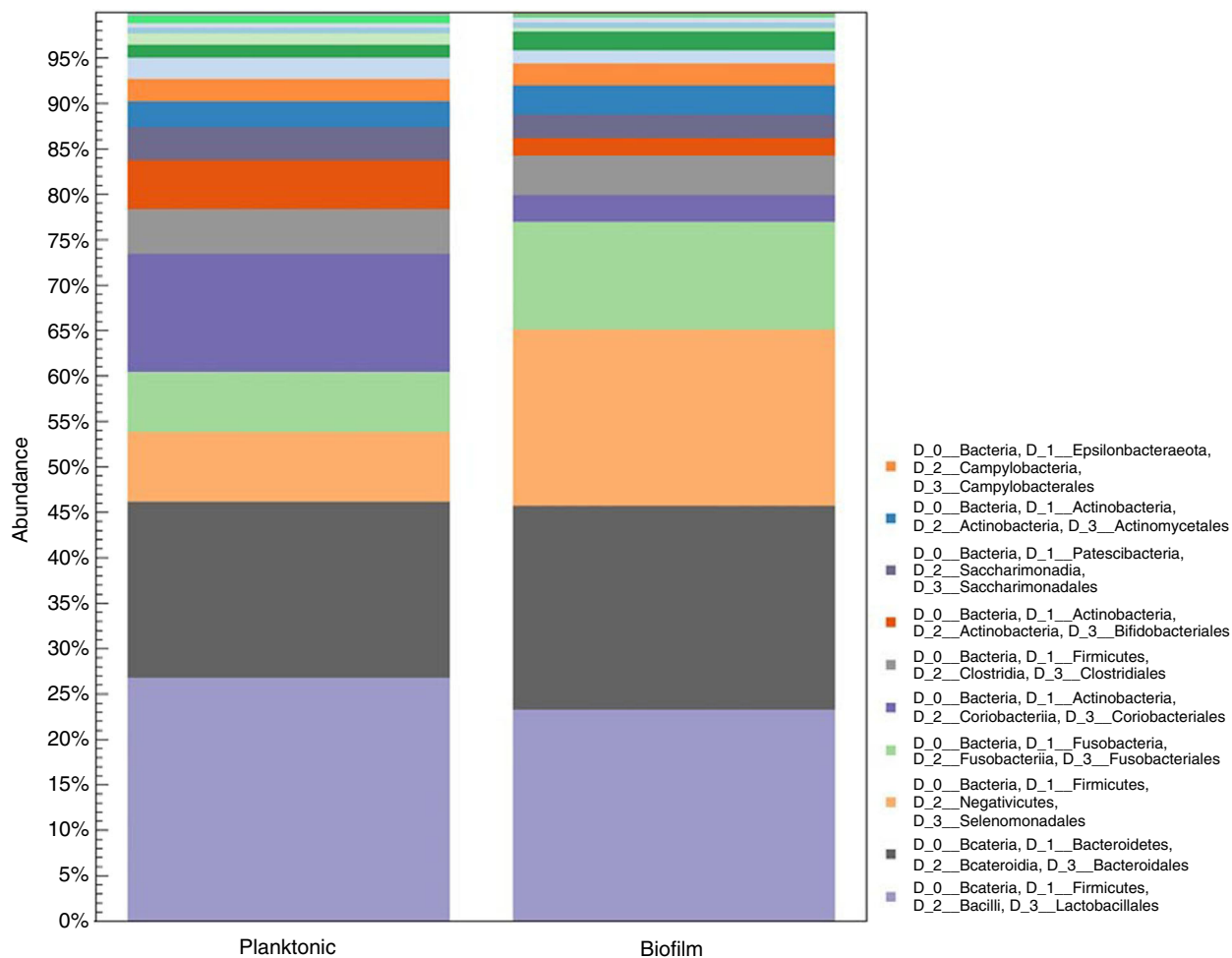
Figure 5 Comparison of bacterial communities identified between biofilm and attached planktonic samples.

System (Illumina, Inc.). We followed sampling and controls procedures described by the Environmental Sequencing Facility (University of Southampton, UK) and Illumina, Inc (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf).

Data and statistical analysis

Bioinformatics analyses of raw data were performed using the Biomedical Genomics Workbench version 4.0 (Qiagen) equipped with the Microbial Genomics Module version 2.0 (Qiagen) plugin. Sequences were imported and processed for optional merge paired reads, adapter trimming, fixed length trimming and then the sequences were filtered based on the number of reads to obtain sequences that were comparable in length and coverage for clustering. Quality and chimera filtering were performed using the recommended programme parameters (for complete

details see www.qiagenbioinformatics.com). Samples with low coverage were removed from further analysis. Operational taxonomic units (OTU) clustering and taxonomic assignment were done using SILVA 16S V132 (97%) as the reference database. New OTUs were indicated when similarity percentage was lower than 80% with a minimum occurrence of 5 reads. Low abundance OTUs were discarded from further analyses (minimum combined abundance was set at 10 OTUs). A summary of processed sequence data is described in Table S1. MUSCLE was used for OTUs alignment to reconstruct a maximum likelihood phylogeny. We compared community structures and diversity across different sample groups to determine whether any differences in the structure were seen. To achieve this a rarefaction sampling analysis was carried out using a standard methodology (Kumar *et al.* 2014). Alpha diversity was calculated using the number of OTUs. Beta diversity was obtained using D_0.5 UniFrac and represented as PCoA. Robustness analysis was performed

using PERMANOVA with UniFrac distances. All sequencing data have been deposited in EMBL-EBI European Nucleotide Archive (ENA) database accession number PRJEB42383(ERP126232).

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions

S-Q.A, R.H, A.M, P. B, J.W and P.S planed the study. S-Q.A and R.H performed the experiments. S-Q.A and R.H wrote the manuscript. A.M, P. B, J.W and P.S reviewed and provided valualbe suggestions to the manuscript. All authors have read and approved the final manuscript.

Data Availability Statement

All sequencing data have been deposited in EMBL-EBI European Nucleotide Archive (ENA) database accession number PRJEB42383 (ERP126232).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Relative abundances of bacterial order identified as operational taxonomic units (OTUs) from the sequence reads generated from all the samples taken at different time points.

Figure S2 Alpha diversity rarefaction curves of samples based on total number of observed operational taxonomic units (OTU).

Table S1 Summary of sequence data used for operational taxonomic units (OTU) clustering and analysis of samples taken at different time points.

Table S2 List of sequence data for individual samples used for operational taxonomic units (OTU) clustering and analysis of samples taken at different time points.

Table S3 PERMANOVA analysis results of testing differences in beta-diversity among different bioreactors.

Table S4 PERMANOVA analysis results of testing differences in beta-diversity among different sampling time points.

Table S5 PERMANOVA analysis results of testing differences in beta-diversity between planktonic and biofilm samples.

Table S6 OTUs identified not based on a sequence from the database.