Title: Development of an *in vitro* Biofilm Model of the Human Supra-gingival Microbiome for Oral Microbiome Transplantation

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Abstract

The high prevalence of dental caries and periodontal disease place a significant burden on society, both socially and economically. Recent advances in genomic technologies have linked both diseases to shifts in the oral microbiota – a community of more than 700 bacterial species that live within the mouth. The development of oral microbiome transplantation draws on the success of fecal microbiome transplantation for the treatment of gut pathologies associated with disease. Many current *in vitro* oral biofilm models have been developed but do not fully capture the complexity of the oral microbiome which is required for successful OMT. To address this, we developed an *in vitro* biofilm system that maintained an oral microbiome with 252 species on average over 14 days. Six human plaque samples were grown in 3D printed flow cells (FCs) on hydroxyapatite discs using artificial saliva medium (ASM). Biofilm composition and growth were monitored by high throughput sequencing and confocal microscopy/SEM, respectively. While a significant drop in bacterial diversity occurred, up to 291 species were maintained in some flow cells over 14 days with 70% viability grown with ASM. This novel *in vitro* biofilm model represents a marked improvement on existing oral biofilm systems and provides new opportunities to develop oral microbiome transplant therapies.

Key words

Dental plaque, DNA sequencing, Oral Microbiome transplantation. Flow cell, Microbiota, Oral bacteria, Species diversity.

Introduction

Globally, 2.5 billion and 743 million people are estimated to suffer from untreated dental caries and severe periodontitis, respectively[1,2]. The highest prevalence of caries occurs in industrialised, middle-income countries as a result of refined sugar consumption (WHO 2017).

This trend is now being observed in developing countries[3], highlighting the need for better therapeutics.

Caries and periodontal disease aetiologies are directly linked to the microbiota present in dental plaque biofilms – diverse, symbiotic microbial communities that live within ecosystems on tooth enamel and dentine. The microbiota contained within plaque biofilms are notoriously difficult to treat using classical methods, such as physical removal or antibiotics[3]. Elsewhere in the body, microbiota have been manipulated using transplantation (*e.g.* Faecal Microbiota Transplantation (FMT) therapy), where microbiota from one individual are placed into another. While FMTs are now commonplace in the gut for certain diseases (*e.g.* *Clostridiodes difficile* infection)[4], oral microbial transplants (OMT) do not exist for the mouth, despite the promise they hold for treating recalcitrant oral diseases[5]. However, several challenges will need to be addressed to develop successful OMT therapies, and one important component is culturing donor dental plaque *in vitro* to increase bacterial biomass and improve safety associated with direct transplant from one person to another.

Several *in vitro* biofilm models have been developed that have significantly broadened our understanding of the bacterial interactions that cause disease[6-8] but they do not always reflect the dynamic nature of the oral environment[9]. For example, the Calgary and Zurich model lack the shear forces associated with saliva flow and the continuous displacement of nutrients typical of the oral environment. Flow cell (FC) systems can address these issues, however, to our knowledge, these models have not been used to grow complex polymicrobial oral biofilms required for OMT.

Here, we developed a 3D printed, flow cell inoculated with human supragingival dental plaque grown in artificial salivary media, (ASM) and cultured over 14 days. We compared bacterial biofilm thickness, volume, composition and diversity after growth and maintained a

core oral microbiome consisting of over 250 bacterial species. The methodology provides the technology essential for future OMT therapies.

Materials and Methods

Collection of supra-gingival plaque

Ethical approval was obtained by the University of Adelaide (#H-2017-108). Dental plaque was collected from six healthy volunteers (aged 25-60 years). Prior to plaque collection, the tip of a sterile Gracey curette (5/6) (Henry Schein, NSW, Australia) was washed in 200µL sterile PBS (pH 7.4) and stored at -80°C to monitor contaminant DNA[10]. Plaque was collected from the buccal interproximal sites of the first two incisors (#31, #32) and the mesio-buccal surface of maxillary first and second molars (#36, #37). Plaque samples from each volunteer were pooled into 200µL sterile PBS.

Flow cell and inoculation

FCs were 3D printed in polypropylene (Fig. A.1) to enable sterilisation. The flow rate of medium was adjusted to 2/5 the total volume of the FC[7]. Each FC contained five hydroxyapatite (HA) discs (D=5mm x H=2mm; Clarkson Chromatography Products, PA, USA). Following sterilisation, FCs were filled with 25% ASM devoid of sucrose 24hrs prior to inoculation to allow pellicle formation and confirm sterility. ASM was modified from the study by Dashper et al, and contained 0.50 g/L tryptone (Oxoid, England), 0.50 g/L neutralised bacteriological peptone (Oxoid), 0.625 g/L type III porcine gastric mucin (Sigma-Aldrich, Germany), 0.25 g/L yeast extract (Oxoid), 0.05 g/L KCl, 0.05 g/L $CaCl_2$, 0.088 g/L NaCl, and 1mg/L haemin (Sigma-Aldrich)[7]. Dental plaque was vortexed in PBS for 30s, and 100µL was added to 1mL of ASM and used to inoculate the FC. Media flow began after 24hrs, and the biofilm was grown at 36 ºC for 14 days. 100µL of the planktonic culture was then removed for sequencing, and each HA disc was removed and placed into a separate sterile 1.5mL tube. Two discs were used for scanning electron microscopy (SEM) and confocal laser scanning

microscopy (CLSM), while three discs were kept for High Throughput Sequencing (HTS) (Table 1).

Biofilm Imaging

SEM

Each disc was prepared for SEM[11], and coated with 2nm platinum, and imaged with a SEM-FEI Quanta 450 FEG. Due to variations in the density of biofilms, micrographs were imaged at three random positions on discs at 1,000x, 5,000x and 20,000x magnification.

CLSM

Each disc was washed in sterile PBS three times for 10 minutes to remove unbound cells. Biofilms were stained using a LIVE/DEAD™ BacLight™ kit (Invitrogen, Vic, Australia), following the manufacturer's instructions. Images were captured at three random positions using an Olympus FV3000 microscope at 60x magnification using the dimensions of 1024 pixels for both axes. Each stack was acquired using a z-step size of 0.5µm. Images were processed using IMARIS software (Bitplane, Zurich, Switzerland, version 7.6) for viability, average biofilm volume (μm^3), and thickness (µm). Collated cells were excluded from the analysis.

16S rRNA amplicon sequencing

DNA was extracted from 100µL taken from controls; plaque used for inoculation of flow cell (inoculated plaque); three HA discs with biofilm growth after 14 days; and planktonic cells (Table 1) using the Qiagen DNeasy PowerSoil® Kit (Qiagen, Maryland, USA), following the manufacturer's instructions. Extraction blank controls (EBCs) were added at the start and end of each extraction to monitor laboratory contamination[12].

DNA sequencing libraries were generated by amplifying the V4 hypervariable region of 16S rRNA gene, as previously described[13], using an Invitrogen Platinum High Fidelity DNA polymerase (Life Technologies, USA). Thermocycling conditions consisted of 6 mins

denaturation at 95°C; 38 cycles of 95°C for 30s, 50°C for 30s and 72°C for 90s; and a final extension of 60°C for 10 minutes[14] (Adler et al. 2013); one no-template control (NTC) was added per amplification batch. Samples were quantified using the Invitrogen Qubit dsDNA BR assay (Life Technologies), purified using 1.1X Axygen AxyPrep Mag™ PCR Clean-up beads, quantified using the D1000 reagents on the Tapestation (Agilent, Santa Clara, CA, USA), and equimolar pooled at 5nM for sequencing at the South Australia Health and Medical Research Institute (SAHMRI, Adelaide, Australia) on an Illumina MiSeq using 150bp paired-end sequencing. The demultiplexed sequencing data was imported into the Quantitative Insights Into Microbial Ecology (QIIME2-2021.4)[15]. Deblurring was performed on merged, demultiplexed, paired-end sequence reads that were quality filtered, and DNA sequences were denoised in amplicon sequence variants (ASVs)[16] and trimmed to 120bp.

Contaminants from laboratory sources (EBCs and NTCs) and FC controls were removed using the R package decontam[17] implemented in phyloseq R[18] using the prevalence based method[17]. Decontam score thresholds were chosen based on a histogram of decontam scores for each filtration step (Appendix Table 1).

Visualization and Statistical analysis

Diversity and compositional analyses were done using QIIME2. Sequences from each sample were rarefied to 1,462 and 26,608 sequences, as the highest number of sequences from ASM samples. α-diversity was calculated using the observed features metric of each sample. The β-diversity of the samples were analysed using weighted and unweighted UniFrac (phylogenetic matrices), Jaccard, and the Bray Curtis indexes and visualised using principal coordinates analysis (PCoA) plots with EMPeror[19]. A Kruskal-Wallis test was used to assess significant differences in α-diversity between sample types. Significant differences in β-diversity was assessed using PERMANOVA. A corrected p-value of <0.05 was considered

significant. Linear discriminant analysis (LDA) effect size (LEfSe)[20] was used to detect significantly abundant bacterial species between groups; an LDA score of >2 was used as the significance threshold. Significantly abundant taxa detected via LefSe analysis were visualised in a heat map using the 'heatmap' function in QIIME2 (Appendix Figure 4).

Results

Viability, thickness and volume of biofilms grown in ASM

Qualitative SEM assessment of biofilms grown on HA discs in ASM were predominantly rod- and cocci-shaped bacteria that grew in 'islands' and varied in biofilm density between donors (Figure 1A, B). CLSM analysis revealed an average $1.57 \times 10^4 \pm 6.08 \times 10^3$ and 4283±2166 live and dead cells from plaque grown in ASM (Table 2), and importantly, >70% of cells were viable (Figure 2A). Lastly, the average thickness and biovolume of ASM-grown biofilms were 30.88±6.11 μm and $4.36 \times 10^5 \pm 8.48 \times 10^4$ μm^3, respectively.

Significant differences in microbial composition between biological and control samples.

Initial analysis aimed to reduce the impact of contaminant DNA introduced during DNA extraction and library preparation, as well as the laboratory environment during collection and growth in the FC system. We examined the composition of the controls alongside the biological samples by conducting PCoA of unweighted UniFrac values (Figure 2B). The microbial composition of laboratory controls (EBCs/NTCs) was significantly different from biological samples, including spent medium, planktonic cells, inoculated samples, and biofilms grown over 14-days in ASM (PERMANOVA, pseudo-F; test: 21.51; $p \leq 0.001$). Contaminants identified in the EBCs/NTCs accounted for 30.55% (42 species) of the total species and were removed from the data using the decontam package. The composition of FC controls (Table 1) was also significantly different from biological samples (PERMANOVA, pseudo-F; test: 17.99; $p \leq 0.001$). As such, we also then removed contaminant

amplicon sequence variants present in the FC controls using decontam, which accounted for 17.63% (35 species) of the remaining sequences in all biological samples (Appendix Table 2) post filtering with EBCs.

α-diversity was reduced in biofilms after 14 days (T14)

We predicted that α-diversity (unweighted UniFrac, i.e. PERMANOVA) may decrease after growth in the FC, as seen with other systems[8]. We compared the α-diversity between inoculated plaque (T0) and T14 biofilms (Table 1). Inoculated plaque samples had an average of 312 species per donor, and after 14 days growth *in vitro*, an average of 252 species were recovered from each donor biofilm (291 species recovered overall). This was a significant decrease in α-diversity compared to the original inoculum (Kruskal-Wallis Pairwise; H test: 6.25, $p<0.05$), but we retained donor specific α-diversity signatures in all sample groups (Kruskal-Wallis; All Groups; H test: 13.05, $p>0.05$) (Appendix Figure 2A, B). Overall, the α-diversity was not significantly different between removal of plaque from the donor and inoculation into the flow cell (Kruskal-Wallis Pairwise; H test: 1.19, $p>0.05$).

β-diversity significantly differed between inoculated plaque and T14 biofilms in ASM

We next examined compositional shifts from the inoculum through to T14 biofilms grown in ASM. Significant differences in bacterial composition were observed after 14 days *in vitro*. Inoculated plaque and T14 biofilms were significantly different across all six donors (PERMANOVA test; pseudo-F; test: 5.68; $p\leq0.001$). However, the microbial composition of biofilms grown on three discs from the same FC clustered closely according to each donor.

Reduction in significantly abundant species at T14 biofilms compared to inoculated plaque

When inoculated plaque was compared to T14 biofilms, the relative abundance of *Firmicutes* and *Bacteriodetes* increased while *Actinobacteria* and *Fusobaceteria* decreased. Specifically, LefSe identified 42 significantly abundant ASVs that were higher in abundance at the time of inoculation (T0) compared to T14, belonging to *Firmicutes* (n=11), *Actinobacteria* (n=9), *Proteobacteria* (n=6), *Fusobacteria* (n=2), *Bacteriodetes* (n=1), *Tenericutes* (n=1), *GN02* (n=1), *Spirochaetes* (n=1) and *Synergistetes* (n=1) phyla (Figure 3). In contrast, 16 differentially abundant genera were more prevalent in T14 biofilms, *Firmicutes* (n=9), *Actinobacteria* (n=3), *Bacteriodetes* (n=3) and *Proteobacteria* (n=1).

Discussion

The present study provides the methodology to culture human plaque biofilms on hydroxyapatite over 14 days. It is thought that ~250-300 bacterial species are thought to be maintained as a core microbiome in the oral cavity[21], a significant challenge for OMT therapy is to reproduce this diversity *in vitro*. Visual analysis of biofilms was consistent with other research which found local variations in the density of biofilms on hydroxyapatite discs[7,22]. These findings highlight our selection to include three random sampling regions for biofilm analysis.

DNA sequencing indicated the α- and β- diversity of T14 biofilms grown with ASM was significantly decreased compared to inoculated plaque with a significant alteration in five phyla between inoculated plaque and T14 biofilms. This was a similar shift in bacterial composition at the phyla level to the study by Du et al. 2017[23]. A significant decrease in α-diversity was observed for biofilms grown in ASM; however, an average of 252 bacterial species across the six donor samples were detected in each plaque sample (275—244 species) after 14 days. To our knowledge, this is the highest oral microbial diversity yet reported using an *in vitro* flow cell model.

Examination of the significantly altered taxa between T0 and T14 biofilms grown in ASM, revealed a reduction in *Actinobacteria* and *Fusobacteria* and an increase in *Bacteriodetes* and *Firmicutes*. The majority of *Actinobacteria* species that reduced in abundance after 14 days were oral commensals[24]. Although this decrease is not ideal, it could be explained by the increase in abundance of *Firmicutes* and *Bacteroidetes*, which is shown to form an alliance against *Actinobacteria*[25]. The reduction of *Fusobacteria*, *Spirochaetes*, and *Synergistetes* at 14 days may be a positive result as these phyla are linked to poor oral health outcomes[26-28]. However, it is unclear when this transition in abundance occurred.

Kistler, et al. (2015) also noted a significant change in bacterial composition over time using saliva as the inoculum, the taxonomic composition of biofilms grown *in vitro* were similar to dental plaque, with the most abundant genera being *Prevotella, Streptococcus*, and *Veillonella*[8]. The shift in bacterial composition at T14 maybe due to the absence of the host-immune interaction[8] or the absence of certain nutrients and growth factors in the medium. ASM can support the growth of a wide range of bacterial species but may decrease metabolic activity in others such as *A. naeslundii*[29]. Previously published research included sucrose in ASM, however sucrose was excluded in this study because it favours the growth of cariogenic bacteria[7,30].

In conclusion, the 3D printed biofilm model was found to be capable of growing oral biofilms with the highest oral microbial diversity reported (average of 252 species) using ASM. This study also demonstrated the ability of ASM to maintain a high species diversity which suggests the methodology has the potential to be used as an antimicrobial testing platform for antimicrobials, oral care products, natural and synthetic compounds, and oral microbiome transplant therapy[31].

Availability of Data and Materials

All QIIME2, code, analysis files, and R code used to plot figures can be found here: https://github.com/DonKevin1994/In_Vitro_Biofilm_Model

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Declaration of Conflicting Interests

All authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Tables and Figure Legends

Table 1. List of samples collected during the study. Biological samples,1-4; Control samples, 5-8.

Sample number	Sample name	Description
1.	Inoculated plaque/T0	Plaque sample mixed with nutrient media prior to FC inoculation
2.	T14 biofilms	Biofilms formed on HA discs after 14 days
3.	Planktonic cells	Planktonic cells taken from the FC after 14 days

4.	Spent medium	Media outflow (waste) from the FC collected for 14 days
5.	FC control	Media collected from FC prior to plaque inoculation
6.	Media control at T0	Nutrient media collected at T0
7.	Media control at T14	Nutrient media collected at T14
8.	PBS control	PBS collected prior to the start of the experiment

Table 2. Number of live and dead cells, biofilm thickness and biovolume across 6 donors

Subject	Number of live cells	Number of dead cells	Biofilm thickness (μm)	Biovolume (μm^3)
1	$1.21 \times 10^4 \pm 4.61 \times 10^3$	3759.33±2162.45	30.75±10.96	$4.34 \times 10^5 \pm 1.52 \times 10^5$
2	$1.78 \times 10^4 \pm 4.52 \times 10^3$	7075.00±1038.48	40.00±3.00	$5.63 \times 10^5 \pm 4.15 \times 10^4$
3	$5.73 \times 10^3 \pm 1.75 \times 10^3$	1973.33±1521.70	22.17±5.11	$3.15 \times 10^5 \pm 7.09 \times 10^4$
4	$1.20 \times 10^4 \pm 8.18 \times 10^3$	2260.33±975.12	35.00±16.52	$4.93 \times 10^5 \pm 2.30 \times 10^5$
5	$1.16 \times 10^4 \pm 2.70 \times 10^3$	6701.00±1102.21	29.17±4.37	$4.12 \times 10^5 \pm 6.06 \times 10^4$
6	$9.76 \times 10^3 \pm 3.65 \times 10^3$	3933.67±1335.28	28.17±1.04	$3.98 \times 10^5 \pm 1.46 \times 10^4$

Figure 1. Supragingival dental plaque biofilm formation on HA discs following 14 days growth using ASM A) SEM images of HA discs grown in ASM from all 6 donors, 1-6. Images were taken from three random regions of each HA disc using the magnifications: 1,000x, 5,000x and 20,000x. Scale bars = 2μm, 10μm and 50μm, respectively.

Figure 2. Cell viability and 16S rRNA sequencing analysis on microbiota grown in ASM for 14-days. A) Percentage viability of supragingival biofilms established in the established

biofilm grown using ASM media. 73.5% and 72.4% of cells in biofilms were alive and 26.5% and 27.6% were dead in ASM media. Error bars represent mean ± S.D. ****p<0.0001, ns: non-significant. B) PCoA plot indicating differentiation of microbiota. Axis 1 explains 31.04% of the variation in the dataset; axis 2 explains 12.00% of the variation and axis 3 explains 7.098% of the variation. The bacterial samples including inoculated and collected plaque, T14 biofilms and planktonic cells, cluster more distantly along the first ordination axis whereas control samples representing the sample blanks and negative controls cluster closely together. C) Comparison of β-diversity of the collected, inoculated and T14 biofilms grown in ASM. Axis 1 explains 51.75% of the variation in the dataset; axis 2 explains 21.36% of the variation and axis 3 explains 11.65% of the variation. D) Change in the dominant phyla from T0 to T14.

Figure 3. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) analysis at species level to compare the oral microbiome profiles of the donors between inoculated plaque (T0) and T14 biofilms grown in ASM.

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