



Development of an *in vitro* biofilm model of the human supra-gingival microbiome for Oral microbiome transplantation

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ARTICLE INFO

Keywords:

Dental plaque
DNA sequencing
Oral microbiome transplantation
Flow cell
Microbiota
Oral bacteria
Species diversity

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 >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 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.

1. Introduction

Globally, 2.5 billion and 743 million people are estimated to suffer from untreated dental caries and severe periodontitis, respectively (Jepsen et al., 2018; Kassebaum et al., 2017). 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 (Bhayade et al., 2016), 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 (Bhayade et al., 2016). Elsewhere in the body, microbiota have been manipulated using transplantation (e.g. Fecal 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. *Clostridioides difficile* infection) (Lee et al., 2016), oral microbial transplants (OMT) do not exist for the mouth, despite the promise they hold for treating recalcitrant oral diseases (Nascimento, 2017). 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 (Belibasakis and Thurnheer, 2014; Dashper et al., 2016; Kistler et al., 2015) but they do not always reflect the dynamic nature of the oral environment (Salli and Ouwehand, 2015). 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 systems can address these issues, however, to our knowledge, these models have not been used to grow

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<https://doi.org/10.1016/j.jmicmeth.2024.106961>

Received 5 April 2024; Received in revised form 21 May 2024; Accepted 21 May 2024

Available online 22 May 2024

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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 Flow cell inoculation
2.	T14 biofilms	Biofilms formed on HA discs after 14 days
3.	Planktonic cells	Planktonic cells taken from the Flow cell after 14 days
4.	Spent medium	Media outflow (waste) from the Flow cell collected for 14 days
5.	Flow cell control	Media collected from Flow cell 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

complex polymicrobial oral biofilms required for OMT.

Flow cell systems have a several major components: A nutrient media source, peristaltic pump, an *in vitro* chamber with surfaces compatible for microbial growth, a heating device and a waste collection source (Dashper et al., 2016). Additionally, systems have an apparatus to prevent the introduction of air bubbles into the chamber (bubble trap) (Zhao et al., 2014) and a flow-break to prevent the back growth of bacteria from the growth chamber to the nutrient media source. Scanning electron (Junka et al., 2015) and confocal laser scanning microscopy (Kommerein et al., 2017) has enabled the qualitative and quantitative analysis of biofilms grown within flow cells. Determining viability of biofilms using confocal microscopy and fluorescent staining, allows validating the number of viable bacterial cells during the preparation of an OMT inoculum. Furthermore, microscopy coupled with the advancement of next generation DNA sequencing technologies to analyse biofilm composition will play a vital role in the development and safety of OMT.

Here, we developed a 3D printed, flow cell inoculated with human supragingival dental plaque grown in artificial salivary media, (ASM) (Dashper et al., 2016) 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.

2. Materials and methods

2.1. 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 200 µL sterile PBS (pH 7.4) and stored at –80 °C to monitor contaminant DNA (Eisenhofer et al., 2019). 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) suspended in 200 µL sterile PBS.

2.2. Flow cell and inoculation

Flow cells were 3D printed in polypropylene (Appendix Fig. 1) to enable sterilisation. The flow rate of medium was adjusted to 2/5 the total volume of the flow cell (Dashper et al., 2016). Each flow cell contained five hydroxyapatite (HA) discs (D = 5 mm x H = 2 mm; Clarkson Chromatography Products, PA, USA). Following sterilisation,

flow cells were filled with 25% artificial saliva medium (ASM) devoid of sucrose 24 h 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 CaCl₂, 0.088 g/L NaCl, and 1 mg/L haemin (Sigma-Aldrich) (Dashper et al., 2016). Dental plaque was vortexed in PBS for 30s, and 100 µL was added to 1 mL of ASM and used to inoculate the flow cell. Media flow began after 24 h, and the biofilm was grown at 36 °C for 14 days. 100 µL of the planktonic culture was then removed for sequencing, and each HA disc was removed and placed into a separate sterile 1.5 mL 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).

2.3. Biofilm imaging

2.3.1. SEM

Each disc was prepared for SEM (Yap et al., 2014), and coated with 2 nm 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 1000×, 5000× and 20,000× magnification.

2.3.2. CLSM

Each disc was washed in sterile PBS three times for 10 min to remove unbound cells. Biofilms were stained using a LIVE/DEAD™ BacLight™ kit (Hayles et al., 2023) (Invitrogen, Vic, Australia), following the manufacturer's instructions. The disc was figuratively divided into 3 equal quadrants and a random spot was selected from each quadrant by the residential microscopist at Adelaide Microscopy who was blinded as to which disc corresponded to which donor. Selected spots were imaged using a confocal laser scanning microscope, (Olympus FV3000), at 60× magnification using the dimensions of 1024 pixels for both axes. Each stack was acquired using a z-step size of 0.5 µm. Images were processed using IMARIS software (Bitplane, Zurich, Switzerland, version 7.6) for viability, average biofilm volume (µm³), and thickness (µm). Collated cells were excluded from the analysis.

2.4. 16S rRNA amplicon sequencing

DNA was extracted from 100 µL 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 (Weyrich et al., 2019).

DNA sequencing libraries were generated by amplifying the V4 hypervariable region of 16S rRNA gene, as previously described (Caporaso et al., 2012), 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 (Adler et al., 2013); one no-template control (NTC) was added per amplification batch to detect downstream contamination or non-specific amplification (Weyrich et al., 2019). Samples were quantified using the Invitrogen Qubit dsDNA BR assay (Life Technologies), purified using 1.1× Axygen AxyPrep Mag™ PCR Clean-up beads, quantified using the D1000 reagents on the TapeStation (Agilent, Santa Clara, CA, USA), and equimolar pooled at 5 nM for sequencing at the South Australia Health and Medical Research Institute (SAHMRI, Adelaide, Australia) on an Illumina MiSeq using 150 bp paired-end sequencing. The demultiplexed sequencing data was imported into the Quantitative Insights Into Microbial Ecology (QIIME2–2021.4) (Bolyen et al., 2019). Deblurring was performed on merged, demultiplexed, paired-end sequence reads that

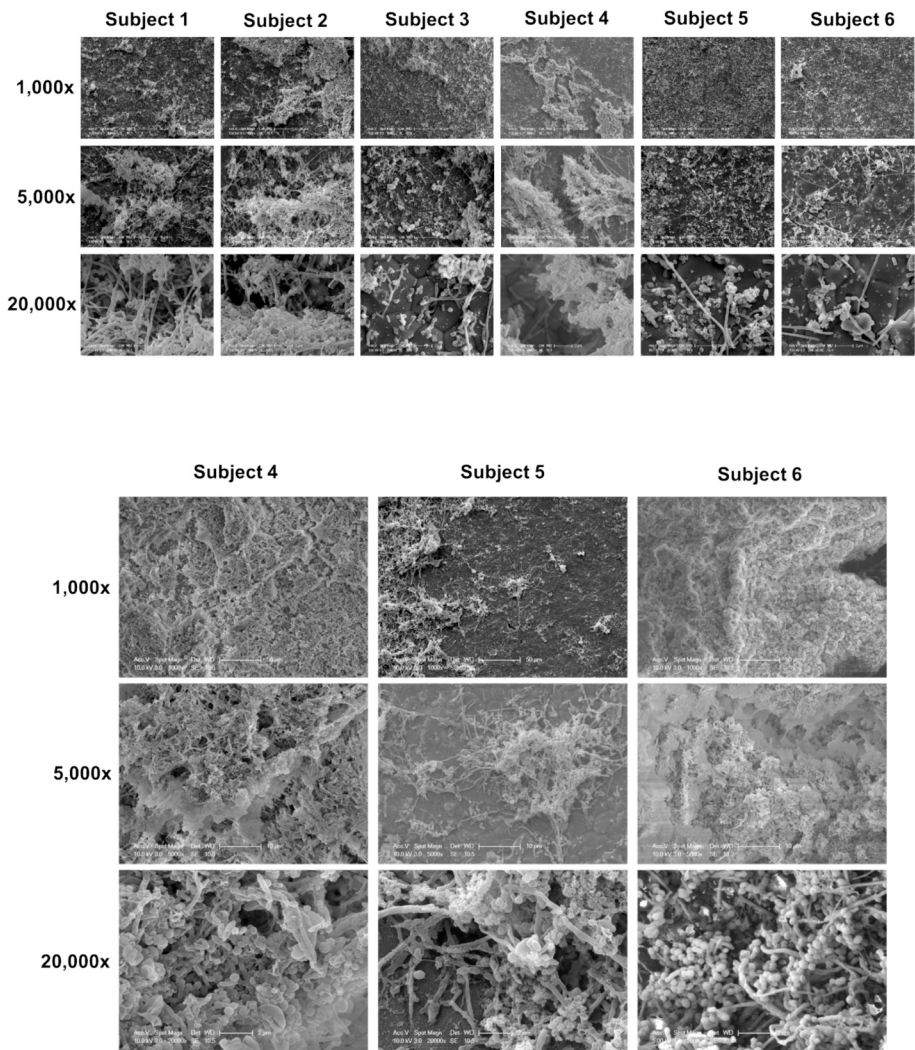


Fig. 1. Supragingival dental plaque biofilm formation on HA discs following 14 days growth using ASM A) SEM images of HA discs grown in ASM from all 6 donors, 1–6. Images were taken from three random regions of each HA disc using the magnifications: 1000 \times , 5000 \times and 20,000 \times . Scale bars = 2 μ m, 10 μ m and 50 μ m, respectively.

were quality filtered, and DNA sequences were denoised in amplicon sequence variants (ASVs) (Amir et al., 2017) and trimmed to 120 bp.

Contaminants from laboratory sources (EBCs and NTCs – referred to as laboratory controls in subsequent paragraphs) and flow cell controls were removed using the R package decontam (Davis et al., 2018) implemented in phyloseq R (McMurdie and Holmes, 2013) using the prevalence based method (Davis et al., 2018). Decontam score

thresholds were chosen based on a histogram of decontam scores for each filtration step (Appendix Table 1).

2.5. Visualization and statistical analysis

Diversity and compositional analyses were performed using QIIME2. Sequences were rarefied to 26,608 from 132,108 sequences. α -diversity was calculated using the Faith's phylogenetic diversity index 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 (Vázquez-Baeza et al., 2013). A Kruskal-Wallis test was used to assess significant differences in α -diversity between sample types. Significant differences in β -diversity were assessed using PERMANOVA. A corrected p -value of <0.05 was considered significant. Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) was used to detect significantly abundant bacterial species by performing a non-parametric factorial Kruskal-Wallis sum-rank test between biologically relevant groups (day 0 and day 14); 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 Fig. 4).

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 (μ m)	Biovolume (μ m ³)
1	$1.21 \times 10^4 \pm 4.61 \times 10^3$	$3.76 \times 10^3 \pm 2.1 \times 10^3$	30.8 ± 11.0	$4.34 \times 10^5 \pm 1.52 \times 10^5$
2	$1.78 \times 10^4 \pm 4.52 \times 10^3$	$7.08 \times 10^3 \pm 1.04 \times 10^3$	40.0 ± 3.0	$5.63 \times 10^5 \pm 4.15 \times 10^4$
3	$5.73 \times 10^3 \pm 1.75 \times 10^3$	$1.97 \times 10^3 \pm 1.52 \times 10^3$	22.2 ± 5.1	$3.15 \times 10^5 \pm 7.09 \times 10^4$
4	$1.20 \times 10^4 \pm 8.18 \times 10^3$	$2.26 \times 10^3 \pm 9.75 \times 10^2$	35.0 ± 16.5	$4.93 \times 10^5 \pm 2.30 \times 10^5$
5	$1.16 \times 10^4 \pm 2.70 \times 10^3$	$6.70 \times 10^3 \pm 1.10 \times 10^3$	29.2 ± 4.4	$4.12 \times 10^5 \pm 6.06 \times 10^4$
6	$9.76 \times 10^3 \pm 3.65 \times 10^3$	$3.93 \times 10^3 \pm 1.33 \times 10^3$	28.2 ± 1.0	$3.98 \times 10^5 \pm 1.46 \times 10^4$

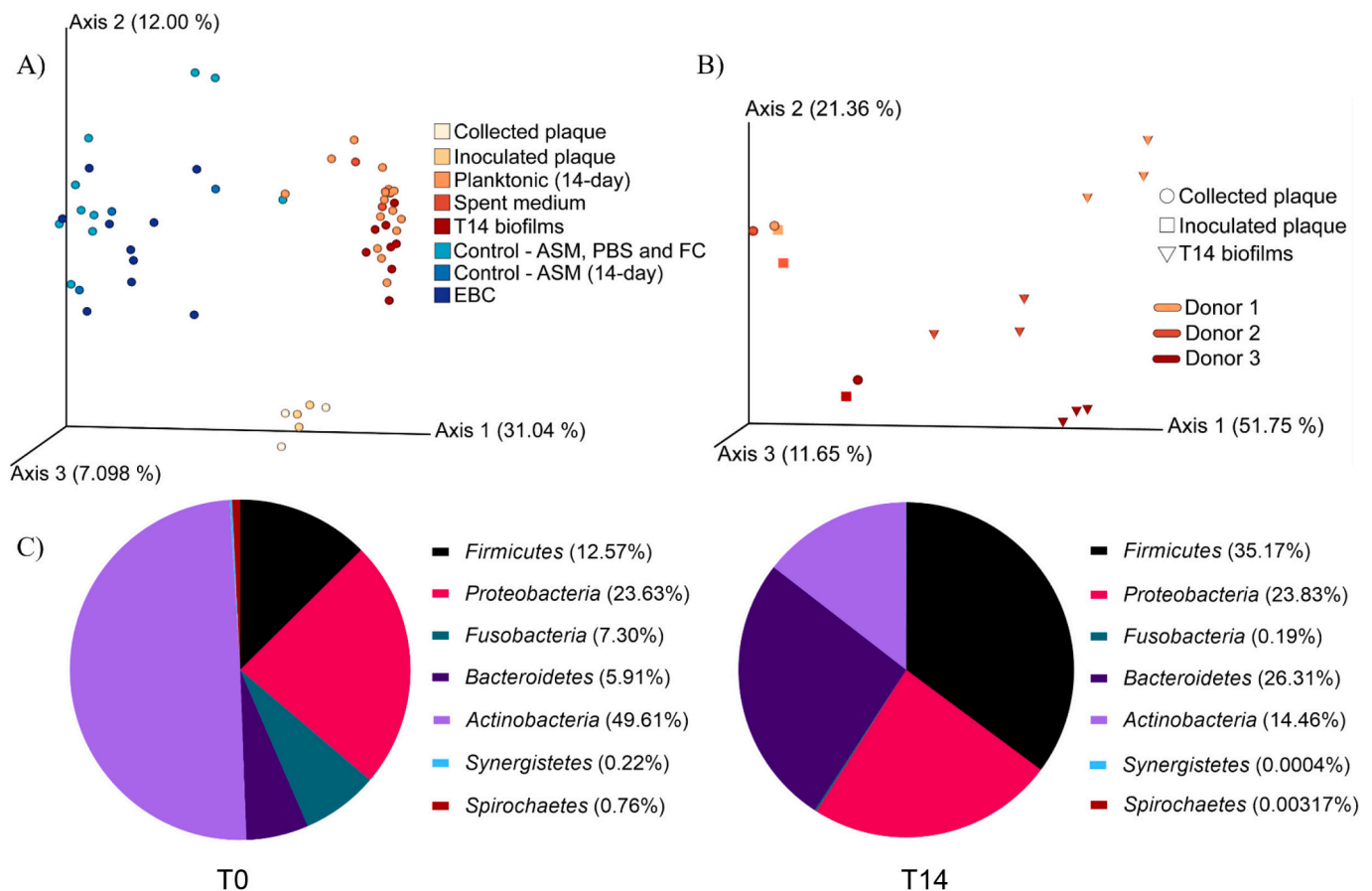


Fig. 2. Cell viability and 16S rRNA sequencing analysis on microbiota grown in ASM for 14-days. A) Comparison of β -diversity between of the biological (shades of red) and control (shades of blue) samples using an unweighted UniFrac algorithm. The bacterial samples including inoculated and collected 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. B) Comparison of β -diversity of the collected, inoculated and T14 biofilms grown in ASM. C) Change in abundance of dominant phyla from inoculation (T0) and 14 days after inoculation (T14). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. 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 (Fig. 1A, B). Confocal microscopy analysis revealed an average 1.57×10^4 (Lee et al., 2016) $\pm 6.08 \times 10^4$ (Bhayade et al., 2016) and 4.28×10^4 (Bhayade et al., 2016) $\pm 2.17 \times 10^4$ (Bhayade et al., 2016) live and dead cells from plaque grown in ASM (Table 2), and importantly, >70% of cells were viable. Lastly, the average thickness and biovolume of ASM-grown biofilms were 3.09×10^4 (Kassebaum et al., 2017) $\pm 0.06 \times 10^4$ (Kassebaum et al., 2017) μm and $4.36 \times 10^5 \pm 8.48 \times 10^4 \mu\text{m}^3$, respectively.

3.2. 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 flow cell system. We examined the composition of the controls alongside the biological samples by conducting PCoA of unweighted UniFrac values (Fig. 2A). The microbial composition of laboratory controls 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 laboratory controls accounted for 30.55% (42 species) of the total species and were removed from the data using the decontam package. The composition of flow cell 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 flow cell controls using decontam, which accounted for 17.63% (35 species) of the remaining sequences in all biological samples (Appendix Table 2) post filtering with laboratory controls.

3.3. 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 flow cell, as seen with other systems (Kistler et al., 2015). We compared the α -diversity between inoculated plaque (T0) and T14 biofilms. 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 (calculated using the Faith's phylogenetic diversity index) compared to the original inoculum (Kruskal-Wallis Pairwise; H test: 6.23, $p < 0.05$), but we retained donor specific α -diversity signatures in all sample groups (Kruskal-Wallis; All Groups; H test: 10.13, $p > 0.05$) (Appendix Fig. 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, $p > 0.05$) but there was a reduction in some anaerobic species in the inoculated plaque.

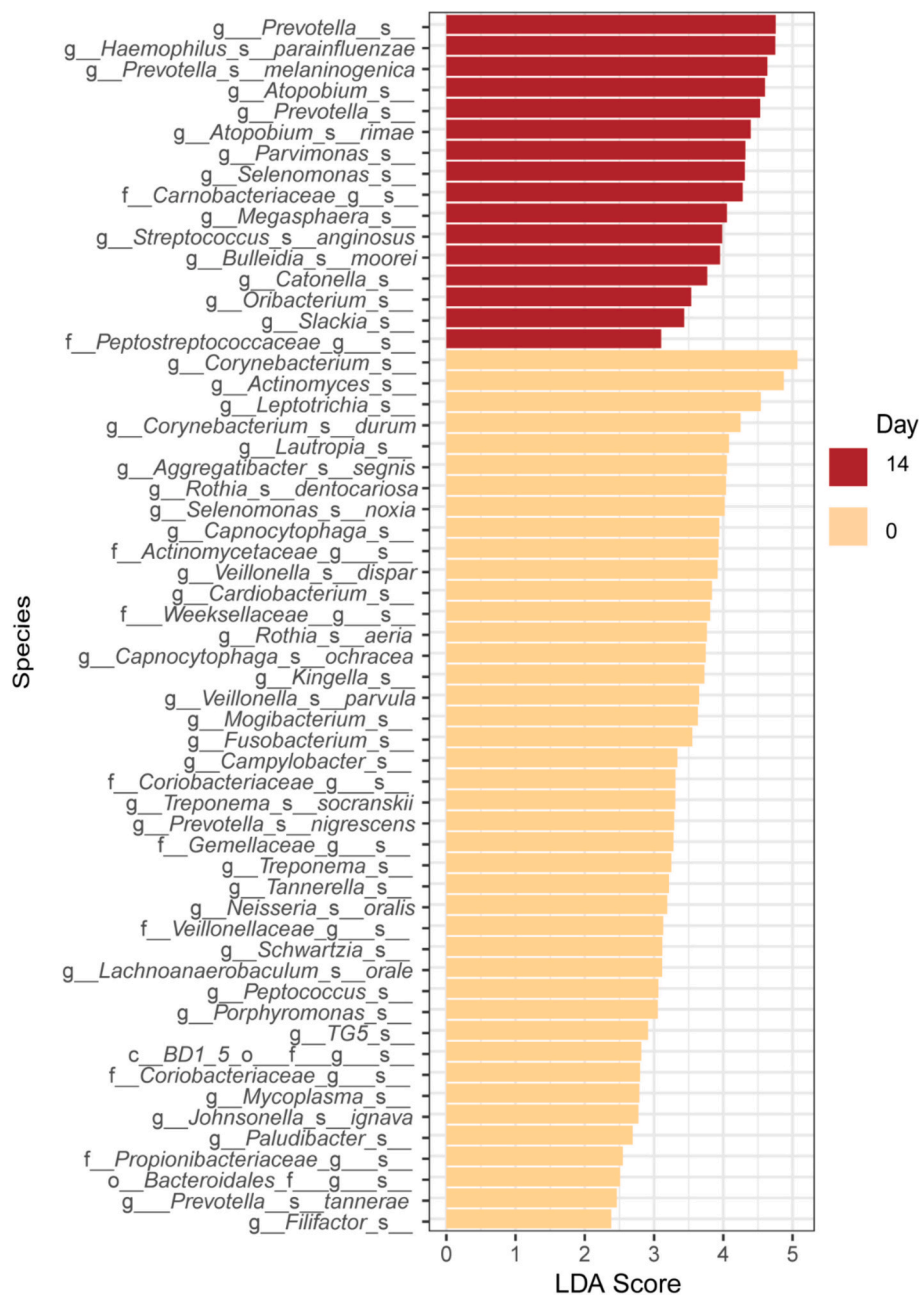


Fig. 3. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) (identifies significantly abundant taxa that are consistent with biologically relevant subclasses) analysis at species level to compare the oral microbiome profiles of the donors between inoculated plaque (T0) and T14 biofilms grown in ASM.

3.4. β -diversity significantly differed between inoculated plaque and T14 biofilms with a reduction in significantly abundant species at T14 biofilms compared to inoculated plaque

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; $p \leq 0.001$) (Fig. 2B). However, the microbial composition of biofilms grown on three discs from the same flow cell clustered closely according to each donor. When inoculated plaque was compared to T14 biofilms, the relative abundance of *Firmicutes* and *Bacteroidetes* increased while *Actinobacteria* and *Fusobacteria* decreased (Fig. 2C). 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* ($n = 2$), *Bacteroidetes* ($n = 1$), *Tenericutes* ($n = 1$), *GN02* ($n = 1$), *Spirochaetes* ($n = 1$) and *Synergistetes* ($n = 1$) phyla (Fig. 3). In contrast, 16 differentially abundant genera were more prevalent in T14 biofilms, *Firmicutes* ($n = 9$), *Actinobacteria* ($n = 3$), *Bacteroidetes* ($n = 3$) and *Proteobacteria* ($n = 1$).

4. 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 (Bik et al., 2010), 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 (Dashper et al., 2016; Nyvad et al., 2013).

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. The significant difference in β - diversity between inoculated plaque and T14 biofilms of the same donor is highlighted by the 51.76% variation along axis 1. This was a similar shift in bacterial composition at the phylum level to the study by Du et al. 2017 (Du et al., 2017) in which an increase in *Bacteroidetes* and *Firmicutes* and a reduction in *Actinobacteria* and *Fusobacteria* was observed in mothers and their children with primary dentition across different age groups. 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.

Significant shifts in β -diversity are not uncommon in *in vitro* biofilms models. Edlund, et al. (2013) reported that there was a 20%–40% reduction of operational taxonomic units of biofilms grown for 48 h in a 24-well plate (static model) in comparison to inoculated saliva from humans (Edlund et al., 2013). A Calgary Biofilm Device (Ceri et al., 1999), a 96-well microtiter plate with a modified lid, was used by Kistler, et al. (2015) to investigate long-term biofilm growth (14 days). It was found that the β -diversity between inoculated saliva and biofilms grown for 14 days was significantly different.

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 *Bacteroidetes* and *Firmicutes*. The majority of *Actinobacteria* species that reduced in abundance after 14 days were oral commensals (Schoilew et al., 2019). 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* (Li and Ma, 2020). 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 (Harrandah et al., 2021; McCracken and Nathalia Garcia, 2021; Visser and Ellen, 2011). However, it is unclear when this transition in abundance occurred.

Kistler, et al. (2015) also noted the taxonomic composition of biofilms grown *in vitro* were similar to dental plaque, with the most abundant genera being *Prevotella*, *Streptococcus*, and *Veillonella* (Kistler et al., 2015). The shift in bacterial composition at T14 maybe due to the absence of the host-immune interaction (Kistler et al., 2015) 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* (Arzmi et al., 2016). Previously published research included sucrose in ASM, however sucrose was excluded in this study because it favours the growth of cariogenic bacteria (Dashper et al., 2016; Rolla et al., 1985).

It is important to note that although flow cell models seem highly beneficial to study oral microbial communities as these systems closely mimic *in vivo* oral conditions, studies utilising advanced next generation sequencing technologies coupled with microscopy are absent in the literature. Most of the research pertaining to these systems involve either a predefined single (Lim et al., 2016; Sliepen et al., 2010; Rath et al., 2017) or multispecies (Foster and Kolenbrander, 2004; Blanc et al., 2014) inoculum which is not representative of the diversity reported *in vivo* (Xiao et al., 2016).

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

(Nath et al., 2021).

Funding

The authors gratefully acknowledge funding by the Australian Dental Research Foundation (Grant 515-2019) and the National Health & Medical Research Council Ideas Grant (2019/GNT1187737).

CRediT authorship contribution statement

Don H.K. Ketagoda: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Peter Varga:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Tracy R. Fitzsimmons:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Nicole E. Moore:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation. **Laura S. Weyrich:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Peter S. Zilm:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

All QIIME2, code, analysis files, and R code used to plot figures can be found here: https://github.com/DonKevin1994/In_Vitro_Biofilm_Model. The sequences were deposited in the NCBI SRA under accession number XXXXXXXXXXXX.

Acknowledgements

The authors would also like to acknowledge the instruments and scientific and technical assistance of Adelaide Microscopy at the University of Adelaide, North Terrace Campus, a facility that is funded by the University and State and Federal Governments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mimet.2024.106961>.

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