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Influence of Substrate Material on the Composition and Diversity of Bacterial Cultures Grown from Human Dental Biofilm Specimens Ex Vivo

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Abstract

Background: Titanium (TI) and yttria stabilized zirconia oxide (YSZ) are materials commonly found at the soft-tissue interface surrounding dental implants. The influence of these surfaces on the processes of bacterial adhesion and biofilm development may affect clinical performance and patient susceptibility to inflammatory peri-implant disease. The purpose of this study was to evaluate the influence of the substrate material on biofilm composition and complexity.

Methods: We cultured biofilms on hydroxyapatite (HA), TI, and YSZ surfaces using dental plaque specimens obtained from three human donors. Average roughness (R_a) values of all surfaces approximated 0.2 μm. Duplicate cultures grew for one, two, three, six or nine days. We then analyzed biofilm composition and diversity using 16S rRNA sequencing. The Shannon Diversity Index (SDI) was calculated for each experimental group, using a linear mixed-model analysis to test for statistically significant differences. Microbial profiles were inter-compared in a pairwise fashion to establish dissimilarity scores, which were recorded in a distance dissimilarity matrix.

Results: Biofilms grown on HA surfaces exhibited significantly higher alpha diversity compared with those formed on TI or YSZ ($p<0.0001$). However, relative abundances of the predominant phyla and genera did not appear to be different across experimental groups.

Conclusions: Under the conditions described, biofilms grown on TI or YSZ appeared significantly less complex than those formed on HA. Limited evidence suggests that high biofilm diversity may be associated with oral health. Thus, compared with periodontal health,

peri-implant health may be more difficult to maintain, due in part to characteristics of the implant/abutment material.

Key Words: Dental implants, biofilms, dental plaque, peri-implantitis, mucositis, titanium

Introduction

The presence of intact skin and mucosal barriers to protect against invading microbial pathogens ranks among the most fundamental requirements for human health. The term "mucosa" refers to an epithelium together with the immediately subjacent lamina propria.[1] Such tissues line the respiratory, urogenital, and alimentary tracts. A compromise of the body's protective epithelial barrier, as occurs in a burn episode for example, renders the host exquisitely susceptible to infection. In perspective, the predominant cause of death in burn patients in the present era remains infection and sepsis, underscoring the consequences often accompanying epithelial damage.[2]

The oral cavity presents a unique challenge to the maintenance of an effective mucosal defense. Only in this region of the body is a mucosal barrier interrupted by the discrete, mineralized ectodermal structures known as teeth. In fact, clinicians have considered plaque infection at the dentogingival interface a *locus minoris resistentiae* (body region more vulnerable than others), highlighting increased potential for microbial ingress and disease progression at this soft-hard tissue boundary.[3] Fortunately, specialized supracrestal attached tissues, comprised of the junctional epithelium (JE) and the connective tissue (CT) attachment, form an effective biologic seal around teeth, supporting health despite discontinuities in the oral mucosa.[1,4,5] During development, the primary epithelial attachment derives from the reduced enamel epithelium, and over time, the JE gradually adopts a stratified squamous appearance.[1,5] Analogous to an epithelium-CT interface, the JE attaches to enamel, cementum, or dentin at the dentogingival junction via hemidesmosomes and a basal lamina.[1,4-6] Deep to the JE, dentogingival and dentoperiosteal fibers insert into the root surface as Sharpey's fibers,

forming the CT attachment.[1,4] The terminal ends of these densely packed extrinsic fibers are invested in cementum and mineralized, firmly anchoring the tooth to the CT.[5]

Dental implants and associated components, which functionally replace missing teeth, also extend through the oral mucosa and thus carry the same requirement for a biological seal. In some respects, the soft tissue interfaces at implant sites and teeth are similar. In both circumstances, hemidesmosomes and basal laminae mediate the epithelial attachment.[5] However, implants are biocompatible inorganic devices installed in host bone, whereas natural teeth are products of a complex developmental process that proceeds in concert with establishment of periodontal tissues. Thus, an implant/abutment lacks a true connective tissue attachment analogous to collagen fibers inserting into the root cementum of natural teeth. Instead, an implant/abutment exhibits a connective tissue adhesion characterized by intimate approximation of collagen fibers predominantly oriented parallel to the surface of the device.[5]

Teeth and implants function within the contaminated oral environment, and the soft tissue interfaces at natural teeth and dental implant sites are under constant microbial challenge. Gingivitis and periodontitis together rank among the most prevalent bacterial diseases affecting humans.[7-9] Peri-implant diseases appear comparably common, meta-analyses having estimated the patient-level prevalences of peri-implant mucositis and peri-implantitis at 43% and 22%, respectively.[10] Periodontal and peri-implant diseases share a common etiology—bacterial species found within oral biofilms—and the pathogenesis of both diseases involves inflammatory destruction of host tissue.[11] In treated periodontitis patients, ample evidence suggests that mechanical disruption of biofilms through sound oral hygiene practices and a monitored professional maintenance program effectively produces periodontal

health and stability for most individuals.[12-17] Likewise, dental plaque removal will reverse or prevent gingivitis in non-periodontitis patients.[18] Effective oral hygiene is also critical for the maintenance of peri-implant health.[19,20] Factors that impede effective plaque removal have been associated with peri-implant mucositis and peri-implantitis.[20-23]

One determinant of oral hygiene effectiveness is the material used in the manufacturing of a dental implant/abutment. The substrate represents a major factor influencing adhesion of both bacterial and host cells. The process of bacterial adhesion involves physicochemical and molecular interactions.[24] Adhesion to abiotic substances typically involves nonspecific interactions, whereas specific ligand-receptor interactions predominate in bacterial adhesion to biological surfaces.[25-27] Bacterial adhesion is an early step in biofilm development, and decreasing bacterial adhesion may promote health and stability of peri-implant tissues. The purpose of this in vitro study was to compare the complete microbiomes cultured on three substrates— HA, TI, and YSZ—subjected to dental biofilms ex vivo.

Methods and Materials

This protocol utilized de-identified dental biofilm and saliva specimens and did not involve contact with patients or patient records. Thus, the Dwight David Eisenhower Army Medical Center Human Research Protections Office determined this research to be exempt from Institutional Review Board review requirements (protocol #20-11855/934337). For this research, we requested use of three de-identified subgingival dental plaque specimens and three matched saliva specimens from the Fort Gordon Saliva and Dental Plaque Repository (protocol #RHC-A-20-059). Specimen donors were periodontally healthy nonsmokers who had

reported no use of any antibiotic or probiotic agent within 90 days of specimen collection. In each experiment, matched plaque and saliva specimens derived from a single donor.

Substrates

The materials evaluated in this investigation were 9.5-mm diameter disks (2mm thickness) composed of HA (Clarkson Chromatography Products, South Williamsport, PA, USA), 99% pure TI (American Elements, Los Angeles, CA, USA, #TI-M-O2-D), and YSZ (American Elements, #ZRO-YSZ-O2R-D). We recorded average surface roughness (R_a) values for all disks using the Perthometer M2 with PFM Drive Unit (Mahr, Providence, RI, USA). Then, we modified R_a values using an aluminum oxide sandblasting unit to an approximate value of 0.2 µm in order to minimize roughness-related adhesion variability across the experimental groups.

Bacterial growth

We gently sonicated plaque specimens in a water bath for 30 seconds to disrupt the biofilm. HA, TI, and YSZ disks were separated and placed into sterile Petri dishes, then coated for 2 hours at room temperature with 1:10 saliva to allow pellicle formation. Next, we removed the saliva and gently added 2 mL sterile tryptic soy broth supplemented with 5 ug/mL hemin and 1 ug/mL menadione (TSB-hk) to the Petri dishes. Plaque specimens were resuspended by briefly vortexing, and we inoculated each Petri dish with 50 uL of the plaque suspension. The dishes then remained in an anaerobic incubator at 37º C (75%N_2/10%CO_2/10%H_2) for static growth. Biofilms from each of the three donors grew for one, two, three, six or nine days before dispersal. We used 23 disks per plaque specimen to ensure adequate bacterial growth for detection and analysis. Ten disks were collected at day one, six at day two, four at day three,

two at day six, and one at day nine. Every 48 hours, growth media was gently removed from wells and replaced by slowly adding a fresh 2 mL of reduced TSB-hk. We duplicated every donor/substrate/growth-period combination.

DNA extraction and 16S rRNA gene sequencing

At the conclusion of the growth period (one, two, three, six or nine days), each disk was removed from the dish and placed in a tube with 1 mL reduced Ringer's solution. The tubes were sonicated in a water bath for 30 seconds and vortexed briefly. The remaining biofilm suspension from each experimental condition was spun at 10,000 rpm for 2 minutes to pellet the cells. The supernatant was removed and the pellet stored at -20º C for DNA extraction. DNA was extracted from the pellet of each biofilm source for all material/growth-period combinations and sent to the Walter Reed Army Institute of Research (WRAIR) for 16S rRNA gene sequencing. MiSeq Reagent Kits (Illumina, San Diego, CA) were used for gene sequencing.

Statistical analyses

Microbiome Insights (Vancouver, British Columbia) completed the statistical analyses. Sequencing quality was first assessed using FastQC 0.11.5. MiSeq-generated Fastq files were quality-filtered, and bacteria were clustered into 97% similarity operational taxonomic units (OTUs) using the mothur software package.[28] The SDI was calculated for each experimental group, and a linear mixed-model tested for statistically significant differences. To evaluate beta diversity, all microbial profiles were inter-compared in a pairwise fashion to establish dissimilarity scores, which were recorded in a distance dissimilarity matrix. Low dissimilarity scores identified similar samples. Abundance-weighted sample pairwise differences were

calculated using Bray-Curtis dissimilarity (the ratio of the summed absolute differences in counts to the sum of abundances in the two samples).[29]

Results

Sequence Quality Determination

The dataset consisted of 56,680 OTUs, not including those exhibiting less than three counts. The samples (donor/substrate/growth-period combinations) generated an average of 265,002 quality-filtered reads. One sample was eliminated from the analysis due to low read counts. Overall sample quality was favorable for comparison of the experimental groups (Figure 1).

Sequence quality was determined by evaluating the number of quality reads per sample, the quality score across all bases, and the distribution of average quality score across all bases. Quality scores took into account the base composition and nucleotide distribution, the guanine-cytosine (GC) content distribution, and the duplication rate. An evaluation of the resulting sequence quality data revealed that there was a near-linear correlation between the number of quality reads and the number of days of biofilm growth for the titanium- and zirconia-based samples with the most pronounced reduction in quality reads occurring between days six and nine. However, this correlation was not found when biofilms were grown on hydroxyapatite (Figure 1). A quality score of 30 indicates that there is an error rate of 1 in 1000 in the reported sequence. A metric known as “Q30” represents the percentage of bases with a quality score of 30 or higher. The Q30 score is often reported in a “traffic light” format, green indicating good quality, orange indicating low quality, and red indicating poor quality. When we plotted Q30

scores against read position, sequence quality appeared high at the beginning of the sequences and lower toward the end (Figure 1). This is a predictable and expected result. However, there were also regions at the beginning of the sequences with poor sequence quality.

Genome Diversity Determination

A total of 16 taxa were identified. A qualitative review of the data indicated that the predominant taxa were members of the genera *Streptococcus* and *Veillonella*. In terms of abundance these were followed by members of the genera *Fusobacterium* and *Lactobacillus*. The least abundant genera included *Stomatobaculum* and *Prevotella*. Relative abundances of the predominant phyla and genera were not significantly different across experimental groups, indicating minimal influence of substrate on taxonomic composition. When we plotted the SDI against days of growth, we noted that diversity increased over time (Figures 2 and 3). However, biofilms grown on HA surfaces demonstrated significantly higher alpha diversity compared with those formed on TI or YSZ ($p<0.0001$). At the genera level we found that, although biofilm complexity tended to increase over time, cultures on HA surfaces displayed the greatest diversity at day one, with diversity of biofilms cultured on the three substrates converging at later time points (Figure 2). Notably, members of the genus *Streptococcus* were predominant at day two on all three materials tested, while members of the genus *Fusobacterium* were predominant on the TI and YSZ based surfaces at day one. Furthermore, there the microbiome profile appeared unique for each individual at each time point. Each donor displayed a distinct taxonomic profile dominated by the *Streptococcus*, *Fusobacterium* and *Veillonella* genera (Figure 4). Similarity between the different groups of samples (HA, TI and YSZ) was estimated by calculating the beta diversity which is an indicator of dissimilarity between groups. When

the diversity of all of the samples in this study were plotted using principle component analysis, the TI and YSZ samples clustered together with HA samples forming a distinct cluster (Figure 5). Permutational analysis of variance (*adonis* R function, or Permanova) determined significant differences in beta diversity associated with substrate (p=0.012) and growth period (p=0.001). In principle component analysis, differences in biofilm diversity by substrate tended to vanish with longer growth periods.

Discussion

Our purpose was to assess the influence of the substrate (HA, TI, or YSZ) on the composition and complexity of bacterial cultures grown from human dental biofilm specimens. We evaluated both alpha and beta diversity values in this study. Methods for measuring and comparing biodiversity were established by Whittaker in 1972.[30,31] Alpha diversity refers to the species diversity or species richness within a particular location or on a particular surface. The SDI, which takes into account the number of species in a sample and the abundance of each species, provides an estimate of the alpha diversity. SDI can range in values from unity (indicating one dominant species) to a value equal to the total number of species in the sample. Biodiversity between groups is known as beta diversity. Theoretically, identical groups would exhibit no dissimilarity, producing a beta diversity value of zero. Completely dissimilar groups would register a maximal beta diversity value of one.

In a previous study, investigators evaluated bacterial cultures—comparable to those used in our research—utilizing confocal laser scanning microscopy with vital fluorescence, scanning electron microscopy, and quantitative polymerase chain reaction.[32] Six species were

selected for analysis, representing initial, early, intermediate, and late colonizers.[32] The authors found that bacterial adherence and biofilm maturation exhibited similar dynamics, irrespective of the surface material.[32] However, the substrate did influence various recorded outcomes—biofilm thickness, deposition of extracellular polysaccharide matrix, and bacterial cell spatial organization.[32] Thus, we hypothesized that 16S rRNA sequencing would identify substrate-related differences in culture composition and complexity. Contrary to this assumption, we observed minimal influence of substrate material on taxonomic composition. We did, however, note statistically significant differences in SDI values (alpha diversity) among biofilms cultured on the three surfaces evaluated, HA disks producing the most diverse microbiota.

In the gut, some evidence suggests that increased diversity may be associated with health.[33,34] Likewise, some conditions which manifest in severe periodontal destruction have been associated with less diverse microbiomes. For example, Moutsopoulos and colleagues compared the composition of subgingival plaque specimens from patients diagnosed with Leucocyte Adhesion Deficiency I (LAD-I), Localized Aggressive Periodontitis (LAP), and periodontal health using comprehensive 16S rRNA gene-based microarrays.[35] The authors found that health-associated microbial communities exhibited increased diversity, with more numerous, less unique, and less invasive species.[35] Periodontitis appears to result from an exaggerated/unbalanced inflammatory response to bacteria commonly found in the oral cavity, with considerable redundancy in disease-associated species and specific microbes associated with severe periodontal disease.[36,37] Thus, focusing on functional rather than phylogenetic diversity may enhance our understanding of metagenomic interactions and lead to new strategies for arresting periodontitis and maintaining periodontal health.[37]

We recognize limitations associated with our investigation. Lack of continuity in workflow was one potential source of bias in our study. Dental biofilm collection occurred in the Periodontics Department, Army Postgraduate Dental School, Fort Gordon, GA. Specimen processing, bacterial growth, and DNA extraction occurred at the Department of Clinical Investigation, Dwight David Eisenhower Army Medical Center, Fort Gordon, GA. 16S rRNA gene sequencing occurred at WRAIR, Silver Spring, MD, and data analysis occurred at Microbiome Insights, Vancouver, British Columbia, Canada. Researchers at Microbiome Insights noticed that the numbers of individual Amplicon Sequence Variants (ASVs) identified in our samples far exceeded counts typically found in fecal samples. This unexpected observation suggested possible errors in the DNA extraction step.

In an ASV-level analysis, investigators sort unique sequences into individual species. Because an ASV analysis is very precise, small variances in sequence identify separate species. In contrast, an analysis completed at the OTU level decreases phylogenetic precision but also reduces "noise" in the data. An error occurring during DNA extraction/purification may give the perception that a separate ASV is present, introducing a misclassification error. However, the same error in the DNA sequence may not manifest as a misclassification in an OTU-level analysis. The OTU approach is permissive of a predetermined variance level in the DNA sequence, classifying closely related species within the same taxonomic unit. However, two individual species that have similar 16S rRNA DNA gene sequences may be grouped together because of the allowed variance. Researchers may apply varying tolerance levels to a given dataset. The selected tolerance level influences the number of OTUs identified and also the likelihood of inappropriately including a species or genus in a particular OTU. For this project,

the sequenced data were quality-filtered and clustered into 97% similarity OTUs, identified to the genus level. While we did not identify specific species, our approach allowed for general insight into various complexes of bacteria present and compare biofilm complexity across experimental groups.

Conclusion

Under the conditions described, biofilms grown on TI or YSZ appeared significantly less complex than those formed on HA. Limited evidence suggests that high biofilm diversity may be associated with oral/periodontal health. Thus, compared with periodontal health, peri-implant health may be more difficult to maintain, due in part to characteristics of the implant/abutment material.

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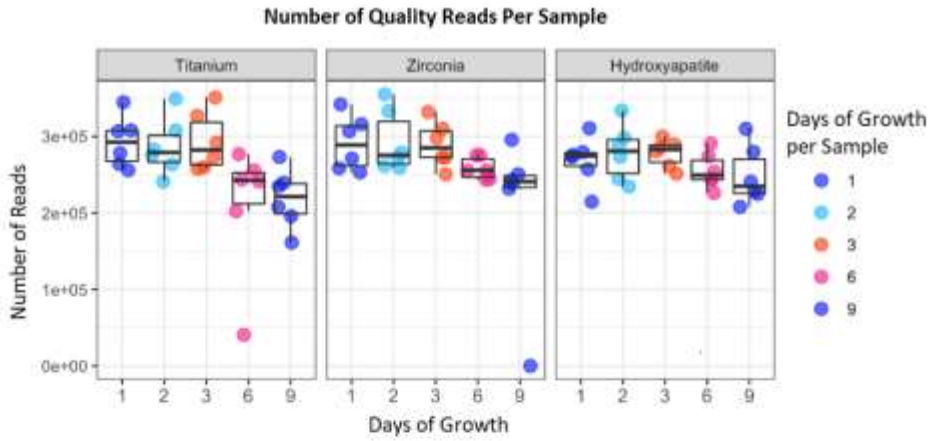
Figures

A.


Number of Quality Reads Per Sample
Titanium
Zirconia
Hydroxyapatite
Number of Reads
3e+05
2e+05
1e+05
0e+00
1 2 3 6 9
Days of Growth
Days of Growth per Sample
1
2
3
6
9

B.


Quality Scores Across All Bases
Forward Read
Reverse Read
Q30 Score (Phred33)
Position in Read
Position in Read

C.

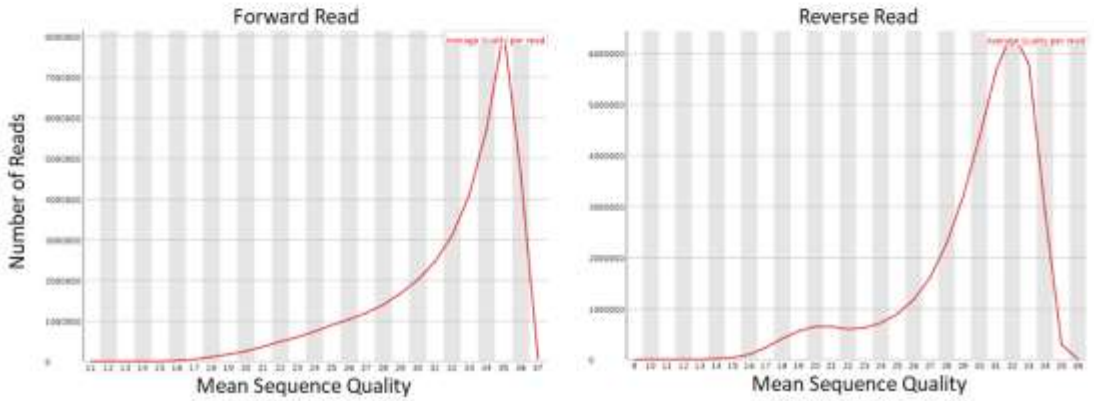

Distribution of Average Quality Scores Across All Bases
Forward Read
Reverse Read
Number of Reads
Mean Sequence Quality
Mean Sequence Quality

Figure 1. Sequencing quality assessment. 1A. Number of quality reads of the 16Sv3v4 region in each sample after discarding poor quality reads. This evaluation confirms adequate sequencing quality to permit further analysis. The number of quality reads per sample was similar among the materials evaluated with a downward trend over nine days of growth. One sample was discarded due to insufficient read data (fewer than 1000 quality reads). 1B. Quality scores across all base pairs. Taken together, the graphs (forward and reverse reads) indicate that an incorrect base pair reading was more likely at the beginning and the end of each DNA strand. 1C. Quality score distribution over all sequences in the forward (R1) and the reverse (R2) directions.

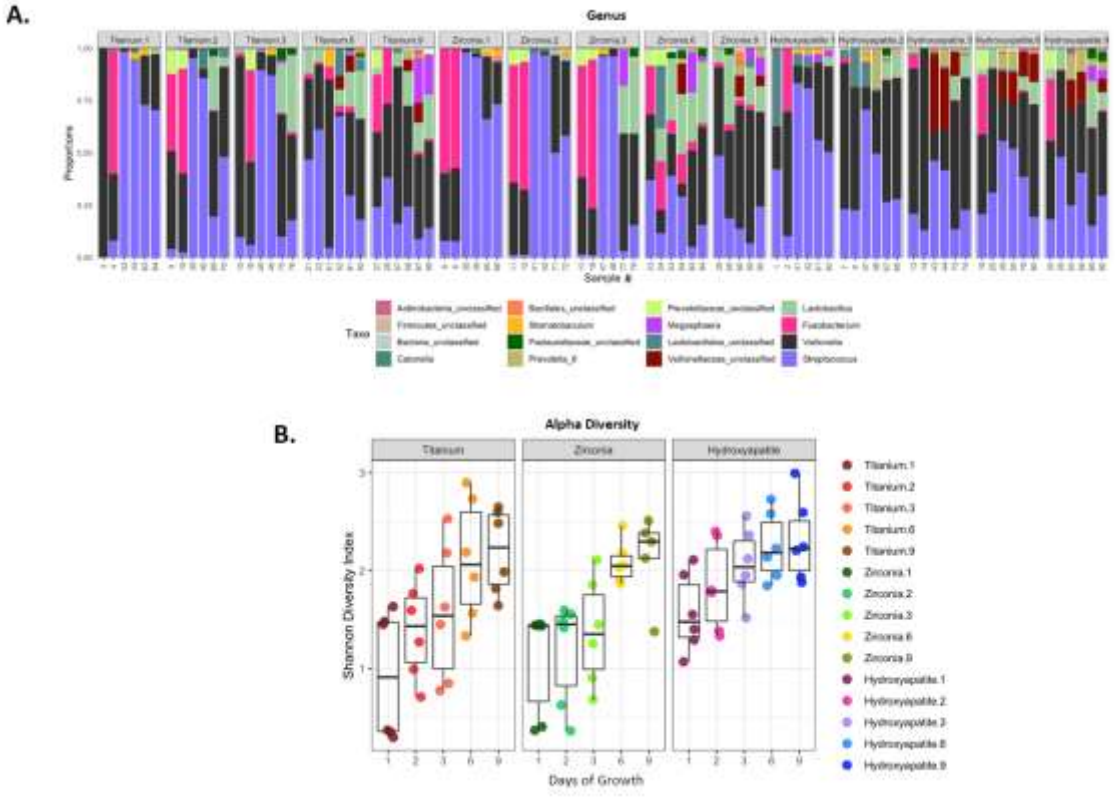


Figure 2. Alpha diversity analysis. 2A. Cumulative bar graph representations of the genera-level microbiome composition. High quality reads were classified using the reference database. OTUs were aggregated into taxonomic ranks and relative abundance values were plotted. Relative abundances of the predominant phyla and genera did not exhibit statistically significant differences across groups, indicating minimal influence of the substrate material on taxonomic composition. 2B. Box and whisker plots of the Shannon Diversity Index (SDI) values (alpha diversity). The linear mixed-model analysis identified significant differences in SDI. Cultures grown on hydroxyapatite exhibited significantly greater alpha diversity ($p<0.0001$) than those formed on zirconia or titanium.

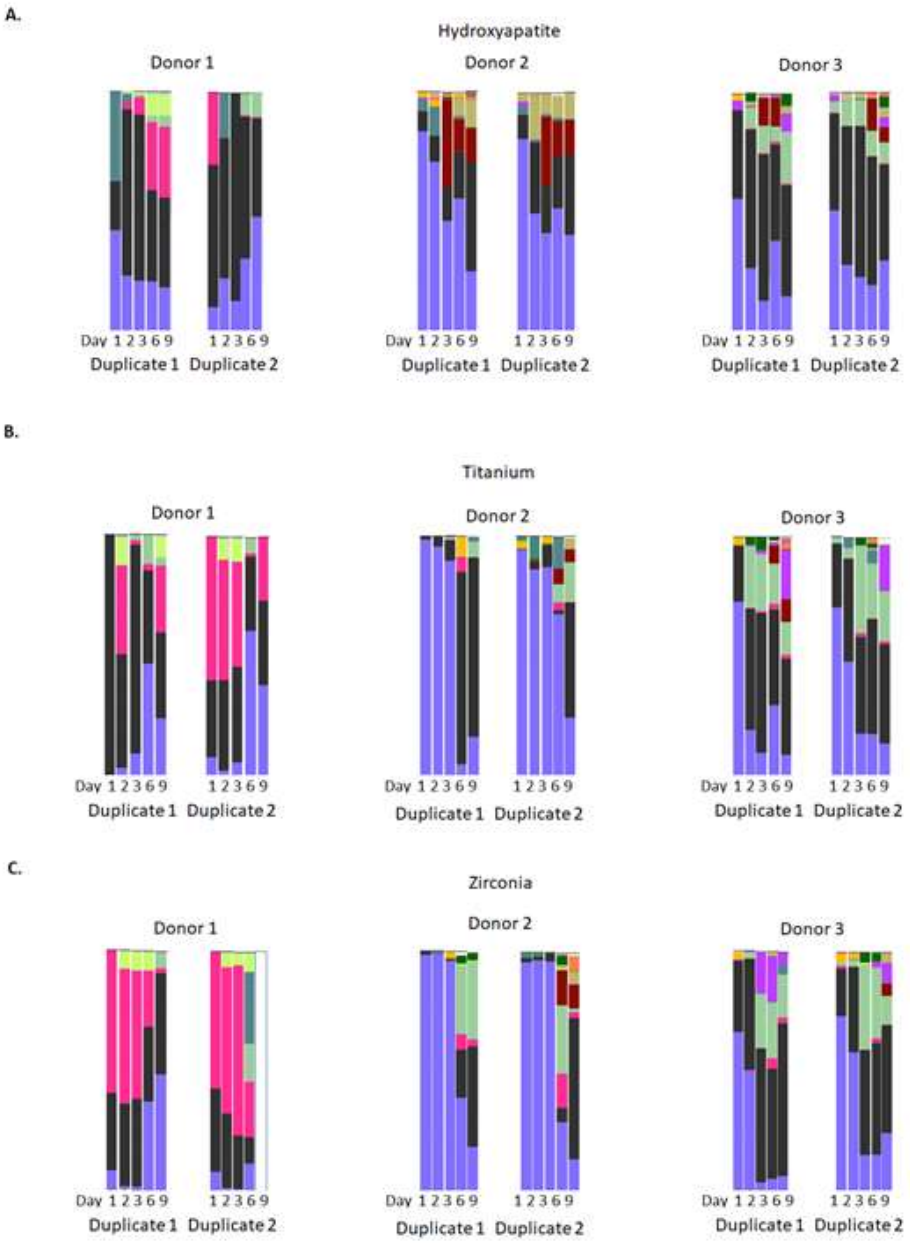


Figure 3. Genera-level microbiome composition by substrate material. Biofilm complexity tended to increase over time, although cultures on hydroxyapatite surfaces were most diverse at day one, with diminished diversity over time. 3A. Hydroxyapatite. 3B. Titanium. 3C. Zirconia.

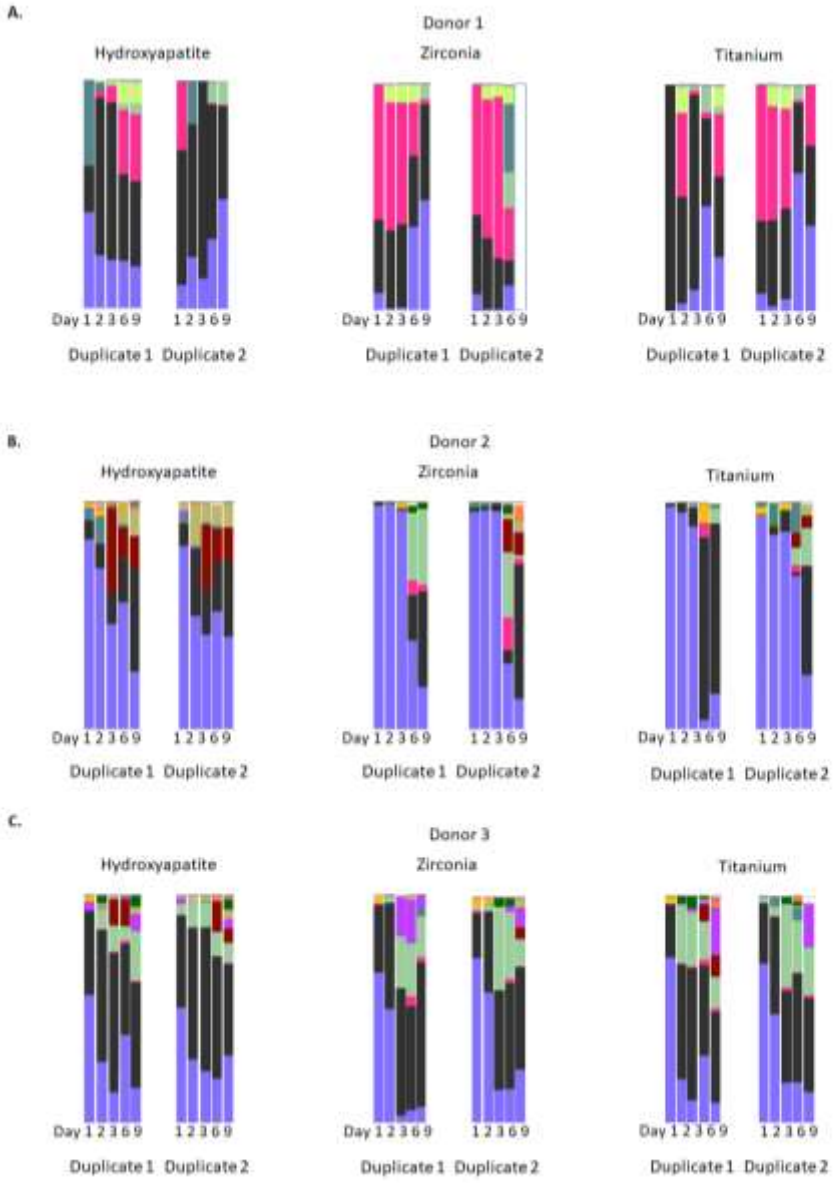


Figure 4. Genera-level microbiome composition by biofilm donor. Subjectively, each specimen exhibited a unique microbial profile. For example, donor 1 exhibited a larger proportion of the genus *fusobacterium* compared with donors 2 and 3. Biofilm complexity tended to increase over time. 4A. Donor 1. 4B. Donor 2. 4C. Donor 3.

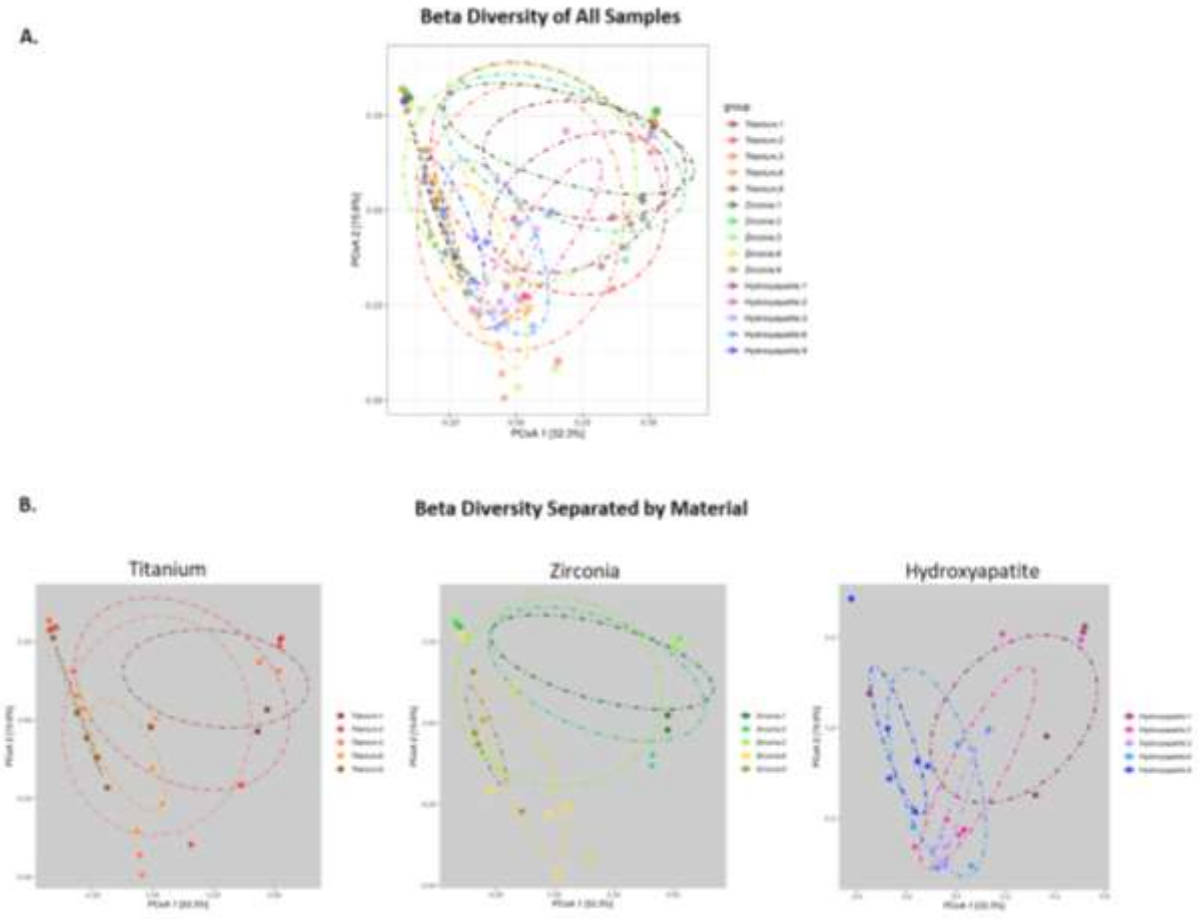


Figure 5. Beta diversity analysis. 5A. Beta diversity calculated for all material/growth-period combinations. We compared all profiles in a pairwise fashion to determine dissimilarity scores, then plotted scores in a distance dissimilarity matrix. Distance functions produce low dissimilarity scores when comparing similar samples. Abundance-weighted sample pairwise differences were calculated using Bray-Curtis dissimilarities (ratios of the summed absolute differences in counts to the sum of abundances in paired samples). This graphic is a visual representation depicting the diversity between all experimental groups. 5B. Beta diversity scores by substrate. Permutational analysis of variance (*adonis* R function, or Permanova)

determined significant differences in beta diversity associated with substrate ($p=0.012$) and growth period ($p=0.001$).