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Shotgun Sequencing Reveals an Altered Microbiome in Failed Dental Implants – A Pilot Study

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Abstract

As implants have become increasingly more common, so have biological complications involving implants, namely peri-implant mucositis and peri-implantitis. Peri-implantitis is a plaque-associated pathological condition occurring in tissues around dental implants, characterized by inflammation in the peri-implant mucosa and subsequent progressive loss of supporting bone. The purpose of our study was to identify the microbiota and the microbiome diversity around healthy and failing implants using deep whole metagenome shotgun sequencing and analysis. Previous studies on the microbiota of peri-implantitis lesions have focused on Gram-negative anaerobes and have identified similar pathogens as those found in periodontitis. Our data shows that the microbiota in failed implants display less community diversity and richness compared to healthy implants. Interestingly, most traditional periodontal pathogens were not found in high quantities around the failed implants. Further studies are needed to clarify the dysbiosis and changes in the microbial composition as the peri-implant environment shifts from one of health to disease.

Keywords: Metagenomics, Microbiota, Peri-Implantitis, Whole genome sequencing, Biofilms, Dysbiosis

Introduction

Interactions between natural resident microbes and the body contribute to health and disease [1,2]. Oral microbiota (e.g., bacteria, fungi and viruses) colonize the oral mucosa as polymicrobial biofilms. Oral mucosal diseases, such as periodontal disease and peri-implantitis, arise from the resident microbiota as a result of a shift in the microbial community that allows pathogenic bacteria to proliferate [3-5]. This causes a disruption in the microbiota-host homeostasis. Tissue inflammation develops with the host's immune response to dysbiosis [6,7]. As a result of the dysbiosis and subsequent host response, destruction of supporting alveolar bone occurs in order to induce tooth loss [8].

Dental implants have become a popular and predictable treatment modality for replacing missing teeth, with well documented long-term success [9]. However, as implants have become increasingly more common, so have biological complications involving implants, namely peri-implant mucositis and peri-implantitis. Peri-implantitis is a plaque-associated pathological condition occurring in tissues around dental implants, characterized by inflammation in the peri-implant mucosa and subsequent progressive loss of supporting bone [10]. The prevalence of peri-implantitis varies greatly depending on the study [11]. but it has been reported to be in between 28% and 56% of subjects and 12% and 43% of implants [12].

Previous studies on the microbiota of peri-implantitis lesions have focused on Gram-negative anaerobes and have identified similar pathogens as those found in periodontitis, such as *Tannerella forsythia*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium* species, *Prevotella intermedia*, and *Prevotella nigrescens* [13-21]. However, the current model of periodontitis has evolved to one of a complex microbial synergy and dysbiosis, consistent with the current understanding of other complex diseases, in contrast to the traditional model in which disease is caused by a select few pathogens. Like teeth, the imbalance of the host-microbial equilibrium with implants manifests as an inflammatory lesion, leading to the rapid progressive destruction of peri-implant bone. Recent studies have suggested that the peri-implantitis lesion has a microbiologic profile that is distinct from periodontitis and it does not fully correspond with disease severity [22]. Species not typically seen in periodontitis, like *Staphylococcus* and *Streptococcus,* have been found in higher counts in diseased implants [16,23-25]. Over 700 bacterial species have been detected in the oral cavity using culture-independent techniques, of which only less than half have been cultivated [26]. With such a diversity in the microbiota around implants, it is important for more studies to focus on the perturbations of the host-microbial equilibrium as the peri-implant environment shifts from a healthy to diseased state. The purpose of this study was to determine the microbiota and its diversity around healthy and failing implants using whole-genome shotgun sequencing and analysis.

Materials and Methods

Samples

Microbial samples were obtained from the surfaces of two failing implants removed from two patients, and from the gingival crevicular fluid/saliva around three control healthy implants in three patients. Samples were collected and frozen at -80°C until further processing.

Failing implants

One failed implant was obtained from a 64-year old Asian Male who presented to the UCSF clinic with a history of multiple failing/mobile implants. A bone level implant (Nobel-Replace Tapered Groovy, Nobel Biocare, Göteborg, Sweden) had been previously placed by an outside referring dentist and restored after three months with a single screw retained restoration in the #14 position (#13 and #15 natural teeth present) before it became mobile less than a year after placement. The implant had fibrous encapsulation with no osseointegration and was removed with reverse torquing.

A second failing implant was obtained from a 51-year old female who had been referred to the UCSF periodontal clinic due to purulence and bone loss around implants #19 and #20. The two implants were transmucosal implants (Straumann® Standard Tissue Level implants, Straumann Group, Basel, Switzerland) that had been restored with individual cemented restorations. The implants were initially treated with non-surgical debridement with titanium curettes and irrigation with chlorhexidine gluconate (0.12%). At the three-month reevaluation, implant #19 was stable, but implant #20 was deemed hopeless with progressive radiographic bone loss and persistent inflammation with purulent exudate and removed with a trephine bur.

Healthy implants

Three healthy implants in three healthy patients, with no signs of inflammation or bleeding on probing, and no signs of attachment loss or radiographic bone loss were used as healthy controls. Saliva and gingival crevicular fluid were collected from the implants with sterile paper points placed into the pocket of the implants.

Preparation of bacterial DNA samples and sequencing

DNA was extracted from samples through a Qiagen DNA microbiome kit (QIAGEN, Inc) according to the manufacturer's protocol. The purity and quantification of DNA was determined by a Qubit dsDNA high sensitivity assay kit (Thermo Fisher Scientific). We then used 200 ng of high-quality DNA for metagenome shotgun sequencing library preparation through KAPA HyperPlus Kits (Roche). Approximately 350 bp-insert-size DNA libraries were prepared, barcoded and the quality of libraries was determined for expected insert size by a Bioanalyzer and High Sensitivity DNA kit (Agilent). Barcoded samples were pooled for sequencing. Illumina platform PE150 (HiSeq4000) was used for sequencing of libraries.

Microbiome data analysis

Metagenomic short reads were trimmed by a Knead Data tool (https://bitbucket.org/biobakery/biobakery/wiki/kneaddata) and were mapped to human microbiome reference and human microbiome project (HMP) data using Metaphlan2/HUMAnN2 (https://bitbucket.org/biobakery/biobakery/wiki/humann2) pipelines in order to identify microbial species. We generated read count tables for microbial species for all the samples for further analysis. Data were analyzed at both the genus and species level. The data for each sample was normalized to have one million reads per million (RPM). Taxa with a mean RPM < 1 were removed from the analysis. Alpha diversity (within sample) for each sample was measured using number of species, Shannon index, Simpson index, and community richness with the Chao1 index.

Beta diversity (between subjects) was computed using the Bray Curtis distance.

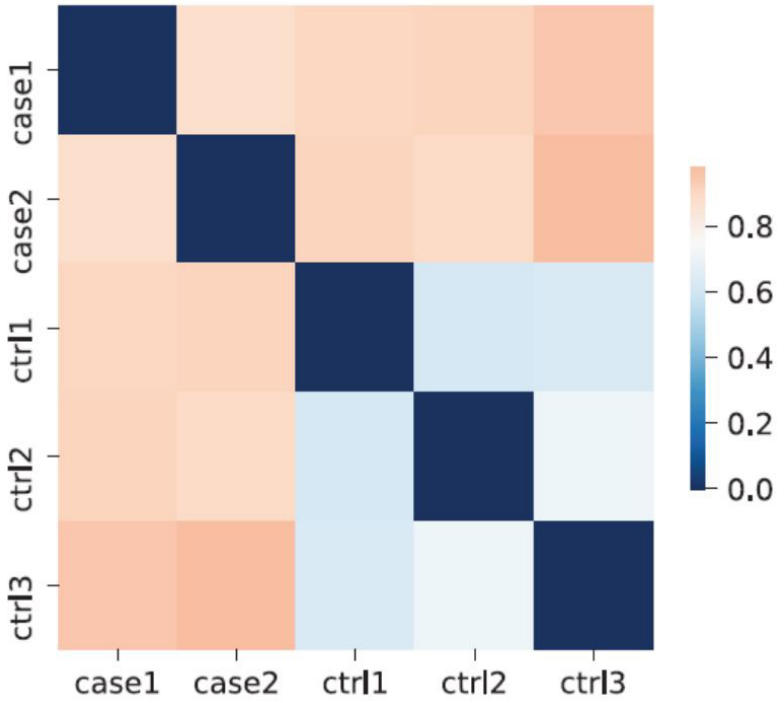


Figure 1: Diseased implants have a lower microbial diversity

The small sample size (2 cases and 3 controls) should not have a major impact on the validity of the results. First, the two-sample t test does not have a minimum sample size requirement (it only requires the assumption of normality). Second, to ensure the results were robust, we compared the results based on two-sample t tests (used in the main analysis) and those based on Wilcoxon rank-sum tests (Supp. Figure 1). We determined that the results were overall consistent. Third, we also visualized individual data points in Figures 3-4.

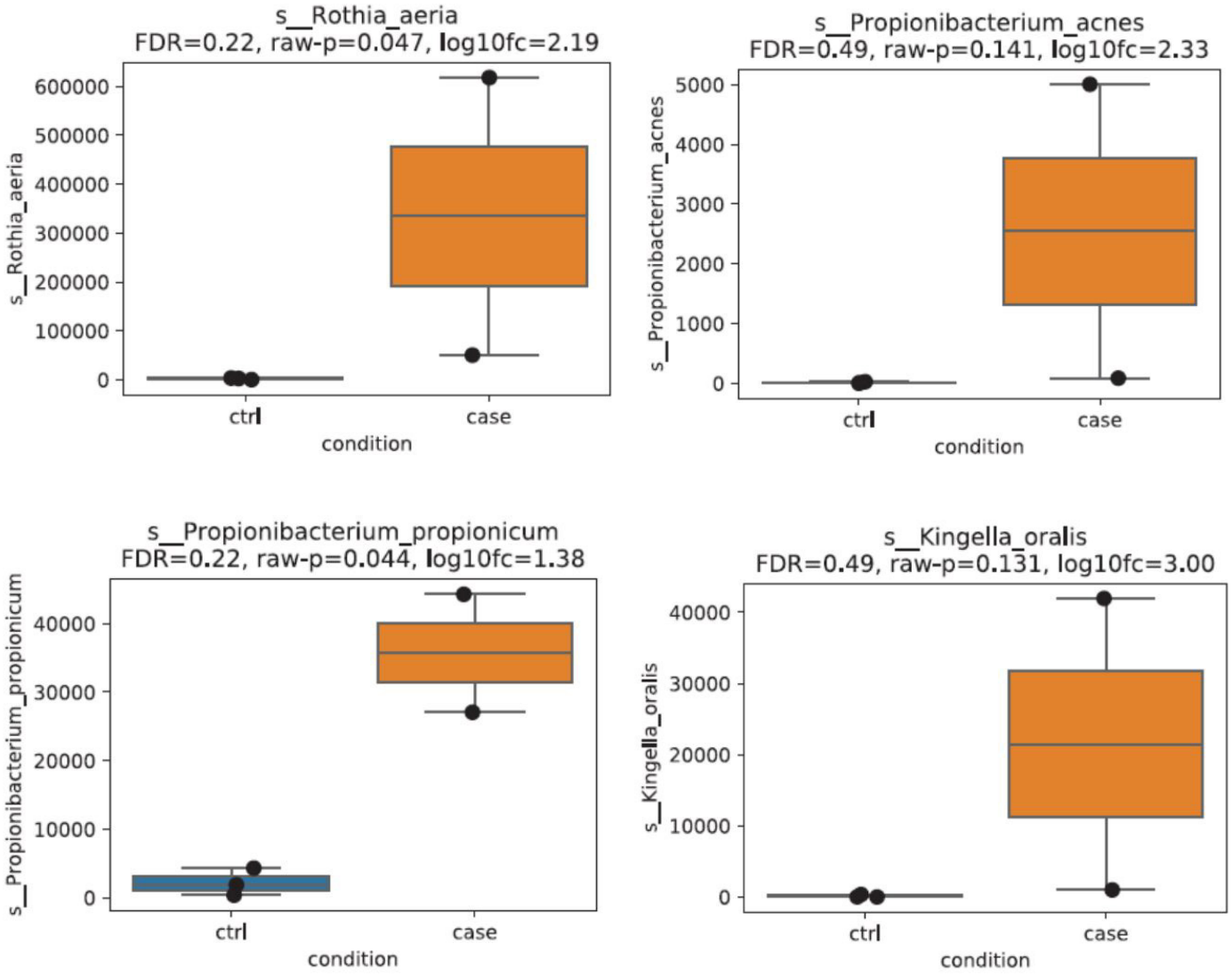
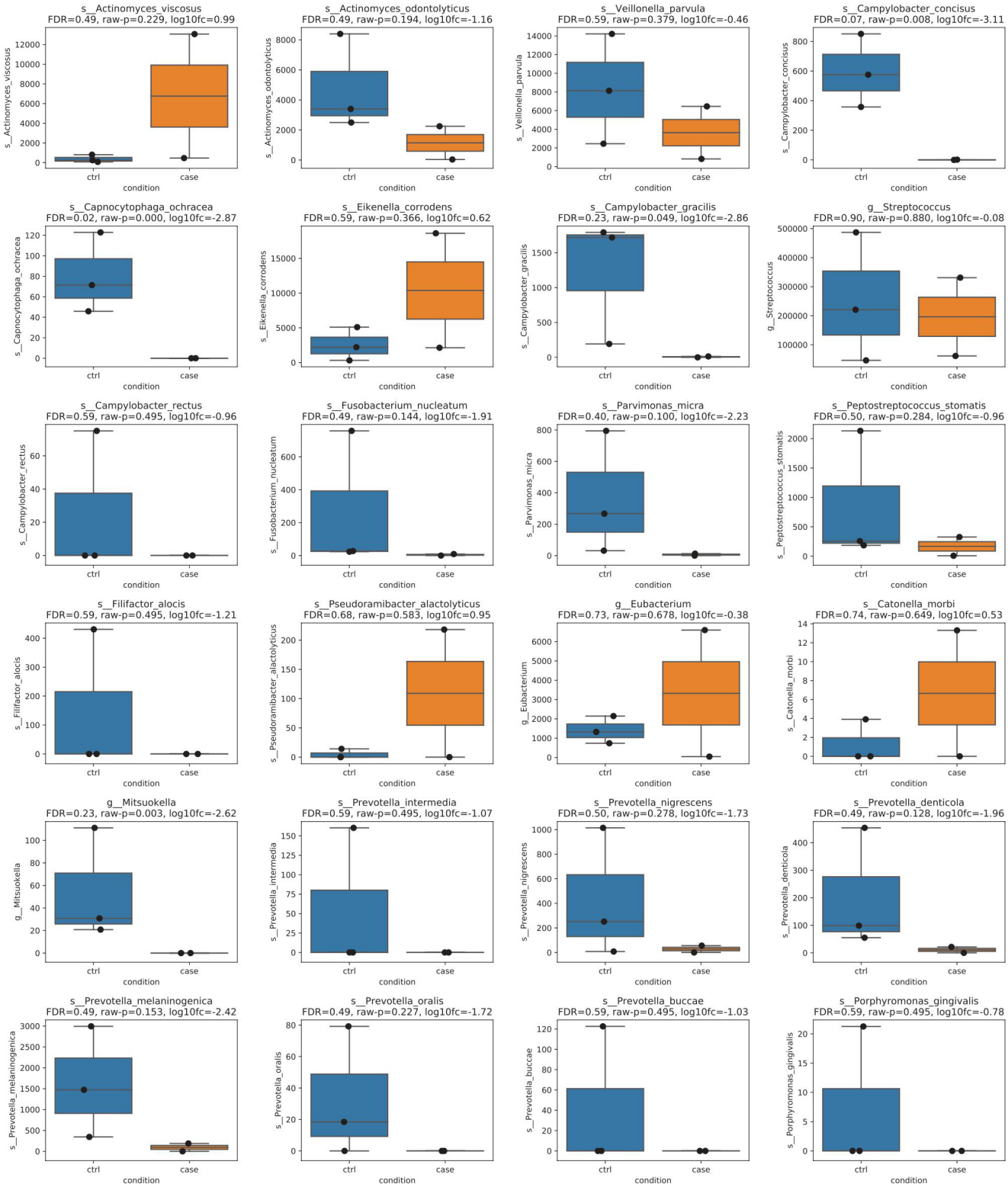


Figure 3: Microbial species unique to peri-implantitis cases

The bar graphs illustrate the microbial species unique to the peri-implantitis cases. The FDR and p values are given for each species


s__Actinomyces_viscosus
FDR=0.49, raw-p=0.229, log10fc=0.99
s__Actinomyces_odontolyticus
FDR=0.49, raw-p=0.194, log10fc=-1.16
s__Veillonella_parvula
FDR=0.59, raw-p=0.379, log10fc=-0.46
s__Campylobacter_concisus
FDR=0.07, raw-p=0.008, log10fc=-3.11
s__Capnocytophaga_ochracea
FDR=0.02, raw-p=0.000, log10fc=-2.87
s__Eikenella_corrodens
FDR=0.59, raw-p=0.366, log10fc=0.62
s__Campylobacter_gracilis
FDR=0.23, raw-p=0.049, log10fc=-2.86
g__Streptococcus
FDR=0.90, raw-p=0.880, log10fc=-0.08
s__Campylobacter_rectus
FDR=0.59, raw-p=0.495, log10fc=-0.96
s__Fusobacterium_nucleatum
FDR=0.49, raw-p=0.144, log10fc=-1.91
s__Parvimonas_micra
FDR=0.40, raw-p=0.100, log10fc=-2.23
s__Peptostreptococcus_stomatis
FDR=0.50, raw-p=0.284, log10fc=-0.96
s__Filifactor_alocis
FDR=0.59, raw-p=0.495, log10fc=-1.21
s__Pseudoramibacter_alactolyticus
FDR=0.68, raw-p=0.583, log10fc=0.95
g__Eubacterium
FDR=0.73, raw-p=0.678, log10fc=-0.38
s__Catonella_morbi
FDR=0.74, raw-p=0.649, log10fc=0.53
g__Mitsuokella
FDR=0.23, raw-p=0.003, log10fc=-2.62
s__Prevotella_intermedia
FDR=0.59, raw-p=0.495, log10fc=-1.07
s__Prevotella_nigrescens
FDR=0.50, raw-p=0.278, log10fc=-1.73
s__Prevotella_denticola
FDR=0.49, raw-p=0.128, log10fc=-1.96
s__Prevotella_melaninogenica
FDR=0.49, raw-p=0.153, log10fc=-2.42
s__Prevotella_oralis
FDR=0.49, raw-p=0.227, log10fc=-1.72
s__Prevotella_buccae
FDR=0.59, raw-p=0.495, log10fc=-1.03
s__Porphyromonas_gingivalis
FDR=0.59, raw-p=0.495, log10fc=-0.78
ctrl
case
condition

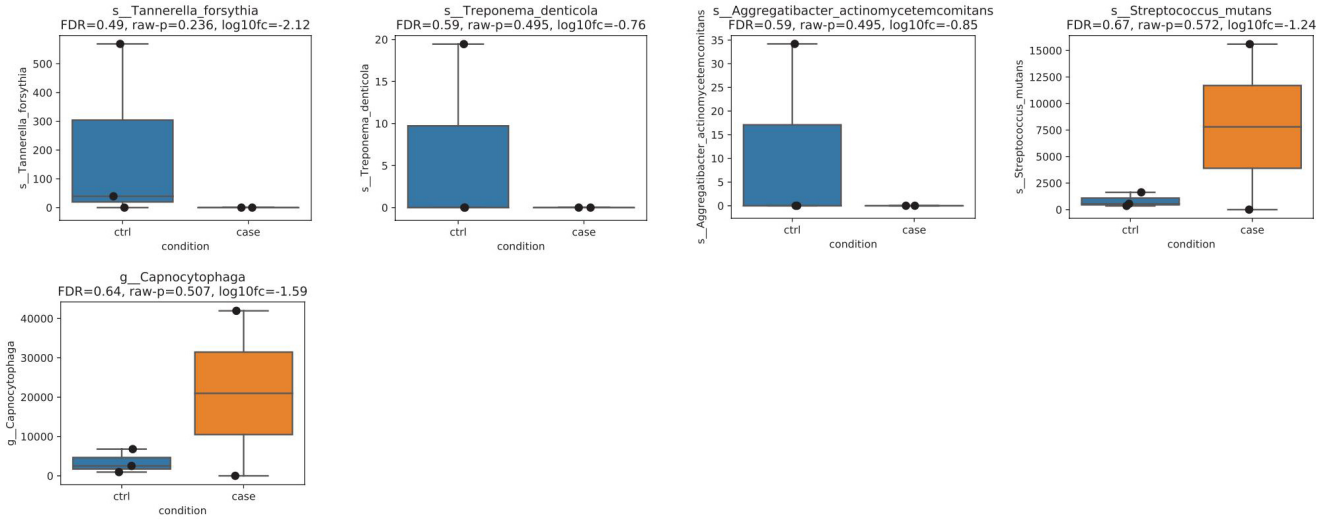


Figure 4: Microbial species related to periodontal disease, caries, and peri-implantitis
The bar graphs illustrate the microbial species related to periodontal disease, caries, and peri-implantitis. The FDR and p values are given for each species

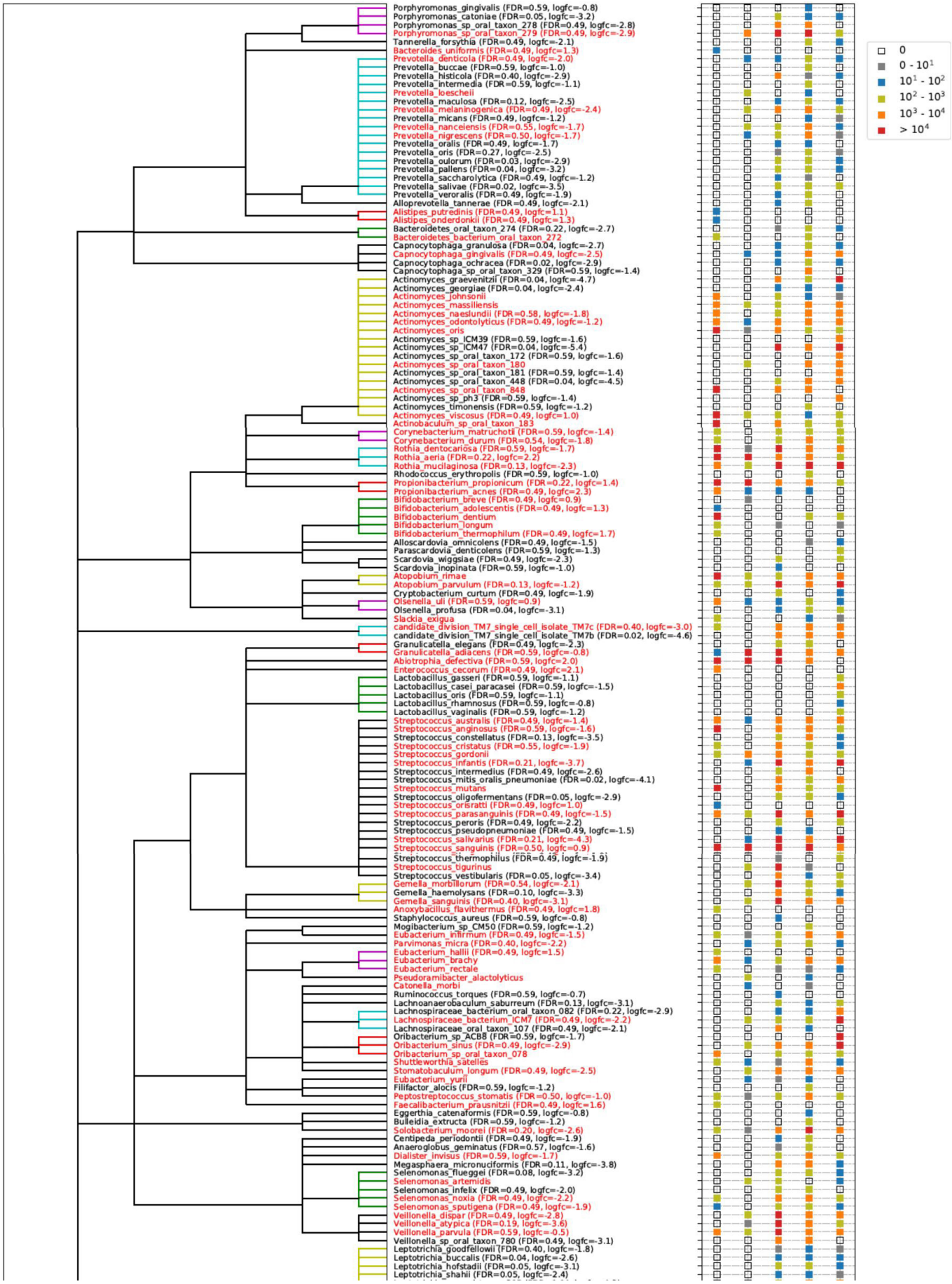
Results

Diseased implants have fewer species and a lower microbial diversity

When considering the number of species and alpha diversity for each sample, control samples had significantly more species and a higher level of alpha diversity (Table 1). The diseased implants had fewer species and a significantly lower level of alpha diversity compared to the healthy controls at both the genus and species level. When evaluating the beta diversity between samples, computed using the Bray Curtis distance, control samples were more similar to each other (Figure 1). Healthy implants shared similar microbiota with little variation between the healthy samples, whereas the diseased implants had a larger variation (Figure 2).

Bacterial species in healthy versus failed implants differ

There were several species significantly more abundant in diseased implant sites. Many species, which are considered part of the normal oral flora, were found significantly or highly elevated in the failed implants, such as *Rothia aeria, Propionibacterium acnes, Propionibacterium propionicum, Kingella oralis, Streptococcus mutans*, and *Actinomyces viscosus* (Figures 3, 4). Some Gram-negative facultative anaerobic bacteria often found in periodontitis, such as *Eikenella corrodens*, and *Capnocytophaga* species, were found at higher levels in the failed implants (Figure 4). However, red complex pathogens and many other periodontal pathogens such as *P. gingivalis, T. forsythia, T. denticola, A. actinomycetemcomitans, F. nucleatum, Campylobacter rectus*, were not found in abundance in any of the failed implant samples but were found in varying prevalence in the healthy controls (Figure 4). Several other genus and species were also related to implant failure.


Porphyromonas_gingivalis (FDR=0.59, logfc=-0.8)
Porphyromonas_catoniae (FDR=0.05, logfc=-3.2)
Porphyromonas_sp_oral_taxon_278 (FDR=0.49, logfc=-2.8)
Porphyromonas_sp_oral_taxon_279 (FDR=0.49, logfc=-2.9)
Tannerella_forsythia (FDR=0.49, logfc=-2.1)
Bacteroides_uniformis (FDR=0.49, logfc=1.3)
Prevotella_denticola (FDR=0.49, logfc=-2.0)
Prevotella_buccae (FDR=0.59, logfc=-1.0)
Prevotella_histicola (FDR=0.40, logfc=-2.9)
Prevotella_intermedia (FDR=0.59, logfc=-1.1)
Prevotella_loescheii
Prevotella_maculosa (FDR=0.12, logfc=-2.5)
Prevotella_melaninogenica (FDR=0.49, logfc=-2.4)
Prevotella_micans (FDR=0.49, logfc=-1.2)
Prevotella_nanceiensis (FDR=0.55, logfc=-1.7)
Prevotella_nigrescens (FDR=0.50, logfc=-1.7)
Prevotella_oralis (FDR=0.49, logfc=-1.7)
Prevotella_oris (FDR=0.27, logfc=-2.5)
Prevotella_oulorum (FDR=0.03, logfc=-2.9)
Prevotella_pallens (FDR=0.04, logfc=-3.2)
Prevotella_saccharolytica (FDR=0.49, logfc=-1.2)
Prevotella_salivae (FDR=0.02, logfc=-3.5)
Prevotella_veroralis (FDR=0.49, logfc=-1.9)
Alloprevotella_tannerae (FDR=0.49, logfc=-2.1)
Alistipes_putredinis (FDR=0.49, logfc=1.1)
Alistipes_onderdonkii (FDR=0.49, logfc=1.3)
Bacteroidetes_oral_taxon_274 (FDR=0.22, logfc=-2.7)
Bacteroidetes_bacterium_oral_taxon_272
Capnocytophaga_granulosa (FDR=0.04, logfc=-2.7)
Capnocytophaga_gingivalis (FDR=0.49, logfc=-2.5)
Capnocytophaga_ochracea (FDR=0.02, logfc=-2.9)
Capnocytophaga_sp_oral_taxon_329 (FDR=0.59, logfc=-1.4)
Actinomyces_graevenitzii (FDR=0.04, logfc=-4.7)
Actinomyces_georgiae (FDR=0.04, logfc=-2.4)
Actinomyces_johnsonii
Actinomyces_massiliensis
Actinomyces_naeslundii (FDR=0.58, logfc=-1.8)
Actinomyces_odontolyticus (FDR=0.49, logfc=-1.2)
Actinomyces_oris
Actinomyces_sp_ICM39 (FDR=0.59, logfc=-1.6)
Actinomyces_sp_ICM47 (FDR=0.04, logfc=-5.4)
Actinomyces_sp_oral_taxon_172 (FDR=0.59, logfc=-1.6)
Actinomyces_sp_oral_taxon_180
Actinomyces_sp_oral_taxon_181 (FDR=0.59, logfc=-1.4)
Actinomyces_sp_oral_taxon_448 (FDR=0.04, logfc=-4.5)
Actinomyces_sp_oral_taxon_848
Actinomyces_sp_ph3 (FDR=0.59, logfc=-1.4)
Actinomyces_timonensis (FDR=0.59, logfc=-1.2)
Actinomyces_viscosus (FDR=0.49, logfc=1.0)
Actinobaculum_sp_oral_taxon_183
Corynebacterium_matruchotii (FDR=0.59, logfc=-1.4)
Corynebacterium_durum (FDR=0.54, logfc=-1.8)
Rothia_dentocariosa (FDR=0.59, logfc=-1.7)
Rothia_aeria (FDR=0.22, logfc=2.2)
Rothia_mucilaginosa (FDR=0.13, logfc=-2.3)
Rhodococcus_erythropolis (FDR=0.59, logfc=-1.0)
Propionibacterium_propionicum (FDR=0.22, logfc=1.4)
Propionibacterium_acnes (FDR=0.49, logfc=2.3)
Bifidobacterium_breve (FDR=0.49, logfc=0.9)
Bifidobacterium_adolescentis (FDR=0.49, logfc=1.3)
Bifidobacterium_dentium
Bifidobacterium_longum
Bifidobacterium_thermophilum (FDR=0.49, logfc=1.7)
Alloscardovia_omnicolens (FDR=0.49, logfc=-1.5)
Parascardovia_denticolens (FDR=0.59, logfc=-1.3)
Scardovia_wiggsiae (FDR=0.49, logfc=-2.3)
Scardovia_inopinata (FDR=0.59, logfc=-1.0)
Atopobium_rimae
Atopobium_parvulum (FDR=0.13, logfc=-1.2)
Cryptobacterium_curtum (FDR=0.49, logfc=-1.9)
Olsenella_uli (FDR=0.59, logfc=0.9)
Olsenella_profusa (FDR=0.04, logfc=-3.1)
Slackia_exigua
candidate_division_TM7_single_cell_isolate_TM7c (FDR=0.40, logfc=-3.0)
candidate_division_TM7_single_cell_isolate_TM7b (FDR=0.02, logfc=-4.6)
Granulicatella_elegans (FDR=0.49, logfc=-2.3)
Granulicatella_adiacens (FDR=0.59, logfc=-0.8)
Abiotrophia_defectiva (FDR=0.59, logfc=2.0)
Enterococcus_cecorum (FDR=0.49, logfc=2.1)
Lactobacillus_gasseri (FDR=0.59, logfc=-1.1)
Lactobacillus_casei_paracasei (FDR=0.59, logfc=-1.5)
Lactobacillus_oris (FDR=0.59, logfc=-1.1)
Lactobacillus_rhamnosus (FDR=0.59, logfc=-0.8)
Lactobacillus_vaginalis (FDR=0.59, logfc=-1.2)
Streptococcus_australis (FDR=0.49, logfc=-1.4)
Streptococcus_anginosus (FDR=0.59, logfc=-1.6)
Streptococcus_constellatus (FDR=0.13, logfc=-3.5)
Streptococcus_cristatus (FDR=0.55, logfc=-1.9)
Streptococcus_gordonii
Streptococcus_infantis (FDR=0.21, logfc=-3.7)
Streptococcus_intermedius (FDR=0.49, logfc=-2.6)
Streptococcus_mitis_oralis_pneumoniae (FDR=0.02, logfc=-4.1)
Streptococcus_mutans
Streptococcus_oligofermentans (FDR=0.05, logfc=-2.9)
Streptococcus_oralis (FDR=0.49, logfc=1.0)
Streptococcus_parasanguinis (FDR=0.49, logfc=-1.5)
Streptococcus_peroris (FDR=0.49, logfc=-2.2)
Streptococcus_pseudopneumoniae (FDR=0.49, logfc=-1.5)
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Streptococcus_sanguinis (FDR=0.50, logfc=0.9)
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Streptococcus_tigurinus
Streptococcus_vestibularis (FDR=0.05, logfc=-3.4)
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Gemella_haemolysans (FDR=0.10, logfc=-3.3)
Gemella_sanguinis (FDR=0.40, logfc=-3.1)
Anoxybacillus_flavithermus (FDR=0.49, logfc=1.8)
Staphylococcus_aureus (FDR=0.59, logfc=-0.8)
Mogibacterium_sp_CM50 (FDR=0.59, logfc=-1.2)
Eubacterium_infirmum (FDR=0.49, logfc=-1.5)
Parvimonas_micra (FDR=0.40, logfc=-2.2)
Eubacterium_hallii (FDR=0.49, logfc=1.5)
Eubacterium_brachy
Eubacterium_rectale
Pseudoramibacter_alactolyticus
Catonella_morbi
Ruminococcus_torques (FDR=0.59, logfc=-0.7)
Lachnoanaerobaculum_saburreum (FDR=0.13, logfc=-3.1)
Lachnospiraceae_bacterium_oral_taxon_082 (FDR=0.22, logfc=-2.9)
Lachnospiraceae_bacterium_ICM7 (FDR=0.49, logfc=-2.2)
Lachnospiraceae_oral_taxon_107 (FDR=0.49, logfc=-2.1)
Oribacterium_sp_ACB8 (FDR=0.59, logfc=-1.7)
Oribacterium_sinus (FDR=0.49, logfc=-2.9)
Oribacterium_sp_oral_taxon_078
Shuttleworthia_satelles
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Bulleidia_extructa (FDR=0.59, logfc=-1.2)
Solobacterium_moorei (FDR=0.20, logfc=-2.6)
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Megasphaera_micronuciformis (FDR=0.11, logfc=-3.8)
Selenomonas_flueggei (FDR=0.08, logfc=-3.2)
Selenomonas_artemidis
Selenomonas_infelix (FDR=0.49, logfc=-2.0)
Selenomonas_noxia (FDR=0.49, logfc=-2.2)
Selenomonas_sputigena (FDR=0.49, logfc=-1.9)
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Leptotrichia_shahii (FDR=0.05, logfc=-2.4)
0
0 - 10^1
10^1 - 10^2
10^2 - 10^3
10^3 - 10^4
> 10^4

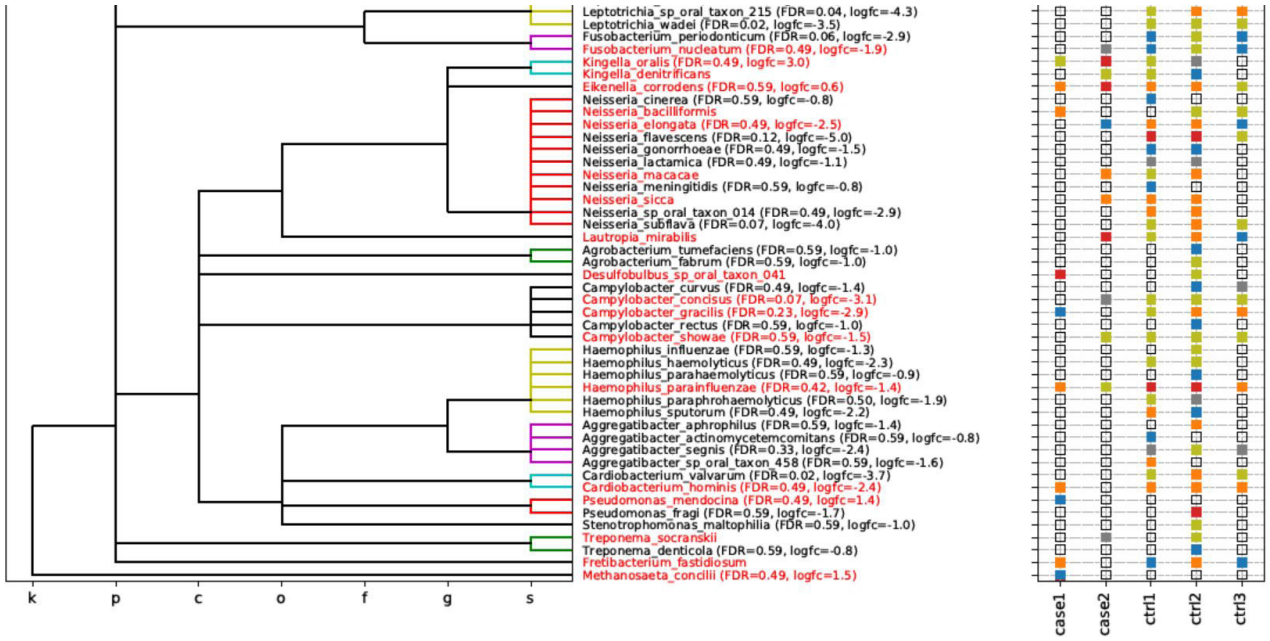

Figure 2: Microbial taxonomy for healthy and diseased implants

The left panel shows the taxonomy for all species, where the species present in the case group (peri-implantitis; may also appear in the control group) are colored red. Thus, the species shown in black are only present in the control group. We also provide the false discovery rate (FDR) and fold change (fc) for species with FDR<0.6. The right panel shows the reads per million (RPM) for the 5 samples respectively. The legend to the right with the color coding provides the RPM

Table 1: Microbiome Diversity Characteristics by Group. Mean values and 95% Confidence Intervals (95%CI)

	No. of Species (95%CI)	Shannon diversity (95%CI)	Simpson diversity (95%CI)	Chao1 diversity (95%CI)
Case/disease	73.0 (71.0-75.0)	2.63 (2.54-2.72)	0.67 (0.54-0.80)	73.00 (71.04-74.96)
Ctrl	73.00 (71.04-74.96)	4.18 (3.14-5.21)	0.88 (0.77-0.99)	0.88 (0.77-0.99)

Discussion

In the present study, the bacterial composition of healthy and failed implants was identified through deep whole metagenome shotgun sequencing. It is noteworthy that most of the "classic" periodontal pathogens were not found at elevated levels on the failed implants and were more abundant around the healthy implants. This is not unusual, as red complex pathogens are found normally in healthy microbiota [23,27]. Current concepts in microbiology and periodontology make distinction between labels such as "pathogens" and "commensals" increasingly complex and unclear. As opposed to the traditional dogma of a select few Gram-negative anaerobic pathogens causing disease, it is thought that disease stems from a dysbiotic shift of the microbial community that favors inflammation and resistance of the host immune response [3,6,7,28]. Indeed, studies on periodontal disease have shown that higher levels of Gram-positive bacteria were associated with diseased sites [29]. Similar studies on peri-implantitis are consistent in showing that peri-implantitis is a polymicrobial infection, with increased numbers of non-traditional bacteria, such as aerobic Gram-negative and anaerobic Gram-positive species [24,25].

Not only was there a shift in the microbes around diseased implants, but there was also a significant decrease in the community diversity and richness. Microbiota that exhibit a reduced species diversity are associated with peri-implantitis [28-30]. However, this is in contrast to what was found in a previous 16S study on peri-implantitis, in which higher diversity and richness was observed in peri-implantitis sites compared to periodontitis and healthy sites. [31,32]. However, given the limitations of 16S sequencing in identifying microbes at the species level, our study is the first to report a comprehensive and deeper microbiome profiling of failed implants by whole genome shotgun sequencing at the species level, albeit of a limited sample size.

Changes in microbial diversity have been linked to many chronic diseases (Human Microbiome Project Consortium 2012) [33]. In particular, low diversity in the gut microbiome has been linked to obesity and inflammatory bowel disease [34-36]. Further, in implant dentistry, larger scale studies are needed to clarify the role of microbial diversity in peri-implant health and disease. Ideally, a longitudinal study that can track microbial shifts over time after placing an implant will reveal new insights on how the oral microbiome evolves around a healthy and diseased dental implant.

One factor that is a limitation of our study is the small sample size. It should be emphasized that with the limited sample size in this study, the results cannot be generalized. Very few studies on peri-implantitis have employed 16S rRNA sequencing to analyze the microbiome of healthy and diseased/failing implants and, to our knowledge, none have employed deep whole metagenome shotgun sequencing as in the current study. With such differences in the oral flora from person to person, it will be important to evaluate the potential shift in microbial composition as the peri-implant environment shifts from one of a healthy state to a diseased state as further and larger studies are completed.

Conclusion

The microbiota in failed implants showed less community diversity and richness compared to healthy implants. Most traditional periodontal pathogens were not found in high quantities around the failed implants. Further studies are needed to clarify the dysbiosis and changes in the microbial composition as the peri-implant environment shifts from one of health to disease.

Author Contributions

C Lee and S Shafa contributed to the design, data acquisition and interpretation, drafted and critically revised the manuscript. MJ Zhang and MR Sailani contributed to the design, data analysis and interpretation, drafted and critically revised the manuscript. P Xenoudi contributed to the design and interpretation and critically revised the manuscript. Y Kapila contributed to conception, design, data interpretation, drafted and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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Supplementary Figure 1

To calculate the differences in species expressed between the test and control groups, two-sample Student's T-tests and Wilcoxon rank-sum tests were both used. Both methods gave similar p-values for all species. The diagonal panels show the histogram of p-values for each species computed by T-test and Wilcoxon test respectively. The off-diagonal panels show the comparison of p-values computed by the two methods respectively, where each dot corresponds to a species.