ANALYSIS OF THE MICROBIOME ASSOCIATED WITH PERI-IMPLANTITIS IN MOROCCAN PATIENTS

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ABSTRACT

Little is known about the microbiome composition associated with peri-implantitis in developing countries. A recent study found a high prevalence of peri-implantitis in a group of Moroccan patients. We hypothesized that a distinct microbiome may be associated with this disease in Moroccan subjects, and the aim of this study was to investigate the composition of the microbiome in peri-implantitis sites and sites without peri-implantitis. The study material consisted of 35 dental patients with dental implants: 22 of these had peri-implantitis, and 13 were without peri-implantitis. Among these subjects, dental plaque samples were collected from 50 peri-implant sites as follows: in the peri-implantitis subjects, 22 samples were from peri-implantitis sites (peri-implantitis patient diseased sites) and 15 samples from sites without peri-implantitis (peri-implantitis patient control sites); and 13 samples from implants from subjects without peri-implantitis (non-peri-implantitis patient control sites). The samples were sequenced for the V1-V3 region of the 16S rRNA gene, and the resultant sequences were classified at the species level using a previously described Blastn-based algorithm. Downstream analysis of the data was performed with Phyloseq, Microbiome, Vegan and MaAsLin packages in R, using a false discovery rate (FDR) cutoff of 0.25. Fifty-six species and 30 genera were identified per sample on average. No significant differences were found between the groups in terms of species richness and alpha diversity. However, beta diversity analysis by PERMANOVA (Adonis) identified a statistically significant difference (FDR=0.024) between the peri-implantitis patient diseased sites and non-peri-implantitis patient control sites. Compared to non-peri-implantitis patient control sites, diseased but not control sites in patients with peri-implantitis showed significantly higher levels of *Peptostreptococcus stomatitis* and *Mogibacterium* spp. However, both diseased and

control sites in patients with peri-implantitis had higher abundance of *Olsenella uli*, *Atopobium spp.* and *Actinomyces spp.* compared to non-peri-implantitis patient control sites. No differences at FDR ≤ 0.25 were found between diseased and control sites in patients with peri-implantitis, but *Porphyromonas endodontalis* tended to be elevated in diseased sites while *Veillonella parvula* tended to increase in control sites. These findings suggest a distinct dysbiotic microbiome is associated with peri-implantitis sites in Moroccan patients.

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LIST OF ABBREVIATIONS

NGS- Next Generation Sequencing

AAGPRs – Anaerobic Asaccharolytic Gram Positive Rods

PI -Peri-Implantitis

Non-PI- Non- Peri-Implantitis

PCR – Polymerase Chain Reaction

CHAPTER 1

INTRODUCTION

1.1. Peri-Implantitis

Treatment with dental implants is a viable option for replacing missing teeth, and in restoring occlusion and the masticatory and aesthetic functions of patients. The long-term success rate of this treatment is high, with some studies in developed countries reporting a success rate of 97% after 5 years (Ribeiro, 2022; Thanissorn, 2022). However, complications following dental implant placement do occur. Peri-implantitis is the most common postoperative complication of implant treatment. The name "peri-implantitis" was first proposed in the year 1994 at the European Workshop on Periodontology. In the 2017 classification of periodontal and peri implant diseases and conditions, peri-implantitis was defined as "a plaque-associated pathological condition that occurs in the tissues around dental implants and is identified by presence of inflammation surrounding the peri-implant mucosa, and succeeding destruction of bone support" (Berglundh, 2018).

In clinical practice, peri-implantitis is diagnosed by presence of inflammation in the peri-implant soft tissues and bleeding on probing/suppuration, increased probing depth, progressive bone loss at 1 year after the delivery of the implant-supported prosthesis, and in cases where baseline radiographs and probing depths are not available, radiographic evidence of bone level ≥3 mm and/or probing depths ≥6mm in conjunction with profuse bleeding on probing(Renvert, 2018). Peri-implant mucositis is a related condition that demonstrates the presence of peri-implant signs of inflammation, profuse bleeding on probing, increase in probing depths compared to baseline but without crestal bone loss (Renvert, 2018).

1.2. Prevalence of Peri-Implantitis

The prevalence of peri-implantitis has been studied extensively. A recent systematic review and meta-analysis (Diaz, 2022) found the prevalence of the peri-implantitis to be 19.6% and 12.39% at the patient level and implant-level, respectively. One study in patients of a U.S. dental school estimated that the prevalence of peri-implantitis over an average follow-up of 2 years was 34% at the patient level and 21% at the implant level (Kordbacheh Changi, 2019). However, these estimates are mostly based on studies performed in developed countries. Studies in developing countries suggest higher prevalence though. For example, (Kissa, 2021), in a study involving follow up of 642 implants and 145 patients in Morocco for a mean 6.4 years, found 41.4% of the subjects had one or more implants with peri-implantitis.

1.3. Etiology and Risk Factors of Peri-Implantitis

The microbial biofilm colonizing the surface of implants is the primary etiological factor of peri-implantitis (Fragkioudakis, 2021). However, peri-implantitis is a multifactorial disease, involving many risk factors, including genetic susceptibility, host immune response, trauma from occlusion, titanium fragments diffusion into surrounding tissues, flow of excess cements post prosthetic installation, history of periodontal disease, inadequate plaque control, cigarette and smokeless tobacco use, hyperglycaemia and obesity, implant's malposition and poor implant prosthesis design (Hashim & Cionca, 2020).

1.4. Microbial Etiology of Peri-Implantitis

Microbiota colonizing the implant surface in the form of biofilm is the primary etiological factor of peri-implantitis, as the case is with periodontitis. However, a study of the initial bacteria adhesion and biofilm formation and deposition on titanium surfaces suggests that this process is different than biofilm formation on teeth (Kotsakis & Olmedo, 2021). Hence, the composition of the biofilm may also be different on dental implants than on teeth. Microbiota implicated in the pathogenesis of peri-include periodontal pathogens, asaccharolytic anaerobic Gram-positive rods (AAGPR), uncultivable Gram-negative rods and opportunistic micro-organisms such as enteric rods and *Staphylococcus aureus* (Lafaurie, 2017). Extensive work has been done on the microbiology of peri-implantitis which is elaborated upon in the subsequent sections.

1.5. Microbiology of Peri-Implantitis – Pre-Sequencing Era

Through early 2000s, the microbiology of peri-implantitis was mostly studied using culture-based techniques, and to a lesser extent, targeted molecular methods, including checkerboard DNA-DNA hybridization, and conventional, real time or nested polymerase chain reaction (PCR). Early studies employing culture techniques demonstrated presence of *Prevotella intermedia* and gram-negative enteric rods with significantly higher numbers in diseased than in healthy implants (Botero, 2005; Leonhardt, 1999). Subsequent studies found a high prevalence of *Porphyromonas*, *Prevotella*, and anaerobic Gram-positive cocci (Neilands, 2015; Peterson, 2009). Similarly, a correlation has been reported between the increase in peri-implant bleeding on probing within 6 months after loading and a rise in counts of *Streptococcus spp.* and *Peptostreptococcus spp*. (Asadzadeh, 2012).

Using Checkerboard DNA-DNA hybridization, the periodontal pathogens *Porphyromonas gingivalis*, *Tanerella forsythia*, and *Treponema denticola* were found at significantly higher counts in both supragingival and subgingival biofilms in peri-implantitis compared to health (Shibli, 2008). Consistently, real-time PCR studies identified *T. denticola, P. gingivalis, and P.intermedia, T. forsythia,* and *Fusobacterium nucleatum* in association with peri-implantitis (Gürlek, 2017; Ito, 2021). Moreover, a systematic review and meta-analysis of PCR-based studies demonstrated a higher prevalence of *Aggregatibacter actinomycetemcomitans* and *P.intermedia* in peri-implantitis than in healthy implants(Sahrmann, 2020). The above-mentioned techniques, however, are closed-ended and involve using preselected probes or primers targeting specific types of bacteria, which may lead to selection bias if the study's purpose is to investigate the composition of the microbiome**.**

1.6. Microbiology of Peri-Implantitis – Current Perspective

Developments in the last two decades, namely the emergence of the concepts of microbiome and microbial dysbiosis and the advent of next-generation sequencing (NGS) methods, has revolutionized the study of oral microbial communities (Kamble, 2020). NGS methods have become the preferable method for studying oral microbiota as they can detect all microorganism within a sample, including those that cannot not be detected using traditional culture techniques or DNA probes, besides its affordability and the possibility to conduct parallel sequencing of multiple samples (Wensel, 2022).

The oral microbiome occupies the second largest microbial community after the gut bacteria. It is described as the collective genome of microbes residing in the

oral cavity (Deo & Deshmukh, 2019). Dysbiosis denotes a disturbance in symbiosis. It usually entails a diminishing number of advantageous bacteria, and expansion of pathogenic bacteria (Berezow & Darveau, 2011; Shaikh, 2018). NGS-based microbial profiling is of two types: 1) profiling based on gene amplicon of marker genes like 16S rRNA, and 2) shotgun metagenomics where all DNA fragments are sequenced. Table 1 summarizes the main studies that used 16S rRNA sequencing to analyze the microbiome associated with peri-implantitis compared to peri-implant health.

Systematic reviews of these studies concluded that a complex and diverse microbiota are associated with peri-implantitis, and that the diversity of the microbiome is positively correlated with the severity of peri-implantitis. Amongst the most common genera found in association with peri-implantitis are *Actinomyces, Eubacterium, Fusobacterium, Mogibacterium, Moraxella, Treponema* and *Porphyromonas.* In contrast, the genera *Streptococcus, Leptotrichia, Prevotella* and *Haemophilus* are more abundant in the dental plaque of healthy implants. A recent systematic review concluded that the genera *Fusobacterium, Treponema*, *Porphyromonas spp.* are associated with peri-implantitis, whereas *Veillonella spp.* and *Neisseria spp.* are associated with healthy implants. In addition, Methanogens and AAGPRs were also detected in peri-implantitis sites. On the other hand, *Streptococcus spp.* is present in both healthy and diseased implant sites (Chun Giok & Menon, 2023; de Melo, 2020; Lafaurie, 2017; Sahrmann, 2020) .

Based on overall evidence, a recent systematic review grouped the oral microbiota into peri-implantitis-associated, peri-implant health-associated, periodontitis-associated, and core taxa (Figure 1) (Belibasakis & Manoil, 2021).

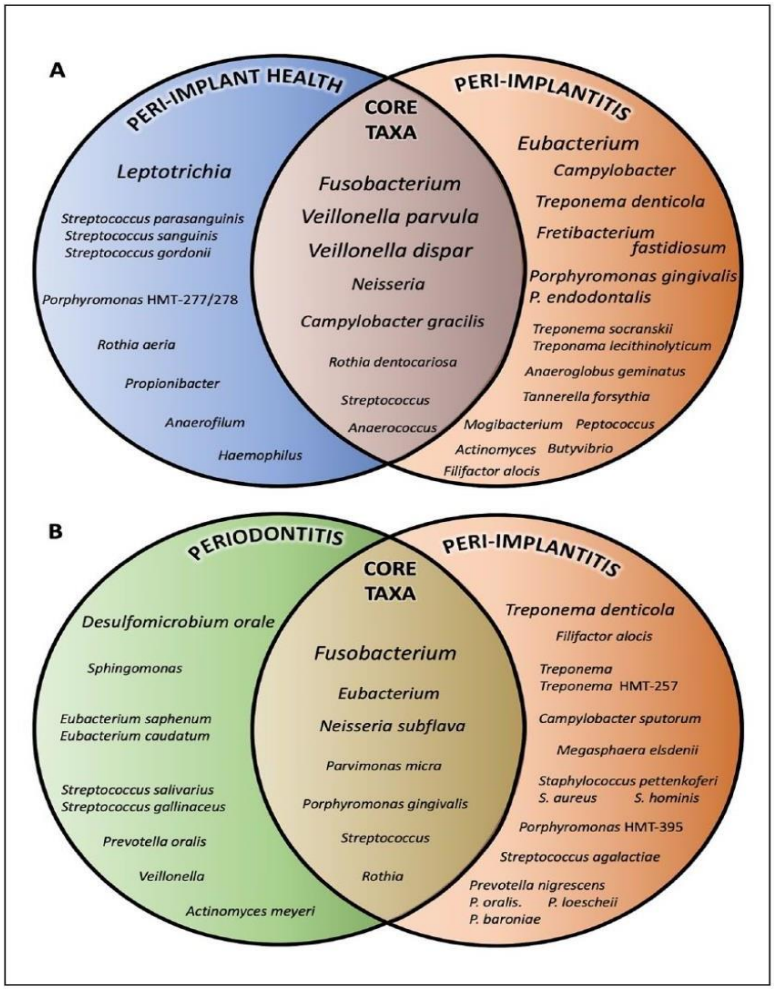

Figure 1. A Venn Diagram. This figure is reproduced from (Belibasakis & Manoil, 2021) under a Creative Commons Attribution 4.0 International License: http://creativecommons.org/licenses/by/4.0/

Table 1. Review of Studies Comparing Peri-Implantitis and Healthy Implants by 16SrRNA Sequencing

Author Name/Yr Country	Participants/Implants/ Study Design	Case Definition	Time of function	Confounders controlled	Hypervariable region, sequencing technology	Taxa associated with health and disease
1. (Sanz-Martin, 2017) Switzerland	82 participants with one implant each Case control retrospective (comparison of sites)	6th European Association definition	One year	Smoking Implant type (included in PCoA model	V1-V3-Illumina Miseq	Species- Peri-implantitis sites- *T. forsythia, T. denticola* and *P. gingivalis.* New pathogens like *Fillifactor alocis, Fretibacterium fastidiosum* and *Treponema maltophilum.* Healthy sites- *Rothia dentocariosa* (p<.05, FDR-adjusted). Core:Health. *Veilonella parvula, S.sanguinis and Rothia sp* .Core periimplantitis: red complex microbes, new pathogens *F.alocis, T.maltophilum* and *F.fastidiosum* played a role as a core of peri-implantitis.
2. (Tamura, 2013) Japan	NR/15 NR/15 Cross sectional/parallel	PD >=4mm,SUP,BOP,visible 3 thread loss of alveolar bone clearly extending around implant as 7isualized on R/G	>=6 months	Antibiotics DM	Full 16S rDNA sequencing of isolated strains	Peri-implantitis sites- *E nodatum (7%), P intermedia (5%), F nucleatum (3%), Filifactor alocis (3%), E brachy (3%), Parascardovia denticolens (3%), and Parvimonas micra (3%* Healthy implant sites -*Veillonella species (spp) (14%), Propionibacterium acnes (5%), Pseudoramibacter alactolyticus (3%), and Parvimonas micra (2%).*

Table 1. Continued.

3. (da Silva, 2014) Brazil	NR/10 NR/10 Cross Sectional /Parallel	Saucer shaped osseous defects >3mm, an inflamed peri-implant mucosa exhibiting BOP and/or SUP and PD>= 5mm, and an inflamed peri-implant mucosa	Not Reported	Antibiotics, anti-inflammatory, peri implant therapy, DM, active periodontal disease, implant with a coated surface, edentulism	Full 16S RNA gene sequencing - Sanger	*F. nucleatum, Dialister invisus, Streptococcus sp. human oral taxon (HOT) 064, F.alocis and Mitsuokella sp. HOT 131* presented a higher mean proportion, while *Veillonella dispar, Actinomyces meyeri, Granulicatella* adiacens showed lower mean proportions – the peri-implantitis sites when compared with healthy implants in both the control and test groups.
4. (Zheng, 2015) China	NR/NR8 Cross sectional /Parallel	Unclear	Not Reported	Not Reported	V1-V3 Pyrosequencing	Peri-implantitis – high phylogenetic microbial diversity *Leptotrichia hofstadii, Eubacterium infirmurn, Kingella denitrificans, Actinomyces cardifensis, Elusimicrobium minutum, Treponema lecithinolyticum, and Globicatella sanguinis* were more in peri-implantitis. *Eubacterium brachy* showed analysis of the co-occurrence –positive correlation between *E. minutum* & Pi., which suggests the association of *Eubacterium* with peri-implantitis

Table 1. Continued.

5. (Gao, 2018) China	Control groups= = 20 Case groups =20 Cross Sectional/ Parallel Total 50 taken, 40 samples used and 10 for backup	Guideline of periodontology; BOP, PPD >= 4mm, with bone tissue loss under first thread of the implant(bone resorption >=2mm	More than half a year	Not Reported	V3-V4, Illumina Miseq	Dominant genera in the cases were *Morallela, Micrococcus and Acinobacter* were dominant. Cluster analysis confirms- *Micrococcus* abundance for cases was significantly higher. *Prevotella* abundance in healthy implants was significantly higher. *GCF showed species of *Hemophilus and Capnocytophagia*. (Not seen in previous studies)
6 (Al-Ahmad, 2018) Germany	Total 10 individuals With at least one implant with periimplantitis and one clinically healthy implant Cross sectional, Split mouth design	Site of peri-implantitis – PD >=5mm, BOP and concomitant radiographic bone loss Healthy- no signs of either bone loss or increased pocket depth (PD<=3)	Not specified	Not Reported	Full 16S RNA gene sequencing, technology not specified (presumably Sanger)	Species *P.gingivalis, T. Forsythia* were present in significantly higher levels in the peri-implantitis group whereas the species *V. dispar,* tended to be present in the healthy implant group. *F. nucleatum* presented a higher relative abundance in the peri-implantitis group. P<0.05

Table 1. Continued

7. (Pallos, 2021) Brazil	Total 42 subjects- 21=Healthy group 21=Peri-implantitis group Case-control Study	Healthy=one healthy implant, PD <4mm,no BOP, Peri-implantitis group= presence of BOP, suppuration, PD<=5mm and bone loss >=3mm	Two years	Not Reported	V4, Ion Torrent PGM system for sequencing	Peri-implantitis: *Faecalibacterium prausnitzii, Haemophilus parainfluenzae, Prevotella copri, Bacteroides vulgatus, and Bacteroides stercoris.* Healthy: *Rothia mucilaginosa , (HMT_681), Haemophilus parainfluenzae (HMT_718),* and *Actinomyces multispecies* (spp120_6) (SP2).
8. (Polymeri, 2021) Netherlands	Total 41 patients Cross sectional split mouth design	Diagnosis for peri implant cases done according to AAP 2017 workshop definition	Wide range of loading - not specific	Not Reported	V5–V7, Pyrosequencing	Deep pockets harboured significantly higher proportions of OTUs: #523 (*F. nucleatum)* (p = .001), #299 (*Prevotella oris*) (p = .001), #427 (*Prevotella nigrescens*) (p = .035), and #495 (*Anaeroglobus geminatus*) (p = .046) compared with shallow pockets that harboured more OTUs#579 (*Rothia mucilaginos*a) (p = .005), #308 (*Neisseria oralis*) (p = .002), #366 (*H. parainfluenzae*) (p = .006), #127 (*Streptococcus oralis*) (p = .009), and #339 (*Strepto- coccus mutans*) (p = .049). Implants diagnosed with peri-implantitis were colonized by higher proportions of OTUs #523 (*F. nucleatum*) (p = .015) and #767 (*T. denticola)* (p = .001) com- pared with healthy implants and peri-implant mucositis which were colonized by higher proportions of#579 (*Rothia mucilaginosa*) (p = .037) and #209 (*Streptococcus sal ivarius*) (p = .015)

1.7. Gap of Knowledge and Study Aims

Most studies that investigated the microbiome of peri-implantitis were conducted in populations from industrialized countries (Lafaurie, 2017; Sahrmann, 2020) and little is known about this topic in developing countries. Given a relatively high prevalence of peri-implantitis reported in Moroccan patients (Kissa, 2021), we hypothesize that a distinctive microbiome may be involved.

The study aims were to:

1. Investigate the microbiome composition according to the peri-implant health status in a group of Moroccan patients.
2. Compare the microbiome composition between sites with and without peri-implantitis in these patients.

CHAPTER 2

METHODS

2.1. Ethical Review

The original study protocol was reviewed and approved by the Pedagogic Committee and the Research Conduct Committee of the Faculty of Dentistry, University of Hassan II, Casablanca, Morocco. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. All the participants were informed about the study, and only consenting patients were included.

2.2. Study Subjects

The study subjects included a subsample of 35 subjects selected from a larger group of 180 subjects that included adult dental implants patients attending a private dental clinic in Casablanca, Morocco. The inclusion criteria of the original group included dental patients who were seen at the clinic for implant maintenance during a 9-month period in 2017 and who had received dental implant treatment at least 1 year earlier. The inclusion criteria of the subjects in this study were age of ≥18 years old, no history of antibiotics or periodontal treatment during the past 6 months, no systemic diseases, no pregnancy (for females), and no history of current smoking. The 35 study subjects included 22 subjects with untreated peri-implantitis, and 13 subjects without peri-implantitis.

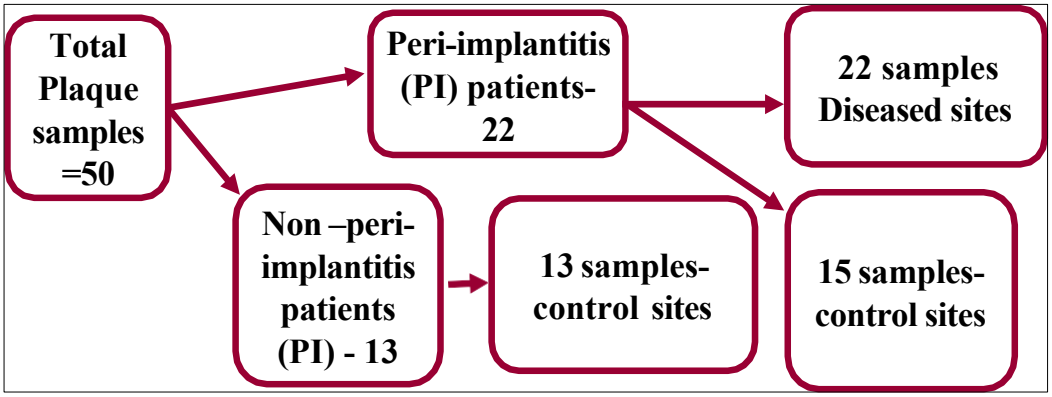

Figure 2. Study Design. Control sites: sites with no peri-implantitis. Diseased sites: sites with peri-implantitis.

2.3. Collection of Dental Plaque Samples

Fifty peri-implant biofilm samples were collected from the study subjects (Figure 2). In the peri-implantitis patients, samples were collected from 1-4 diseased sites (with ≥6 mm pocket depth and bleeding and/or suppuration), and when available, from implant sites with no signs of peri-implantitis. In subjects with no peri-implantitis the samples were collected from non-diseased implants.

In each patient, the implant sites were isolated using cotton rolls, then cotton pellets were used to remove the supramucosal biofilms. A sterile curette was used to collect submucosal biofilm from the sites and placed in an Eppendorf tube containing 500 µl TE buffer (Invitrogen, USA). In addition, a sterile endodontic paper point (size #40) was placed in each pocket for 10 seconds and then transferred to the same Eppendorf tube containing the TE buffer. Similar procedures of biofilm collection were followed for the control sites that showed no clinical or radiographic signs of peri-implantitis.

A total of 50 plaque samples were collected, comprising 37 samples in peri-implantitis patients (22 and 15 samples from diseased and control sites, respectively), and 13 samples from control sites in patients without peri-implantitis.

2.4. Sample Preparation and DNA Extraction

The plaque samples were stored at -80° until processed. Prior to DNA extraction, the samples were thawed and vortexed on the vortex mixer at a high speed for 30 seconds, after which the paper points were discarded, and the samples were then subjected to centrifugation at 14,000 rpm for 10 minutes to pellet bacterial cells using Sorvall Legend Micro 21R centrifuge (Thermo Fisher Scientific, Waltham, Massachusetts, USA). DNA was extracted from the pellets using Purelink Microbiome DNA extraction kit (Figure 3; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to manufacturer's instructions with a few modifications to maximize the DNA Yield. Namely the volume of S1 and S2 lysis buffers in which the pellets were suspended prior to transferring to bead tubes was adjusted to 600ul and 75 ul, respectively. Incubation was performed at -95°C for ten minutes on a Isotemp (Thermo Fisher Scientific, Waltham, Massachusetts, USA), followed by three cycles of 1-minute bead beating at 6.0 M/s using a Fast prep bead beating system (MP Biomedicals Santa Ana, California, USA). The remaining steps performed a described in the kit's manual. DNA was eluted in 25µl of low EDTA buffer. DNA yield was measured using Qubit dsDNA High Sensitivity kit on a Qubit 2.0 Fluorometer (Invitrogen,Thermo Fisher Scientific, Waltham, Massachusetts, USA).

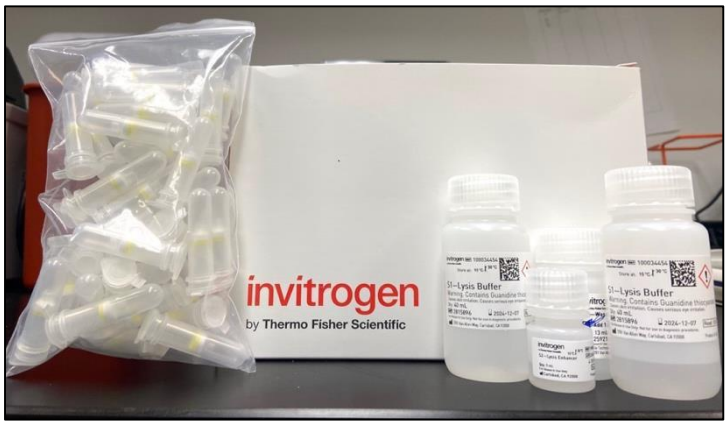

Figure 3. Pure Link Microbiome DNA Extraction Kit (Invitrogen, USA)

2.5. Pre-Amplification of 16S Target Sequences Using 27FYM and 519R

The V1-V3 region of the 16S rRNA gene was pre-amplified using the degenerate primers 27FYM (Frank, 2008) and 519R (Lane, 1985). The reaction mix comprised 12.5µl platinum superFi green mix, 1µl primer mixes at 0.4 µM each, 5 µl template DNA and 7.5 µl PCR grade water (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Thermal cycling protocol with Veriti 96 well thermal cycler (Figure 4; Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was as follows: Denaturation at 98O C for 30 sec, 25 cycles of 98°C for 10 s, 53°C for 10 s,72°C for 15 s and final extension of 72O C for 5 min. Successful amplification was confirmed with electrophoresis as a ~ 520 bp band.

Figure 4. A Thermal Cycler for Pre-Amplification of Target Genes

2.6. Sequencing

The amplicons were sent to integrated Microbiome Resource (IMR) facility, Dalhousie University, Canada for library preparation and sequencing. Briefly, a second round of PCR was performed to add index and adaptor sequences to the amplicons. The libraries were then mixed in equimolar concentrations and sequenced using 2X300 bp chemistry on an Illumina MiSeq instrument.

2.7. Bioinformatic Analysis – Data Preprocessing

Data preprocessing including primer trimming, merging of reads, quality-filtration, and chimera removal was performed with previously described procedure (Al-Hebshi, 2017). Briefly, the software PEAR (Zhang, 2014) was employed to stitch paired sequences using the following parameters: minimum amplicon length = 432 bp; maximum amplicon lengths = 522 bp; and P-value = 0.001. The mothur software

package version 1.38.1, (Schloss, 2009) was used to process the merged reads as follows: reads with ambiguous bases, with homopolymers >8 bases long, that did not achieve a sliding 50-nucleotide Q-score average of ≥35 were filtered out; the remaining reads were checked for chimeras with Uchime (Edgar, 2011) using the self-reference approach. A script of the commands used with PEAR and mothur are included in Appendix A and B, respectively.

2.8. Bioinformatic Analysis -Taxonomy Assignment, Diversity and Differential Abundance Analysis

The high-quality, merged reads were classified to the species level using a previously described Blastn-based algorithm (Al-Hebshi, 2015). Briefly, each read was searched against four curated reference 16S rRNA gene ranked by their biological relevance. A hit was defined as a match at alignment coverage of ≥98% and sequence similarity ≥98%. The read was then assigned the taxonomy of the hit reference sequence with the highest percent identity and bit score belonging to the highest priority reference set. Unmatched reads were subjected to *de novo* operational taxonomic unit (OTU) analysis and each OTU with more than 100 reads was assumed to be a novel species and assigned to the closest known species.

The taxonomic profiles were reviewed and environmental contaminants were removed. Observed species, Chao index and Shannon's alpha diversity for each group using were then computed using Phyloseq (McMurdie & Holmes, 2013) in R. Differences between the groups were assessed using Mann-Whitney test or Wilcoxon signed rank test as appropriate. For assessing beta diversity, principal component analysis (PCA) was performed on centred log-ratio (CLR) species counts followed by Adonis PERMANOVA (permutational analysis of variance) using Phyloseq and Microbiome (Xia, 2018) packages in R. Outliers observed in PCA were excluded from

the downstream analysis. MaAsLin2 (Microbiome Multivariable Associations with Linear Models) package in R (Mallick, 2021) was used to identify differentially abundant genera and species between the study groups, using a false discovery rate cut-off (FDR) of 0.25.

The scripts of the commands used with R for alpha diversity, beta diversity, differential abundance and plotting are included Appendices C-F.

CHAPTER 3

RESULTS

3.1 Average Microbiome Profiles

A total of 249 species belonging to 97 genera and 9 phyla were detected in the samples. The average number of phyla per sample was 7 for all groups. Figure 5 shows the average relative abundances of phyla in the groups and the corresponding detailed data are presented in Appendix G. Actinobacteria was the most abundant phylum in all three groups. It accounted for 43% and 50% of the average microbiome in the peri-implantitis patient diseased and control sites, respectively, and for 37% of the control sites in non-peri-implantitis patients. It was followed by Firmicutes, which accounted for 40%, 34% and 28% of the average microbiome in the 3 aforementioned groups, respectively.

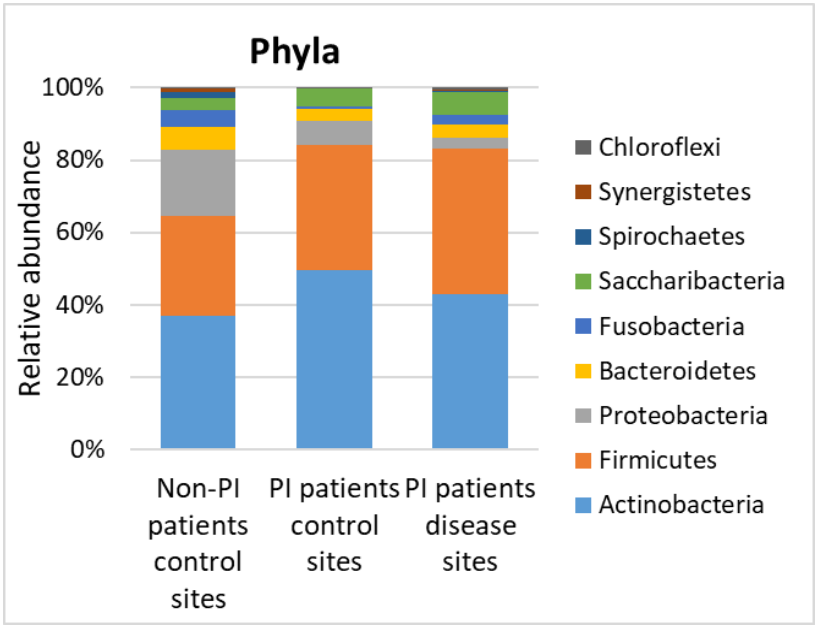

Figure 5. Stacked Bars Depicting Phylum-Level Taxonomic Profiles in the Study Groups. DNA extracted from the samples was sequenced for V1-V3 region of the 16S rRNA gene, merged, quality filtered and assigned to species level taxonomies using a BLASTn-based algorithm.

The average number of genera per sample was 31 in the peri-implantitis subjects (for both diseased sites and control sites), and 27 in the control sites in non-peri-implantitis patients. Figure 6 presents the top 14 genera with an overall average relative abundance ≥ 2 %; the corresponding detailed data are shown in and Appendix H. *Actinomyces* was the most abundant genus. It made up 22% of the microbiome of diseased sites and 37% of control sites in peri-implantitis patients, and 26% of control sites in non-peri-implantitis patients. Other abundant genera, in descending order, included *Brevundimonas, Parvimonas, Olsenella, Mogibacterium*, and *Streptococcus*, accounting together for 36%, 29% and 33% of the microbiome in the 3 study groups, respectively.

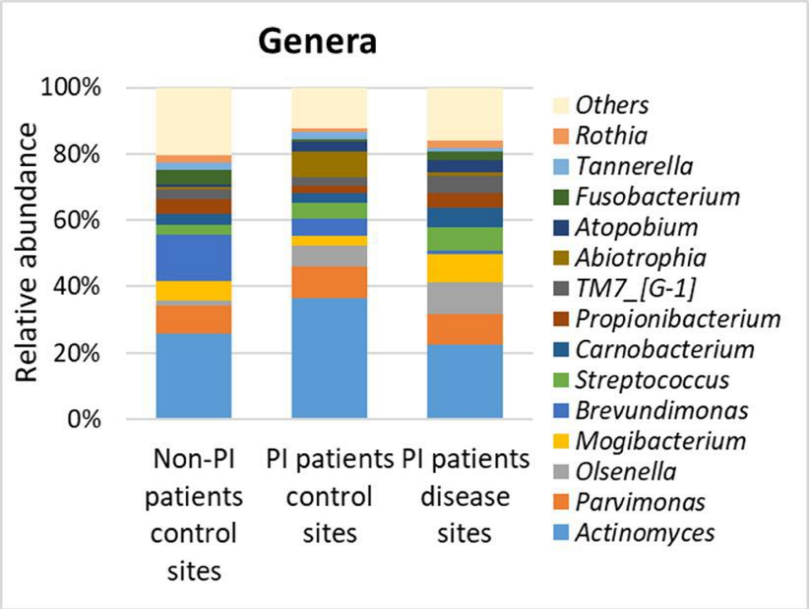

Figure 6. Stacked Bars Depicting the Genus-Level Taxonomic Profiles in the Study Groups. Top 14 genera are highlight. DNA extracted from the samples was sequenced for V1-V3 region on the 16S rRNA gene, merged, quality filtered and assigned to species level taxonomies using a BLASTn-based algorithm.

On the species level, on average 58, 60, and 48 species were identified in the 3 groups, respectively. The most abundant species were *Parvimonas micra, Brevundimonas diminuta, Mogibacterium timidum*, *Olsenella uli, Carnobacterium*

divergence, Actinomyces israelii and *Actinomyces sp. OT 525* accounting for 37%-42% of the microbiome, but the relative abundances varied substantially among the three groups. Figure 7 presents the top 20 species with an overall average relative abundance ≥ 2 %; the corresponding detailed data are shown in and Appendix I.

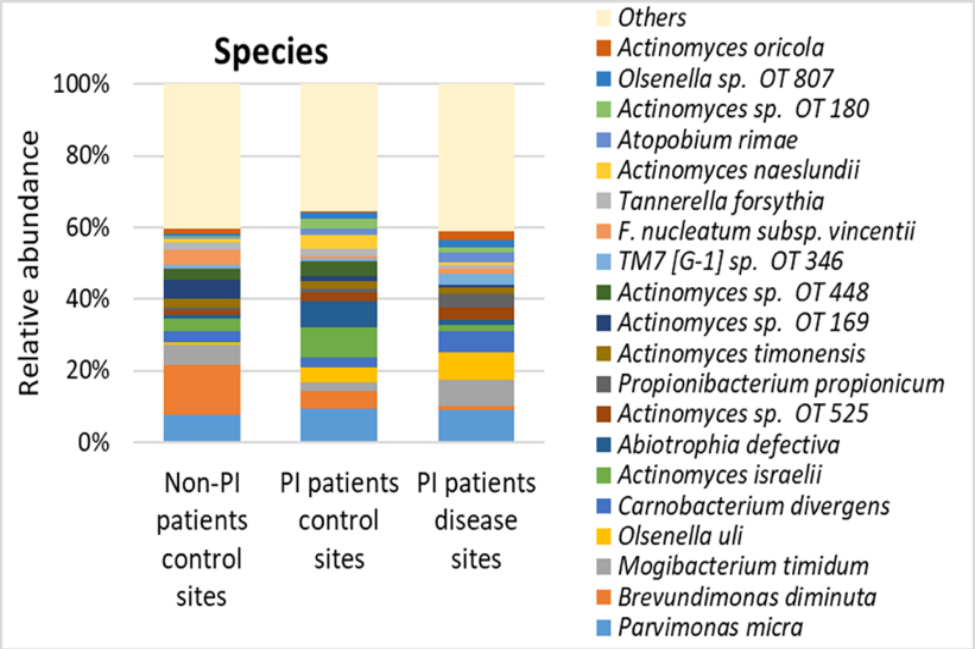

Figure 7. Stacked Bars Depicting the Species Level Taxonomic Profiles in the Study Groups. Top 20 species are highlighted. DNA extracted from the samples was sequenced for V1-V3 region of the 16S rRNA gene merged, quality filtered and assigned to species level taxonomies a BLASTn-based algorithm.

3.2 Outlier Analysis

To identify presence of outliers, a preliminary PCA plot was generated. IM21 and IM22 were identified as outliers, and these were excluded from further analysis as seen in figure 8.

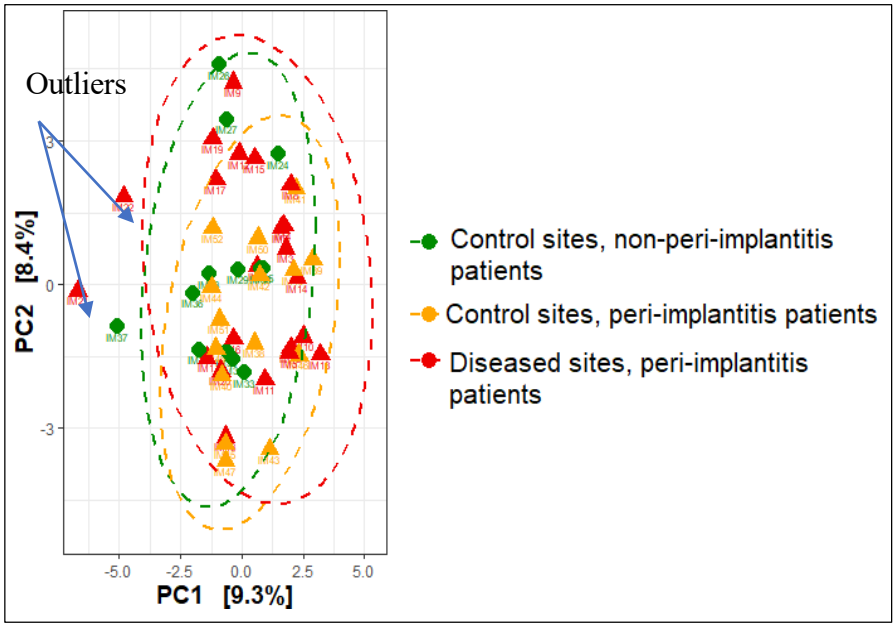

Figure 8. Preliminary Principal Component Analysis (PCA) Plots to Identify Outliers. Plots were generated from centred log-ratio transformed taxonomic profiles to identify outliers. IM21 and IM22 are outliers.

3.3 Species Richness and Alpha Diversity Analysis

Species richness and alpha diversity are represented in figure 9. No significant differences were found in observed species and the Chao index (expected species) between the three groups, indicating similar species richness. Similarly, there were no significant differences between the groups in alpha diversity measured using the Shannon index.

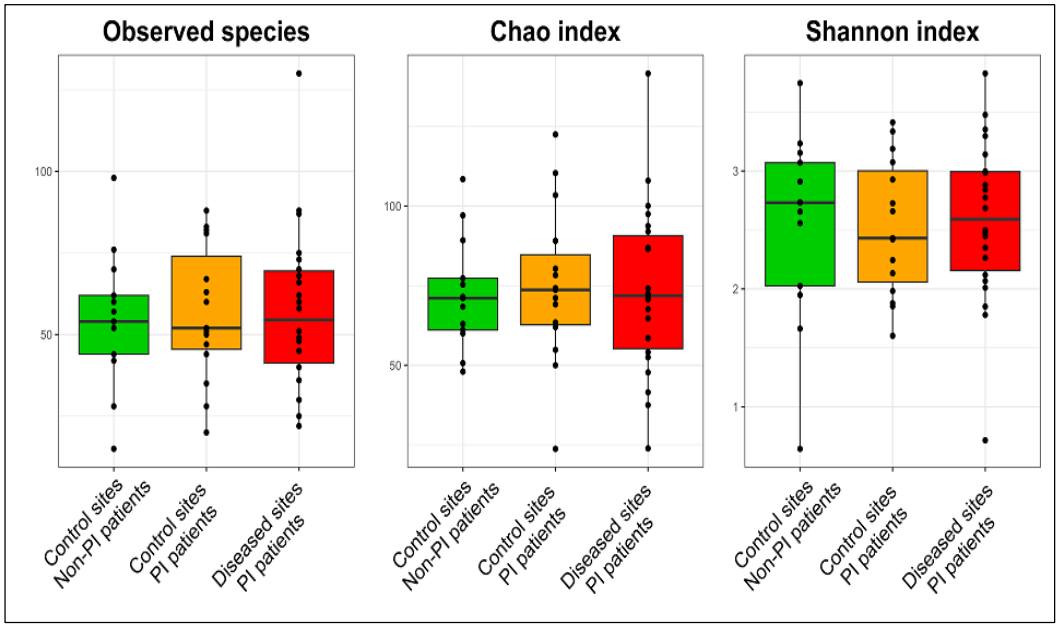

Figure 9. Species Richness and Alpha Diversity in the Study Groups. Taxonomic profiles were used to calculate observed species, Chao index (expected richness) and Shannon's index (alpha diversity) for each group. Statistical significance was assessed with Mann-Whitney or Wilcoxon signed rank test, as appropriate.

3.4 Beta Diversity Analysis

Beta diversity was assessed using PCA followed by Adonis (PERMANOVA) test. There was a statistically significant separation among the groups (P=0.024) indicating differences in microbial composition as given in figure 10. However, pairwise comparisons showed that the difference was statistically significant only for the comparison of the diseased sites in peri-implantitis patients versus the control sites in non-peri-implantitis patients as evident from table 2.

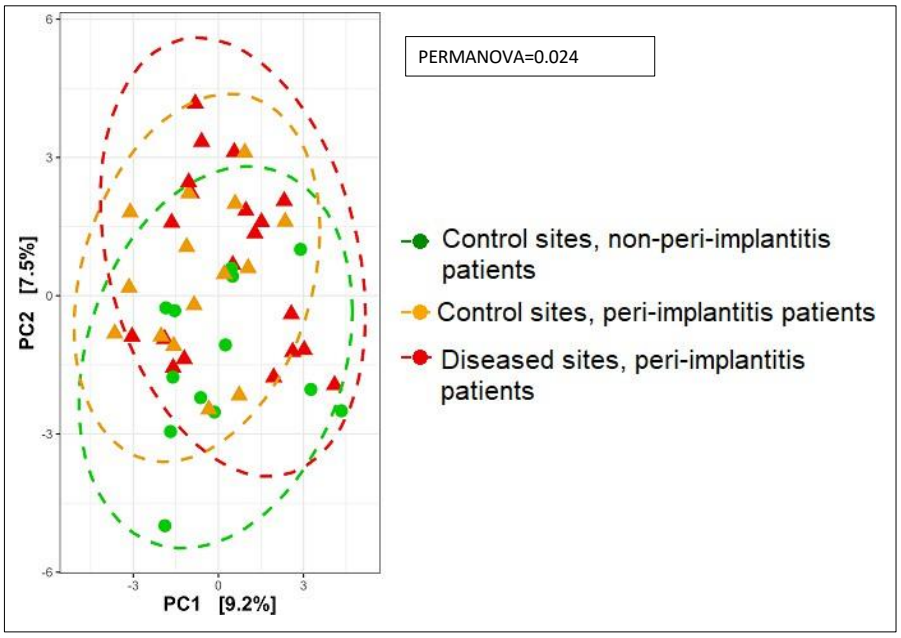

Figure 10. Principal Component Analysis (PCA) Plot for the Beta Diversity. Plots were generated from centred log-ratio transformed taxonomic profiles.

Table 2. Pairwise Adonis Test

Pairs	R2	P value	P adjusted
Diseased sites in peri-implantitis patients vs. control sites in patients without peri-implantitis	0.05	0.008	0.024
Diseased sites vs. control sites in patients with peri-implantitis	0.03	0.491	1
Control sites: peri-implantitis patients vs. patients without peri-implantitis	0.05	0.029	0.087

3.5 Dispersion Within Groups

Dispersion analysis explains how the data are dispersed around the central value of a group. The dispersion of the samples in each of the study groups is presented in figure 11.. There were no significant differences in dispersion between the groups ($p=0.66, p>0.05$) table 3.

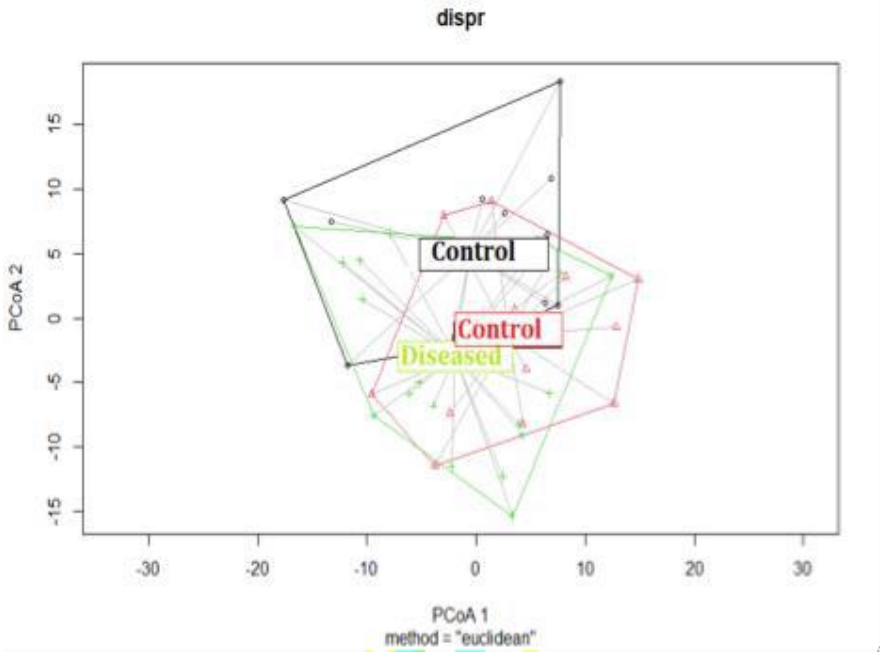

Figure 11. A Principle Component Analysis(PCA) Plot Showing Dispersion Within Each of the Study Groups. Black- non-peri-implantitis patients control sites. Green -peri-implantitis patients control sites. Red- peri-implantitis patients diseased sites.

Table 3. ANOVA Test for Dispersion

Df	Sum Sq	Mean Sq	F value	P value
2	13.04	6.52	0.42	0.66

3.6 Differentially Abundant Genera Among the Study Groups

Differentially abundant genera were identified using MaAsLin analysis applied to CLR-transformed data, setting FDR cut off to 0.25. The non-diseased sites in peri-implantitis patient showed significantly higher abundances of the genera *Atopobium, Olsenella, Veillonella,* and *Actinomyces* compared to the control sites in non-peri-implantitis patient, while the latter group had higher abundance of the genus *Lautropia* (Figure 12).

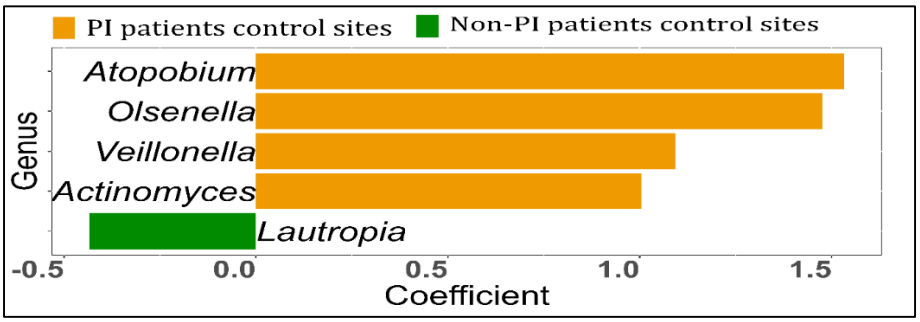

Figure 12. Differentially Abundant Genera Between the Control Sites in the Peri-Implantitis and Non-Peri-Implantitis Patients. Significant differences were identified using MaAsLin analysis applied to CLR-transformed data (FDR ≤0.25).

The disease sites in peri-implantitis patient showed higher abundance of *Olsenella, Atopobium*, and *Actinomyces* compared to the control sites in non-peri-implantitis patient, but also higher abundances of *Mogibacterium and Peptostreptococcus.* Among all genera, *Olsenella* showed the strongest association with the diseased sites, and *Brevundimonas* was associated with the non-peri-implantitis patient control sites (Figure 13)

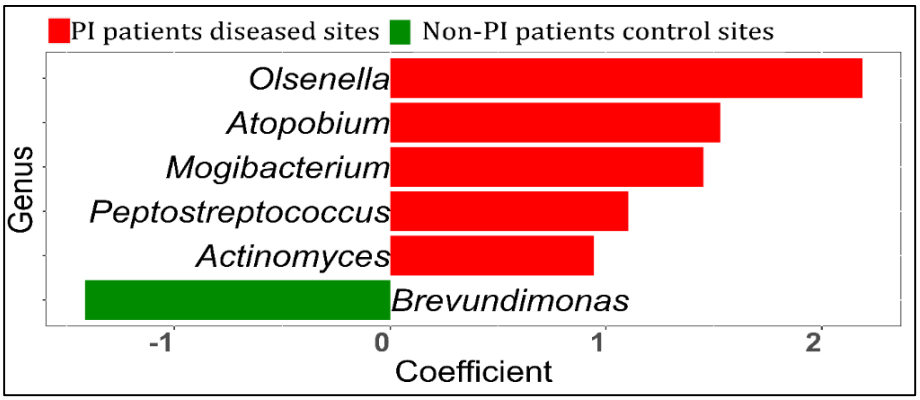

Figure 13. Differentially Abundant Genera Between the Non-Peri-Implantitis Patients Control Sites and Peri-Implantitis Patients Diseased Sites. Significant differences were identified using MaAsLin analysis applied to CLR-transformed data (FDR ≤0.25).

At a FDR threshold of ≤ 0.25, there were no significant differences between the control and diseased sites in peri-implantitis patient. However, using nominal P-value≤ 0.05, some trends were identified. These included detecting the genus *Porphyromonas*

in higher abundance in the diseased sites, and the genus *Brevundinomonas* more enriched in the peri-implantitis patients control sites (Figure 14).

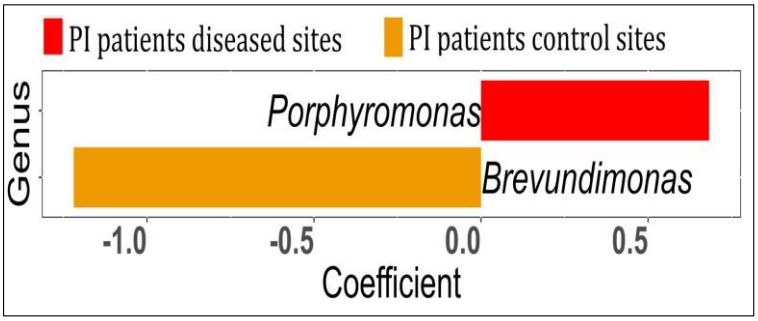

Figure 14. Trends of Differences in Genera Between the Control and Diseased Sites in Peri-Implantitis Patients. MaAsLin analysis was performed on CLR-transformed data, accounting for mixed effect. There were no significant differences at FDR≤ 0.25, but trends at nominal P-value are presented (P<0.05).

3.7 Differentially Abundant Species Between the Study Groups

Differentially abundant species were identified using MaAsLin analysis applied to CLR-transformed data, setting FDR cutoff to 0.25. There were significantly higher abundance of the following species in the diseased sites in peri-implantitis patients compared to the control sites in non-peri-implantitis patients: *Olsenella uli, Actninomyces sp._oral_taxon _525, Atopobium_sp._oral_taxon_199* and *Peptostreptococcus_stomatis.* On the other hand, *Brevundimonas_diminuta* was over-represented in the control sites (Figure 15).

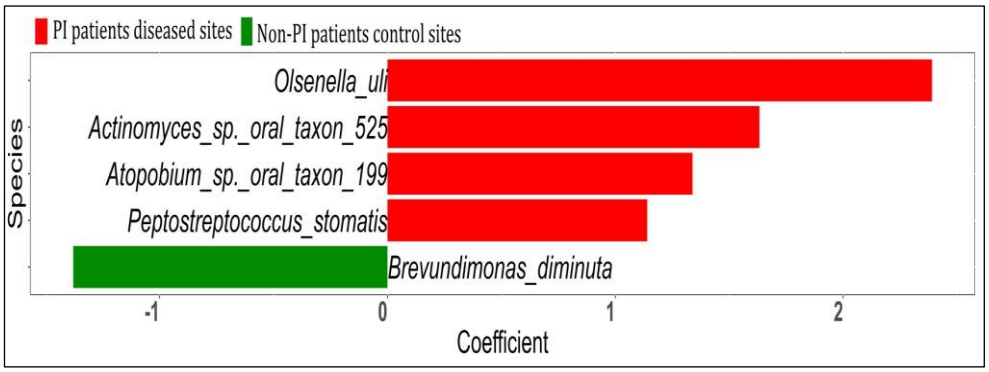

Figure 15. Differentially Abundant Species Between the Non-Peri-Implantitis Patients Control and Peri-Implantitis Patients Diseased Sites. Significant differences were identified using MaAsLin analysis applied to CLR-transformed data (FDR≤0.25).

Comparison of the control sites between the groups showed that the over-represented species were *Actinomyces_sp._oral_taxon_180* and *Veillonella_parvula* in the peri-implantitis patients' group, and *Actinomyces_massiliensis* in the non-peri-implantitis group (Figure 16).

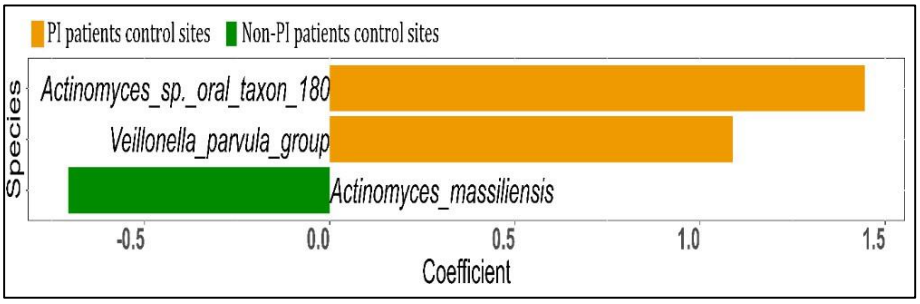

Figure 16. Differentially Abundant Species Between the Control Sites in the Peri-Implantitis and the Non-Peri-Implantitis Patients. Significant differences were identified using MaAsLin analysis applied to CLR-transformed data (FDR ≤0.25).

No significant differences at FDR≤ 0.25 were found between the diseased and control sites in peri-implantitis patients. However, at nominal P-value ≤0.05, *Porphyromonas* endodontalis showed a pattern of being more enriched in diseased sites, and the species *Veillonella_parvula_group, Actinomyces_naeslundii* and

Brevudimonas_diminuta were over-represented in the control sites in peri-implantitis patients (Figure 17).

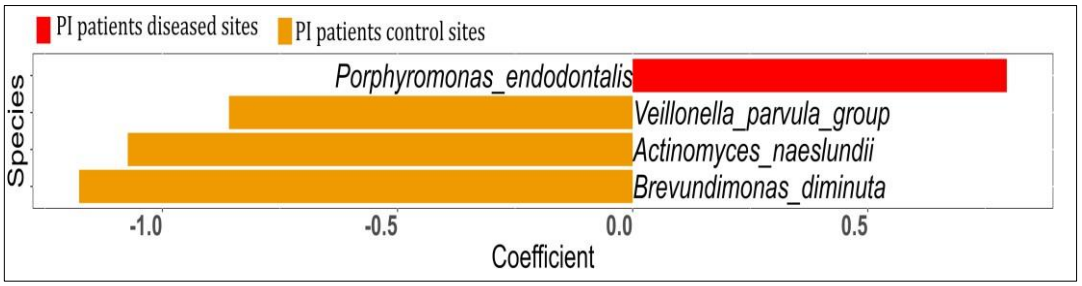

Figure 17. Trends of Differences in Species Between the Peri-Implantitis Patients Diseased and Control Sites. Significant differences were identified using MaAsLin analysis applied to CLR-transformed data and accounting for mixed effect. No significant difference at FDR≤ 0.25, but trends at nominal P-value are presented ($P<0.05$).

CHAPTER 4

DISCUSSION

The microbiome of the peri-implant tissues has been extensively studied. Findings suggest that its composition is somewhat different than that of subgingival sites, and insofar as for peri-implantitis the microbiome has been described as a mixed infection comprising periodontopathogens and other uncommon bacterial species (Lafaurie et al. 2017, Sahrmann et al. 2020).

The advent of novel open-ended microbial detection techniques has provided a better insight into microbial etiology and the pathogenesis of peri-implantitis. However, it is unclear if patients residing in different geographic regions may present different microbial profiles. In this study, we used the 16S rRNA sequencing technique to investigate the microbial profiles of healthy and diseased peri-implant sites at the phylum, genus, and species levels in a group of Moroccan patients. We hypothesized that a distinct microbiome may be present in these subjects, and that this may be one of the factors contributing to a relatively high prevalence of peri-implantitis among Moroccan patients which has been reported recently (Kissa, 2021).

There are diverse microenvironments in the oral cavity which may influence the colonization of microorganisms at specific oral sites, and these are influenced by various factors, such as the surface characteristics of the substrate, gradients of oxygen and nutrients, proximity to salivary glands, and other factors (Proctor & Relman, 2017). Microorganisms are continuously shed into saliva and distributed throughout the oral cavity. These microorganisms are detected at various oral sites, although higher relative abundance is usually present where the proper niche belongs (Baker, 2024).

In this study we used the 16S rRNA sequencing technique that identifies a nucleotide sequence of the conserved 16S rRNA gene, which contains approximately

1,500 base pairs belonging to the 30S ribosomal subunit. (Srinivasan, 2015). This technique has several advantages, but also some challenges. The challenges of using this technique include: low taxonomic resolution in the 16S rRNA reads (not ideal for detection of certain species and strains), complex bioinformatic analysis of the results that needs specific training in use of bioinformatic software like FastQC, Pear, mothur, qiime2, expensive laboratory equipment, maintenance, reagent procurement, limiting sample quantity for repeated testing or conducting antibiotic sensitivity testing, and varied microbiology laboratory protocols requiring validation (Muhamad Rizal, 2020). Despite these limitations, 16S rRNA sequencing technique has the following advantages: identification of unculturable and fastidious bacteria, shorter and predictable turn-around-time with streamlined identification protocol, accurate results, data portability, and technology transition readiness (Muhamad Rizal, 2020) .

In this study population we found similar bacterial species richness (Chao and Shannon's alpha diversity index) among the three study groups. In contrast, systematic reviews have reported that peri-implantitis sites have higher bacterial diversity than healthy implant sites (de Melo, 2020; Lafaurie, 2017).

With regards to the mean microbial profiles at the phylum level in the present Moroccan patients, Actinobacteria, Firmicutes and Proteobacteria were the main phyla in all three groups. These results for the healthy sites are consistent with the findings of (Sanz-Martin, 2017). In contrast, some studies reported a higher abundance of Actinobacteria in peri-implantitis sites compared to healthy implants (Gao, 2018; Polymeri, 2021). Yet, other studies reported that Firmicutes is associated with both diseased as well as healthy implant sites (Al-Ahmad, 2018; Zheng, 2015).

Actinomyces was the most abundant genus in the present Moroccan patients. Similar findings have been reported in Japanese and Brazilian patients (Pallos, 2021; Tamura, 2013) .

At the species level, *O. uli., P. endodontalis and P. stomatitis* were found at higher level in peri-implantitis sites compared to control sites, in both peri-implantitis and non-peri-implantitis patients. A study in a Korean population showed similar results of a higher relative abundance of *Olsenella* species in sites with peri-implantitis than in periodontitis (Kim, 2023).

Notably, the presence of *B. diminuta* species in the control sites in this study population is a novel finding and has not been reported previously. *B. diminuta* is a gram-negative, aerobic bacterium, and has previously been reported in blood and urine samples, and may be associated with nosocomial infections in immunocompromised patients (Lupande-Mwenebitu, 2021). There have also been a few reports of detecting this species in polluted environments and urban streams in South Africa and Nigeria (Lukhele, 2021; Timilehin, 2019). Occurrence of *Veillonella spp.* in non-peri-implantitis control sites agrees with previously published studies.(Sanz-Martin, 2017; Tamura, 2013). The association of the red complex pathogens with peri-implantitis has been well documented in the literature (Rajasekar & Varghese, 2023). However, in the present study we did not detect any of the red complex species *P. gingivalis, T. denticola, or T. forsythia* which have been associated with peri-implantitis in previous studies before the availability of open-ended microbial detection techniques. The different distribution among the varied patient groups at the phylum, genus and species levels could be attributed to the diversity of the population studied, genetic factors, sample size, experimental setups, healthcare systems, and the brand, material or design of the dental implants used in this patient population.

This is the first study that analyzed the microbial makeup of peri-implant tissues of Moroccan patients. Although, this study detected only a few of the bacterial species that were reported in previous studies, the pathogenic potential of the taxa identified cannot be ignored. According to the keystone pathogen hypothesis, certain low-abundance microbial pathogens can orchestrate inflammatory disease by remodeling a benign microbiota into a dysbiotic one (Hajishengallis, 2012; Sanz-Martin, 2017). Moreover, certain bacteria may be detected in diseased as well as healthy sites, which may be related to the type of strain and diversity in the virulence within the population structure of the specific pathogen. This might also justify the molecular and functional characterization of the key virulence factors produced by the keystone pathogens. Certain disease modifiers, such as genetic and environmental factors may also affect susceptibility or resistance to peri-implant disease. Identification of such modifiers in the Moroccan population may be challenging and may also be comprised of certain protective members of the microbiota that balance out the effect of keystone pathogen, hyporesponsive polymorphisms that control inflammation, or polymorphisms that mitigate microbial immune evasion (Hajishengallis, 2012; Hajishengallis & Lambris, 2012) .

While there is evidence that the peri-implant microbiome is distinct from the periodontal infection; (Kotsakis & Olmedo, 2021), the bacterial infection affects the implants secondarily after the rejection reaction by the immune system is triggered. Some local bacterial reactions might occur due to leakage from adjacent implant abutment connections. The process of implant integration is an “immunoinflammatory process” instead of only an “inflammatory process” (Albrektsson, 2022). For the Moroccan patients, inadequate plaque control, peri-implant inflammation, history of previous implant failures, and pain/discomfort at the implant sites were significantly

associated with increased probing depths and reduced bone levels as reported in previously published study (Kissa, 2021).

To conclude, this first study on Moroccan patients revealed a distinct microbiome in association with peri-implantitis. *Brevundimonas diminuta* showed the strongest association with healthy peri-implant sites while *Olsenella uli was* on the top of species enriched in diseased peri-implant sites.

In the future, studies with a larger sample size and diverse populations should be conducted to better understand the microbial features. Techniques like Shotgun metagenomics may be used to dive deeper into the strain-level microbial analysis. Incorporating patient data like disease status, probing depths, and implant surface structure will aid in co-relating the metadata with microbial profiles. Considering immune response biomarkers among the peri-implant patients in Morocco may help further understand their higher prevalence of peri-implantitis.

BIBLIOGRAPHY

Al-Ahmad, A., Muzafferiy, F., Anderson, A. C., Wölber, J. P., Ratka-Krüger, P., Fretwurst, T., . . . Hellwig, E. (2018). Shift of microbial composition of peri-implantitis-associated oral biofilm as revealed by 16S rRNA gene cloning. *J Med Microbiol, 67*(3), 332-340. doi:10.1099/jmm.0.000682

Al-Hebshi, N. N., Nasher, A. T., Idris, A. M., & Chen, T. (2015). Robust species taxonomy assignment algorithm for 16S rRNA NGS reads: application to oral carcinoma samples. *J Oral Microbiol, 7*, 28934. doi:10.3402/jom.v7.28934

Al-Hebshi, N. N., Nasher, A. T., Maryoud, M. Y., Homeida, H. E., Chen, T., Idris, A. M., & Johnson, N. W. (2017). Inflammatory bacteriome featuring Fusobacterium nucleatum and Pseudomonas aeruginosa identified in association with oral squamous cell carcinoma. *Sci Rep, 7*(1), 1834. doi:10.1038/s41598-017-02079-3

Albrektsson, T., Tengvall, P., Amengual, L., Coli, P., Kotsakis, G. A., & Cochran, D. (2022). Osteoimmune regulation underlies oral implant osseointegration and its perturbation. *Front Immunol, 13*, 1056914. doi:10.3389/fimmu.2022.1056914

Asadzadeh, N., Naderynasab, M., Fard, F. G., Rohi, A., & Haghi, H. R. (2012). The microbiology of the peri-implant sulcus following successful implantation of oral prosthetic treatments. *Indian J Dent Res, 23*(6), 753-757. doi:10.4103/0970-9290.111253

Baker, J. L., Mark Welch, J. L., Kauffman, K. M., McLean, J. S., & He, X. (2024). The oral microbiome: diversity, biogeography and human health. *Nat Rev Microbiol, 22*(2), 89-104. doi:10.1038/s41579-023-00963-6

Belibasakis, G. N., & Manoil, D. (2021). Microbial Community-Driven Etiopathogenesis of Peri-Implantitis. *J Dent Res, 100*(1), 21-28. doi:10.1177/0022034520949851

Berezow, A. B., & Darveau, R. P. (2011). Microbial shift and periodontitis. *Periodontol 2000, 55*(1), 36-47. doi:10.1111/j.1600-0757.2010.00350.x

Berglundh, T., Armitage, G., Araujo, M. G., Avila-Ortiz, G., Blanco, J., Camargo, P. M., . . . Zitzmann, N. (2018). Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol, 89 Suppl 1*, S313-s318. doi:10.1002/jper.17-0739

Botero, J. E., González, A. M., Mercado, R. A., Olave, G., & Contreras, A. (2005). Subgingival microbiota in peri-implant mucosa lesions and adjacent teeth in partially edentulous patients. *J Periodontol, 76*(9), 1490-1495. doi:10.1902/jop.2005.76.9.1490

Chun Giok, K., & Menon, R. K. (2023). The Microbiome of Peri-Implantitis: A Systematic Review of Next-Generation Sequencing Studies. *Antibiotics (Basel), 12*(11). doi:10.3390/antibiotics12111610

da Silva, E. S., Feres, M., Figueiredo, L. C., Shibli, J. A., Ramiro, F. S., & Faveri, M. (2014). Microbiological diversity of peri-implantitis biofilm by Sanger sequencing. *Clin Oral Implants Res, 25*(10), 1192-1199. doi:10.1111/clr.12231

de Melo, F., Milanesi, F. C., Angst, P. D. M., & Oppermann, R. V. (2020). A systematic review of the microbiota composition in various peri-implant conditions: data from 16S rRNA gene sequencing. *Arch Oral Biol, 117*, 104776. doi:10.1016/j.archoralbio.2020.104776

Deo, P. N., & Deshmukh, R. (2019). Oral microbiome: Unveiling the fundamentals. *J Oral Maxillofac Pathol, 23*(1), 122-128. doi:10.4103/jomfp.JOMFP_304_18

Diaz, P., Gonzalo, E., Villagra, L. J. G., Miegimolle, B., & Suarez, M. J. (2022). What is the prevalence of peri-implantitis? A systematic review and meta-analysis. *BMC Oral Health, 22*(1), 449. doi:10.1186/s12903-022-02493-8

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics, 27*(16), 2194-2200. doi:10.1093/bioinformatics/btr381

Fragkioudakis, I., Tseleki, G., Doufexi, A. E., & Sakellari, D. (2021). Current Concepts on the Pathogenesis of Peri-implantitis: A Narrative Review. *Eur J Dent, 15*(2), 379-387. doi:10.1055/s-0040-1721903

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., & Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl Environ Microbiol, 74*(8), 2461-2470. doi:10.1128/AEM.02272-07

Gao, L., Xu, T., Huang, G., Jiang, S., Gu, Y., & Chen, F. (2018). Oral microbiomes: more and more importance in oral cavity and whole body. *Protein Cell, 9*(5), 488-500. doi:10.1007/s13238-018-0548-1

Gürlek, Ö., Gümüş, P., Nile, C. J., Lappin, D. F., & Buduneli, N. (2017). Biomarkers and Bacteria Around Implants and Natural Teeth in the Same Individuals. *J Periodontol, 88*(8), 752-761. doi:10.1902/jop.2017.160751

Hajishengallis, G., Darveau, R. P., & Curtis, M. A. (2012). The keystone-pathogen hypothesis. *Nat Rev Microbiol, 10*(10), 717-725. doi:10.1038/nrmicro2873

Hajishengallis, G., & Lambris, J. D. (2012). Complement and dysbiosis in periodontal disease. *Immunobiology, 217*(11), 1111-1116. doi:10.1016/j.imbio.2012.07.007

Hashim, D., & Cionca, N. (2020). A Comprehensive Review of Peri-implantitis Risk Factors. *Current Oral Health Reports, 7*(3), 262-273. doi:10.1007/s40496-020-00274-2

Ito, T., Mori, G., Oda, Y., Hirano, T., Sasaki, H., Honma, S., . . . Yajima, Y. (2021). Clinical evaluation of periodontal pathogen levels by real-time polymerase chain reaction in peri-implantitis patients. *Int J Implant Dent, 7*(1), 105. doi:10.1186/s40729-021-00385-0

Kamble, A., Sawant, S., & Singh, H. (2020). 16S ribosomal RNA gene-based metagenomics: A review. *Biomedical Research Journal, 7*(1), 5-11. doi:10.4103/bmrj.Bmrj_4_20

Kim, H. J., Ahn, D. H., Yu, Y., Han, H., Kim, S. Y., Joo, J. Y., . . . Lee, J. Y. (2023). Microbial profiling of peri-implantitis compared to the periodontal microbiota in health and disease using 16S rRNA sequencing. *J Periodontal Implant Sci, 53*(1), 69-84. doi:10.5051/jpis.2202080104

Kissa, J., El Kholti, W., Chemlali, S., Kawtari, H., Laalou, Y., & Albandar, J. M. (2021). Prevalence and risk indicators of peri-implant diseases in a group of Moroccan patients. *J Periodontol, 92*(8), 1096-1106. doi:10.1002/jper.20-0549

Kordbacheh Changi, K., Finkelstein, J., & Papapanou, P. N. (2019). Peri-implantitis prevalence, incidence rate, and risk factors: A study of electronic health records at a U.S. dental school. *Clin Oral Implants Res, 30*(4), 306-314. doi:10.1111/clr.13416

Kotsakis, G. A., & Olmedo, D. G. (2021). Peri-implantitis is not periodontitis: Scientific discoveries shed light on microbiome-biomaterial interactions that may determine disease phenotype. *Periodontol 2000, 86*(1), 231-240. doi:10.1111/prd.12372

Lafaurie, G. I., Sabogal, M. A., Castillo, D. M., Rincón, M. V., Gómez, L. A., Lesmes, Y. A., & Chambrone, L. (2017). Microbiome and Microbial Biofilm Profiles of Peri-Implantitis: A Systematic Review. *J Periodontol, 88*(10), 1066-1089. doi:10.1902/jop.2017.170123

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A, 82*(20), 6955-6959. doi:10.1073/pnas.82.20.6955

Leonhardt, A., Renvert, S., & Dahlén, G. (1999). Microbial findings at failing implants. *Clin Oral Implants Res, 10*(5), 339-345. doi:10.1034/j.1600-0501.1999.100501.x

Lukhele, T., Nyoni, H., Mamba, B. B., & Msagati, T. A. M. (2021). Unraveling bacterial diversity in oil refinery effluents. *Archives of Microbiology, 203*, 1231-1240.

Lupande-Mwenebitu, D., Tshiyongo, R. K., Lunguya-Metila, O., Lavigne, J.-P., Rolain, J.-M., & Diene, S. M. (2021). First Isolation and Clinical Case of Brevundimonas diminuta in a Newborn with Low Birth Weight, in Democratic Republic of Congo: A Case Report. *Medicina, 57*(11), 1227.

Mallick, H., Rahnavard, A., McIver, L. J., Ma, S., Zhang, Y., Nguyen, L. H., . . . Huttenhower, C. (2021). Multivariable association discovery in population-scale meta-omics studies. *PLoS Comput Biol, 17*(11), e1009442. doi:10.1371/journal.pcbi.1009442

McMurdie, P. J., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One, 8*(4), e61217. doi:10.1371/journal.pone.0061217

Muhamad Rizal, N. S., Neoh, H. M., Ramli, R., PR, A. L. K. P., Hanafiah, A., Abdul Samat, M. N., . . . Khor, B. Y. (2020). Advantages and Limitations of 16S rRNA Next-Generation Sequencing for Pathogen Identification in the Diagnostic Microbiology Laboratory: Perspectives from a Middle-Income Country. *Diagnostics (Basel), 10*(10). doi:10.3390/diagnostics10100816

Neilands, J., Wickström, C., Kinnby, B., Davies, J. R., Hall, J., Friberg, B., & Svensäter, G. (2015). Bacterial profiles and proteolytic activity in peri-implantitis versus healthy sites. *Anaerobe, 35*(Pt A), 28-34. doi:10.1016/j.anaerobe.2015.04.004

Pallos, D., Sousa, V., Feres, M., Retamal-Valdes, B., Chen, T., Curtis, M., . . . Shibli, J. A. (2021). Salivary Microbial Dysbiosis Is Associated With Peri-Implantitis: A Case-Control Study in a Brazilian Population. *Front Cell Infect Microbiol, 11*, 696432. doi:10.3389/fcimb.2021.696432

Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., Schloss, J. A., . . . Guyer, M. (2009). The NIH Human Microbiome Project. *Genome Res, 19*(12), 2317-2323. doi:10.1101/gr.096651.109

Polymeri, A., van der Horst, J., Buijs, M. J., Zaura, E., Wismeijer, D., Crielaard, W., . . . Brandt, B. W. (2021). Submucosal microbiome of peri-implant sites: A cross-sectional study. *J Clin Periodontol, 48*(9), 1228-1239. doi:10.1111/jcpe.13502

Proctor, D. M., & Relman, D. A. (2017). The landscape ecology and microbiota of the human nose, mouth, and throat. *Cell host & microbe, 21*(4), 421-432.

Rajasekar, A., & Varghese, S. S. (2023). Bacterial Profile Associated with Peri-Implantitis: A Systematic Review. *J Long Term Eff Med Implants, 33*(3), 9-20. doi:10.1615/JLongTermEffMedImplants.2022044320

Renvert, S., Persson, G. R., Pirih, F. Q., & Camargo, P. M. (2018). Peri-implant health, peri-implant mucositis, and peri-implantitis: Case definitions and diagnostic considerations. *J Periodontol, 89 Suppl 1*, S304-S312. doi:10.1002/JPER.17-0588

Ribeiro, M. C. O., Vargas-Moreno, V. F., Gomes, R. S., Faot, F., Del Bel Cury, A. A., & Marcello-Machado, R. M. (2022). Implant-supported crowns with locking taper implant-abutment connection: A systematic review and meta-analysis. *J Prosthet Dent*. doi:10.1016/j.prosdent.2022.06.005

Sahrmann, P., Gilli, F., Wiedemeier, D. B., Attin, T., Schmidlin, P. R., & Karygianni, L. (2020). The Microbiome of Peri-Implantitis: A Systematic Review and Meta-Analysis. *Microorganisms, 8*(5). doi:10.3390/microorganisms8050661

Sanz-Martin, I., Doolittle-Hall, J., Teles, R. P., Patel, M., Belibasakis, G. N., Hämmerle, C. H. F., . . . Teles, F. R. F. (2017). Exploring the microbiome of healthy and diseased peri-implant sites using Illumina sequencing. *J Clin Periodontol, 44*(12), 1274-1284. doi:10.1111/jcpe.12788

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., . . . Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol, 75*(23), 7537-7541. doi:10.1128/AEM.01541-09

Shaikh, H. F. M., Patil, S. H., Pangam, T. S., & Rathod, K. V. (2018). Polymicrobial synergy and dysbiosis: An overview. *J Indian Soc Periodontol, 22*(2), 101-106. doi:10.4103/jisp.jisp_385_17

Shibli, J. A., Melo, L., Ferrari, D. S., Figueiredo, L. C., Faveri, M., & Feres, M. (2008). Composition of supra- and subgingival biofilm of subjects with healthy and diseased implants. *Clin Oral Implants Res, 19*(10), 975-982. doi:10.1111/j.1600-0501.2008.01566.x

Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S., . . . Lynch, S. V. (2015). Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One, 10*(2), e0117617. doi:10.1371/journal.pone.0117617

Tamura, N., Ochi, M., Miyakawa, H., & Nakazawa, F. (2013). Analysis of bacterial flora associated with peri-implantitis using obligate anaerobic culture technique and 16S rDNA gene sequence. *Int J Oral Maxillofac Implants, 28*(6), 1521-1529. doi:10.11607/jomi.2570

Thanissorn, C., Guo, J., Jing Ying Chan, D., Koyi, B., Kujan, O., Khzam, N., & Miranda, L. A. (2022). Success Rates and Complications Associated with Single Immediate Implants: A Systematic Review. *Dent J (Basel), 10*(2). doi:10.3390/dj10020031

Timilehin, A. A., Olufemi, O. I., & Adeniyi, O. A. (2019). Genotypic characterization of aminoglycoside resistance genes from bacteria isolates in selected municipal drinking water distribution sources in southwestern Nigeria. *Ethiopian journal of health sciences, 29*(3).

Wensel, C. R., Pluznick, J. L., Salzberg, S. L., & Sears, C. L. (2022). Next-generation sequencing: insights to advance clinical investigations of the microbiome. *J Clin Invest, 132*(7). doi:10.1172/jci154944

Xia, Y., Sun, J., & Chen, D. G. (2018). *Statistical Analysis of Microbiome Data with R*: Springer Nature Singapore.

Zhang, J., Kobert, K., Flouri, T., & Stamatakis, A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics, 30*(5), 614-620. doi:10.1093/bioinformatics/btt593

Zheng, H., Xu, L., Wang, Z., Li, L., Zhang, J., Zhang, Q., . . . Chen, F. (2015). Subgingival microbiome in patients with healthy and ailing dental implants. *Sci Rep, 5*, 10948. doi:10.1038/srep10948

APPENDIX A

SCRIPT OF THE COMMANDS USED WITH PEAR SOFTWARE

Pear
Merging paired reads

$ pear -f sampleA_R1.fastq -r sampleA_R2.fastq -n 460 -m 570 -p 0.001 -v 30 -o sampleA.assembled

Where f is the R1 fastq file for sampleA
r is the R2 fastq file for sampleA
n is minimum amplicon length
m is maximum amplicon length
p significance
v is minimum overlap between R1 and R2
o is output file name

APPENDIX B

SCRIPT OF THE COMMANDS USED WITH MOTHUR SOFTWARE

Windows version

mothur v.1.42.0
Last updated: 5/8/19
by
Patrick D. Schloss

Department of Microbiology & Immunology

University of Michigan
http://www.mothur.org

When using, please cite:
Schloss, P.D., et al., Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol, 2009. 75(23):7537-41.

Distributed under the GNU General Public License

Type 'help()' for information on the commands that are available

For questions and analysis support, please visit our forum at https://forum.mothur.org

Type 'quit()' to exit program
[NOTE]: Setting random seed to 19760620.

Interactive Mode

mothur >
trim.seqs(fasta=IMmerged.fasta, qfile=IMmerged.qual, oligos=oligos.oligos, minlength=420, maxambig=0, maxhomop=8, qwindowaverage=30, qwindowsize=50)

summary.seqs(fasta=IMmerged.trim.fasta)

list.seqs(fasta=IMmerged.trim.fasta)

get.seqs(group=IM.groups, accnos=IMmerged.trim.accnos)

count.groups(group=IM.pick.groups)

unique.seqs(fasta=IMmerged.trim.fasta)

count.seqs(name=IMmerged.trim.names, group=IM.pick.groups)

chimera.vsearch(fasta=IMmerged.trim.unique.fasta, count=IMmerged.trim.count_table, dereplicate=t)

classify.seqs(fasta=IMfinal.fasta, count=IMfinal.count_table, reference=HOMDEXTGGNCBI.fasta, taxonomy=HOMDEXTGGNCBI.taxonomy, method=wang, relabund=F, cutoff=80, probs=F)

remove.lineage(fasta=IMfinal.fasta, count=IMfinal.count_table, taxonomy=IMfinal.HOMDEXTGGNCBI.wang.taxonomy, taxon=Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadales_unclassified;Pseudomonadales_unclassified;Pseudomonadales_unclassified;-
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Massilia;-
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter;-
Bacteria;Bacteria_unclassified;Bacteria_unclassified;Bacteria_unclassified;Bacteria_unclassified;Bacteria_unclassified;-
Bacteria;Proteobacteria;Proteobacteria_unclassified;Proteobacteria_unclassified;Proteobacteria_unclassified;Proteobacteria_unclassified;-
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;-
Bacteria;Proteobacteria;Betaproteobacteria;Betaproteobacteria_unclassified;Betaproteobacteria_unclassified;Betaproteobacteria_unclassified;-
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Bradyrhizobiaceae;Bradyrhizobiaceae_unclassified;-
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiales_unclassified;Burkholderiales_unclassified;-
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Moraxellaceae_unclassified;-
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Oxalobacteraceae_unclassified;-
Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;Sphingomonas;-
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;-
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiaceae;Burkholderia;-
Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;Sphingomonadaceae_unclassified;)

count.groups(count=IMfinal.pick.count_table)

remove.groups(count=IMfinal.pick.count_table, fasta=IMfinal.pick.fasta, taxonomy=IMfinal.HOMDEXTGGNCBI.wang.pick.taxonomy, groups=IMD-I

APPENDIX C

SCRIPT OF THE COMMANDS USED WITH MASLIN IN R (DIFFERENTIAL ABUNDANCE ANALYSIS)

```
# Install MaAslin2
if(!requireNamespace("BiocManager", quietly = TRUE))
install.packages("BiocManager")
BiocManager::install("Maaslin2")
install.packages("tidyverse")
#load libraries
library(Maaslin2)
library(data.table)
library(tibble)
library(tidyverse)
input_data = read.table(file = 'species.txt', sep = '\t',  header = TRUE, row.names = 1, stringsAsFactors = FALSE)
input_metadata = read.table(file = 'metadata.txt', sep = '\t',  header = TRUE, row.names = 1, stringsAsFactors = FALSE)
Maaslin2(input_data = input_data, input_metadata = input_metadata, "species", min_abundance = 0.0001,
        min_prevalence = 0.01,
        min_variance = 0.0,
        normalization = "CLR",
        transform = "NONE",
        analysis_method = "LM",
        max_significance = 0.25,
        random_effects = NULL,
        fixed_effects = c("Group1"),
        correction = "BH",
        standardize = FALSE,
        cores = 1,
        plot_heatmap = TRUE,
        plot_scatter = TRUE,
        heatmap_first_n = 50,
        reference = c("Group1,Healthy_Control"))
```

APPENDIX D

SCRIPT OF THE COMMANDS USED WITH R SOFTWARE FOR ALPHA DIVERSITY

```
# analysis of taxonomic data to asses alpha diversity
# Install required packages
install.packages('patchwork')
install.packages('FSA')
#load libraries
library(vegan)
library(phyloseq)
library(tidyverse)
library(patchwork)
library(tibble)
library(tidytext)
library (FSA)

data_otu <- read.csv("OTU_for_alpha.csv", header = TRUE)
data_grp <- read.csv("metadata_SO.csv", header=TRUE, stringsAsFactors = TRUE)
data_taxo <- read.csv("tax_table_all.csv", header = TRUE)

data_otu <- data_otu %>%
tibble::column_to_rownames("otu")
data_taxo <- data_taxo %>%
tibble::column_to_rownames("otu")
data_grp <- data_grp %>%
tibble::column_to_rownames("sampleid")
data_grp$Group1 <- factor(data_grp$Group1, levels = c("Healthy_Control",
"Patient_no_peri-implantitis", "Patient_peri-implantitis") ,ordered = TRUE)

OTU = otu_table(as.matrix(data_otu), taxa_are_rows = FALSE)
SAM = sample_data(data_grp, errorIfNULL = TRUE)
TAX = tax_table(as.matrix(data_taxo))
data_phylo <- phyloseq(OTU, TAX, SAM)

# calculate alpha div indices using vegan package
data_richness <- estimateR(data_otu)
data_evenness <- diversity(data_otu) / log(specnumber(data_otu))
data_shannon <- diversity(data_otu, index = "shannon")
data_alphadiv <- cbind(data_grp, t(data_richness), data_shannon, data_evenness)
write.csv(data_alphadiv, file = "alphadiv.csv")
p1<- ggplot(data_alphadiv, aes(x=Group1, y=S.obs)) +theme_bw() +
geom_boxplot(fill=c("green3","orange", "red")) + theme(axis.text.x=
element_text(size=13, face = "bold", angle=45, vjust=0.5), plot.title =
element_text(size=20, face = "bold", hjust = 0.5)) +
  labs(title= 'Observed species', x= ' ', y= '') +
  geom_point()
p2 <- ggplot(data_alphadiv, aes(x=Group1, y=S.chao1)) + theme_bw()+
```

```
  geom_boxplot(fill=c("green3","orange", "red")) + theme(axis.text.x=
element_text(size=13, face = "bold", angle=45, vjust=0.5), plot.title =
element_text(size=20, face = "bold", hjust = 0.5)) +
  labs(title= 'Chao index', x= ' ', y= '') +
  geom_point()
p3 <- ggplot(data_alphadiv, aes(x=Group1, y=data_shannon)) +theme_bw() +
geom_boxplot(fill=c("green3","orange", "red")) + theme(axis.text.x=
element_text(size=13, face = "bold", angle=45, vjust=0.5), plot.title =
element_text(size=20, face = "bold", hjust = 0.5)) +
  labs(title= 'Shannon index', x= ' ', y= '') +
  geom_point()
(p1 | p2 | p3 )
ggsave("Alphadiv_IM.tiff", width = 12, height = 10, units = c("in"), dpi = 800,
compression = "lzw")
kruskal.test(data_shannon ~ Group1, data = data_alphadiv)
kruskal.test(data_evenness ~ Group1, data = data_alphadiv)
kruskal.test(S.chao1 ~ Group1, data = data_alphadiv)
kruskal.test(S.obs ~ Group1, data = data_alphadiv)

#Posthoc
dunnTest(data_alphadiv$data_shannon, data_alphadiv$Group1, method='by')
```

APPENDIX E

SCRIPT OF THE COMMANDS USED IN R FOR PRINCIPAL COMPONENT ANALYSIS AND PERMANOVA

```
# Install required packages

if (!requireNamespace("BiocManager", quietly = TRUE))
install.packages("BiocManager")
BiocManager::install("phyloseq")
BiocManager::install("microbiome")
install.packages('devtools')
install_github("pmartinezarbizu/pairwiseAdonis/pairwiseAdonis")

library(tidyverse); packageVersion("tidyverse")
library(phyloseq); packageVersion("phyloseq")
library(microbiome); packageVersion("microbiome")
library(vegan); packageVersion("vegan")
library("tibble")
library(tidytext)
library(devtools)
library(pairwiseAdonis)

# load your OTU table
otu_mat <- read.csv("OTU.csv")

tax_mat <- read.csv("tax_table.csv")

samples_df <- read.csv("metadata.csv")


# 1. create a phyloseq object

# define row names for each data frame
otu_mat <- otu_mat %>%
tibble::column_to_rownames("otu")

tax_mat <- tax_mat %>%
  tibble::column_to_rownames("otu")

samples_df <- samples_df %>%
  tibble::column_to_rownames("ID")

# change data frame to matrix
otu_mat <- as.matrix(otu_mat)
tax_mat <- as.matrix(tax_mat)


# transform to phyloseq objects
```

```
OTU = otu_table(otu_mat, taxa_are_rows = TRUE)
TAX = tax_table(tax_mat)
samples = sample_data(samples_df)

mm <- phyloseq(OTU, TAX, samples)
mm # optional


#CLR transform


mm_clr <- microbiome::transform(mm, "clr")

mm_clr <- transform(mm, "clr")# alternatively use this command

# display the original and clr transformed data

otu_table(mm)[1:5, 1:5]

phyloseq::otu_table(mm_clr)[1:5, 1:5]


#PCA via phyloseq
ord_clr <- phyloseq::ordinate(mm_clr, "RDA")

#include label below to identify outliers

phyloseq::plot_ordination(mm, ord_clr,  color="Group3", shape = 'Gender') +
  scale_colour_manual('Group_1', values = c('orange2','red2', 'blue', 'purple'))+
  geom_point(size = 4) +
  theme_bw()+  # optional to change the theme from grey scale to black and white
  stat_ellipse(aes(group = Group3), linetype = 2, linewidth= 1)+
  theme(legend.key.size = unit(1, 'cm'),
        legend.text                    =                    element_text(size=15),
axis.title=element_text(size=14,face="bold"), legend.title = element_text(size = 18) ) +
guides(shape = guide_legend(override.aes = list(size = 5)))


ggsave("pca.tiff", width = 12, height = 9, units = c("in"), dpi = 800, compression =
"lzw" )


#### Optional analyses

#Plot scree plot
phyloseq::plot_scree(ord_clr) +
  geom_bar(stat="identity", fill = "blue") +
  labs(x = "\nAxis", y = "Proportion of Variance\n")

#Examine eigenvalues and % prop. variance explained
```

```
head(ord_clr$CA$eig)

sapply(ord_clr$CA$eig[1:5], function(x) x / sum(ord_clr$CA$eig))


set.seed(1000)


#Generate distance matrix
clr_dist_matrix <- phyloseq::distance(mm_clr, method = "euclidean")

#ADONIS test

c <- adonis2(clr_dist_matrix ~ Group3, data = samples_df)
c <- vegan::adonis2(clr_dist_matrix ~ phyloseq::sample_data(mm_clr)$Group3,
permutations = 1000)
write.table(c, file="Adonis2.csv", sep=",")
p <- pairwise.adonis(clr_dist_matrix,phyloseq::sample_data(mm_clr)$Group3)
write.table(p, file="PairwiseAdonis2.csv", sep=",")

#Dispersion test and plot
dispr <- vegan::betadisper(clr_dist_matrix, phyloseq::sample_data(mm_clr)$Group3)
dispr

#statistical test
a <- anova(dispr)
write.table(a, file="dispersionanova.csv", sep=",")
permutest(dispr)

#pairwise test
HSD <- TukeyHSD(dispr)
write.table(HSD, file="dispersionTukeyHSD.csv")
#plotting
plot(dispr)
boxplot(dispr)
```

APPENDIX F

SCRIPT OF THE COMMANDS USED WITH R SOFTWARE FOR PLOTTING DIFFERENTIALLY ABUNDANT FEATURES

```
install.packages("ggplot2")
library(ggplot2)
library("tidyverse")

# use data.table library for keeping greek letters unchanges
library('data.table')
Sys.setlocale("LC_ALL", "Greek")
options(encoding = "UTF-8")
install.packages("tidytext")
library(tidytext)

## species bar plots healthy vs no pocket
df <- read.table("species_peri-implantitis_vs_HC.txt", header = TRUE, sep = "\t", check.names = FALSE )

df$Disease_status <- factor(df$Disease_status,levels = c('Healthy Control', 'Patient peri-implantitis site'),ordered = TRUE)

ggplot(data = df, aes(x=reorder_within(feature, coef, feature), y= coef, fill = Disease_status)) +
  geom_bar(aes(fill = Disease_status), stat="identity")+
  scale_fill_manual(values=c("green4", "red"))+
  coord_flip() +  # for fliping the plot
  ylab("Coefficient") + xlab("Species") +
  labs( title = "Test plot", subtitle = "Bar plot")+  # Title and subtitle can be added using labs function
  geom_text(aes(y = 0,  label = feature, hjust = as.numeric(coef > 0)), size =9, fontface ="italic") +
  theme(panel.background = element_rect(fill ="white", colour= "black"),
        axis.text.x = element_text(size = 22, face = "bold", hjust = 1),  # angle and hjust for giving any angle and hjust helps adjusting the distance from baseline
        axis.text.y = element_blank(), axis.title.x = element_text(size = 25), axis.title.y = element_text(size = 25),
        legend.text = element_text(size=24), legend.title = element_text(size=24, face ="bold"))


ggsave("species_peri-implantitis_vs_HC.tiff", width = 20, height = 4, units = c("in"), dpi = 800, compression = "lzw")
```

```
# clustered bar plots
df <- read.table("both_species.txt", header = TRUE, sep = "\t", check.names = FALSE
)

df$Group <- factor(df$Group,levels = c('Tumor_vs_HC', 'AdjN_vs_HC'),ordered = TRUE)

## For individual plots
ggplot(data = df, aes(x=reorder_within(feature, coef, feature), y= coef, fill = Group)) +
geom_bar(aes(fill = Group), stat="identity", width=0.7, position=position_dodge(width=0.8)) + # removing position_dodge will result in stacked bars instead of grouped bars
  scale_fill_manual(values=c("red3", "darkblue"), labels=c( 'T_vs_HC', 'AdjN_vs_HC')) +
  coord_flip() +
  ylab("coefficient") + xlab("feature") +
  geom_text(aes(y = 0,  label = feature, hjust = as.numeric(coef > 0)), size =7.8, fontface ="italic") +
  theme(panel.background = element_rect(fill ="white", colour= "black"), axis.text.x = element_text(size = 20, face = "bold"),
        legend.text = element_text(size=24), legend.title = element_text(size=24, face ="bold"),
        axis.text.y = element_blank(), axis.title.x = element_text(size = 25), axis.title.y = element_text(size = 25) ) +
  guides(fill=guide_legend(title="Comparison"))


ggsave("comparison_sample.tiff", width = 18.5, height = 25, units = c("in"), dpi = 800, compression = "lzw")

ggplot(data = df, aes(x=reorder_within(feature, coef, feature), y= coef, fill = Group)) +
geom_bar(aes(fill = Group), stat="identity",width=0.7, position=position_dodge(width=0.8))+
  scale_fill_manual(values=c("red3", "darkblue"), labels=c( 'T_vs_HC', 'AdjN_vs_HC'))+
  coord_flip() +
  ylab("coefficient") + xlab("Genus") +
  geom_text(aes(y = 0,  label = feature, hjust = as.numeric(coef > 0)), size =7, fontface ="italic") + ylim(-12.5,9.5) +
  theme(panel.background = element_rect(fill ="white", colour= "black"), axis.text.x = element_text(size = 20, face = "bold"),
```

```
      axis.text.y = element_blank(), axis.title.x = element_text(size = 25), axis.title.y =
element_text(size = 25) ) +
  guides(fill=guide_legend(title="Comparison")) +
  facet_wrap( ~ Type, scales="free")+
  theme(strip.text = element_text(size=25, face = "bold"), legend.text =
element_text(size=24), legend.title = element_text(size=24, face ="bold"))


ggsave("comparison_both_species.tiff", width = 22.5, height = 16, units = c("in"), dpi
= 800, compression = "lzw")
```

APPENDIX G

RELATIVE ABUNDANCES FOR THE THREE GROUPS AT THE PHYLUM LEVEL

Table 4. The relative abundances for 9 phyla

Phyla	Non-PI patients control sites	PI patients control sites	PI patients disease sites	Over all
Actinobacteria	37%	50%	43%	43%
Firmicutes	28%	34%	40%	35%
Proteobacteria	18%	7%	3%	8%
Bacteroidetes	6%	3%	4%	4%
Fusobacteria	5%	1%	3%	3%
Saccharibacteria	3%	5%	7%	5%
Spirochaetes	2%	0%	0%	1%
Synergistetes	1%	0%	0%	0%
Chloroflexi	0%	0%	1%	0%

APPENDIX H

RELATIVE ABUNDANCES FOR THE THREE GROUPS AT THE GENUS LEVEL

Table 5. The relative abundances for top 14 genera

Genera	Non-PI patients control sites	PI patients control sites	PI patients disease sites	Over all
Actinomyces	26%	37%	22%	28%
Parvimonas	8%	10%	9%	9%
Olsenella	1%	6%	10%	6%
Mogibacterium	6%	3%	8%	6%
Brevundimonas	14%	5%	1%	6%
Streptococcus	3%	5%	7%	5%
Carnobacterium	3%	3%	6%	4%
Propionibacterium	4%	2%	4%	4%
TM7_[G-1]	3%	3%	5%	4%
Abiotrophia	1%	8%	1%	3%
Atopobium	0%	3%	4%	3%
Fusobacterium	5%	1%	2%	2%
Tannerella	2%	2%	1%	2%
Rothia	2%	1%	2%	2%
Others	20%	12%	16%	

APPENDIX I

RELATIVE ABUNDANCES FOR THE THREE GROUPS AT THE SPECIES LEVEL

Table 6. The relative abundances for top 20 species

Species	Non-PI patients control sites	PI patients control sites	PI patients disease sites	Over all
Parvimonas micra	8%	9%	9%	9%
Brevundimonas diminuta	14%	5%	1%	6%
Mogibacterium timidum	6%	3%	7%	5%
Olsenella uli	1%	4%	8%	5%
Carnobacterium divergens	3%	3%	6%	4%
Actinomyces israelii	3%	8%	2%	4%
Abiotrophia defectiva	1%	8%	1%	3%
Actinomyces sp. OT 525	1%	2%	4%	3%
Propionibacterium propionicum	1%	1%	4%	2%
Actinomyces timonensis	2%	2%	2%	2%
Actinomyces sp. OT 169	5%	1%	0%	2%
Actinomyces sp. OT 448	3%	4%	0%	2%
TM7 [G-1] sp. OT 346	1%	1%	3%	2%
F. nucleatum subsp. vincentii	4%	1%	1%	2%
Tannerella forsythia	2%	2%	1%	2%
Actinomyces naeslundii	1%	4%	1%	2%
Atopobium rimae	0%	2%	3%	2%
Actinomyces sp. OT 180	1%	3%	1%	2%
Olsenella sp. OT 807	1%	2%	2%	2%
Actinomyces oricola	1%	0%	3%	2%
Others	40%	35%	41%	