The Role of the Oral Microbiota and Intrinsic Host Factors in Peri-Implant Diseases:

Peri-Implant Mucositis versus Peri-Implantitis

By

Amit Rozenblit

Performed under the supervision of committee members:

Dr. Chrysi Stavropoulou (Advisor)

Dr. Anastasia Kelekis-Cholakis

Dr. Prashen Chelikani

Written report of research project submitted for completion of course DDSS 7220 Essay/Research Project

Division of Periodontics

Department of Dental Diagnostics and Surgical Sciences

Dr. Gerald Niznick College of Dentistry

University of Manitoba

Winnipeg, Manitoba

Abstract

Aim: To characterize and compare the role of the oral microbiota and host biomarkers in patients with peri-implant mucositis and peri-implantitis.

Methods: Patients diagnosed with either peri-implant mucositis (N=13) or peri-implantitis (N=20) were recruited from the Dr. Sam Borden Graduate Periodontics Clinic at the University of Manitoba. Subgingival plaque samples and crevicular fluid samples were collected from diseased implant sites and healthy natural teeth to study the local microbiome and host biomarker profiles, respectively. 16 rRNA and ITS rRNA amplicon sequencing were performed to detect bacterial and fungal species. Eight different cytokines were isolated and analyzed: IL-1β, IL-2, IL-4, IL-6, IL8, IL-10, IFN-γ, TNF-α.

Results: Bacterial differential abundance analysis indicated statistically significant differences in the abundance of 17 different bacterial taxa and species between sites with peri-implant mucositis and peri-implantitis, most notably *Tannerella forsythia* was significantly more abundant at sites with peri-implantitis compared to sites with peri-implant mucositis. Fungal differential abundance analysis indicated that *Malassezia* species were significantly reduced in sites with peri-implant mucositis compared to sites with peri-implantitis. Cytokine analysis indicated no statistically significant differences in the crevicular concentrations of any cytokines analyzed between the peri-implant mucositis and peri-implantitis groups.

Conclusion: Peri-implant mucositis and peri-implantitis have similar microbial communities and cytokine profiles; however, the presence of certain bacterial and fungal species may play a role in the transition between peri-implant diseases.

Keywords: Periodontal disease, peri-implantitis, peri-implant mucositis, biomarkers, microbiome

Introduction

Implant-supported prostheses provide patients with significant advantages over conventional fixed or removable options, and have shown excellent long term success rates.[1] They have quickly evolved from an experimental treatment to the gold standard for predictable replacement of missing teeth in modern dental practice.[1] Dental implant prevalence in the United States was estimated to be roughly 0.7% in 1999 and increased 8-fold to 5.7% in 2016, with a projected prevalence in 2026 of up to 23%, representing a rapidly growing market projected to reach a global market share over $8 billion USD.[2] Despite the widespread use and great success of dental implants as a treatment modality, they are susceptible to numerous biological and mechanical complications that can jeopardize their survival.

Peri-implant mucositis is a condition characterized by inflammation of the peri-implant mucosa without the loss of supporting bone.[3] Clinical diagnosis is based on the presence of bleeding on probing, erythema, edema, possible suppuration, and/or increased probing depths.[4] There is strong evidence from animal and human experimental studies suggesting that plaque is the etiological factor for peri-implant mucositis, while there is limited evidence for non-plaque-induced peri-implant mucositis.[5] Peri-implant mucositis is fairly common, with an estimated prevalence of 30% at the implant level and 47% at the patient level.[6] Peri-implant mucositis is reversible when managed appropriately and is assumed to precede peri-implantitis; however, specific features or conditions characterizing the transition to peri-implantitis have not yet been identified.[3,7]

Peri-implantitis represents a more advanced pathological entity characterized by inflammation of the peri-implant mucosa and progressive loss of supporting bone, and is a major cause of implant failure.[7] Diagnosis is based on similar signs as seen in peri-implant mucositis with the addition of radiographic evidence of marginal bone loss beyond the initial remodeling phase.[7] The estimated prevalence for peri-implantitis is 9% at the implant level and 20% at the patient level.[6] Although various surgical and non-surgical modalities exist to treat peri-implant diseases, they may add significant costs and morbidity to the overall implant treatment and have limited predictability. Early detection and prevention of peri-implant disease is therefore important to minimize the need for costly and unpredictable intervention.

The pathogenesis of peri-implant mucositis and peri-implantitis both involve an inflammatory reaction to local microbial plaque biofilm accumulation.[8] Initially, there is a host response involving the activation of the innate immune system manifesting in recruitment of neutrophils and production of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF- α), in an attempt to contain the microbial infection. If the microbial plaque biofilm is not removed, the initial inflammatory reaction transitions to a more chronic response, characterized predominantly by lymphocytes and plasma cells, which produce a wide range of pro-inflammatory mediators capable of degrading the peri-implant mucosa and bone.[7,8]

The transition from peri-implant health to peri-implant mucositis and then to peri-implantitis can be characterized by a shift in local microbial dynamics.[8] Healthy sites have predominantly gram-positive cocci and facultative anaerobic rods in a stable community that prevents overgrowth of pathogenic species.[8] Sites with peri-implant mucositis exhibit more pathogenic bacteria, including those found in the orange- and red-complexes.[8] Sites with peri-implantitis exhibit significantly higher microbial diversity and high amounts of *P. gingivalis*, *T. forsythia*, *T. denticola*, *F. nucleatum,* and *C. rectus,* among others.[7,8] Certain fungal and viral entities, such as *Candida albicans,* Herpes Simplex Virus and Epstein-Barr Virus, have also been implicated in peri-implant disease; however, there is no direct evidence confirming a direct role in the etiopathogenesis of peri-implantitis.[7-9]

The inflammatory nature of peri-implant diseases suggests that diseased sites would be associated with higher levels of pro-inflammatory cytokines, as detected in the peri-implant crevicular fluid (PICF). Indeed, it has been shown that the levels of Interleukin-1β (IL-1β), Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Interleukin-17 (IL-17), and TNF-α are significantly higher in sites with peri-implantitis compared to healthy implants.[10-11] However, most studies report combined data for peri-implant mucositis and peri-implantitis so there has been no unique biomarker associated with either disease alone.[10-11] Determining a potential biomarker associated more strongly with one disease or the other may allow for the development of new methods to diagnose disease early and implement appropriate management to prevent disease progression.

There remains a gap in the literature regarding which factors may characterize the transition from peri-implant mucositis to peri-implantitis, possibly due to a sparsity of studies that have drawn comparisons between these two stages of peri-implant disease. The aim of this study is to characterize and compare the role of the oral microbiota in patients with peri-implant mucositis and peri-implantitis by determining the composition of the bacteriome and mycobiome isolated from dental plaque on diseased implants. The study also aims to characterize and compare the host innate immune profile isolated from the crevicular fluid around diseased implants.

Methods and Materials

The cross-sectional pilot study was conducted following the protocol approved by the University of Manitoba's Health Research Ethics Boards (HREB HS22900-H2019:229). Patients were recruited from the University of Manitoba, Dr. Gerald Niznick College of Dentistry, Graduate Periodontics Clinic between January 2022 to December 2023 if they met the following inclusion criteria: (1) 18 years or older, (2) presence of at least one implant with peri-implant mucositis (Group PM) or peri-implantitis (Group P) as defined by the American Academy of Periodontology 2017 Classification of Periodontal and Peri-implant Disease and Conditions[4] (3) otherwise healthy periodontium or gingivitis defined by a Periodontal Screening and Recording Score of 0, 1, or 2, and (4) the presence of at least one natural tooth demonstrating clinical gingival health.[12] Patients were excluded from the study if they demonstrated antibiotic use within three months before sample collection, uncontrolled diabetes (defined as HbA1C >7%), daily corticosteroid and/or non-steroidal anti-inflammatory drug intake, or periodontal scaling completed within 30 days prior to sample collection. Patients who were recruited were asked to review and sign an informed consent form to be part of the study.

Sample Collection

Questionnaires regarding general health, dental health and taste preferences were distributed to each participant. To eliminate any residual food debris, each participant rinsed with clean water for 30 seconds 5 minutes before spitting into a sterile 50mL disposable tube until a minimum of 2-3mL of saliva was collected. For each participant, crevicular fluid and subgingival plaque samples were collected from an implant exhibiting either peri-implant mucositis or peri-implantitis and for a control tooth (defined as a periodontally-healthy tooth, preferably a canine) in the same arch. Cotton roll isolation was used to minimize sample contamination prior to inserting Periopaper® (OraFlow, PlainView, New York, NY, USA) into four different sites per control tooth and implant to obtain gingival and peri-implant crevicular fluid samples, respectively. Supragingival plaque was removed using a sterile, sharp titanium curette or sharp stainless steel curette for implants and control teeth, respectively. Subgingival plaque collection was then performed using a separate sterile, sharp titanium curette or sharp stainless steel curette for implants and control teeth, respectively. Plaque samples were dislodged from the curette via agitation for one minute in tubes containing 1mL of sterile RNAprotect Bacteria Reagent (Qiagen, Cat.#76506, Hilden,

Germany). All samples were labeled with the participant ID number, collection date, and group number, and stored at -80°C until analysis.

Sample Analysis

DNA Extraction for 16S and ITS1 rRNA Amplicon Sequencing

Genomic DNA was extracted from subgingival plaque samples using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The V4 hypervariable region of the bacterial *16S rRNA* and the fungal *Internal Transcribed Spacer (ITS)* 1 region was sequenced by Génome Québec Innovation Center (Montreal, Canada), using the Illumina NextSeq PE300. The microbiome data was analyzed using QIIME2 2018.11 (Quantitative Insights into Microbial Ecology).[13,14] The demultiplexed pair-end sequences were filtered and merged using DADA2 implemented in QIIME2 and the table of amplicon sequence variants (ASVs) was obtained.[15] The UNITE database (version 8.2; QIIME developer release) and the Human Oral Microbiome Database (HOMD, version 15.1) were used for the taxonomic assignment, with classify-sklearn naïve Bayes taxonomy classifier.[16, 17] The data was imported into R using the R package "qiime2R" (version 0.99.6) and additional filtering was performed using "phyloseq" (version 1.46.0) to remove ASVs present only in 1 sample and samples with less than 100 reads.[18,19]

Cytokine Analysis from Peri-Implant and Gingival Crevicular Fluid

Protein extraction from periopaper samples and cytokine analysis for the detection of the following pro- or anti-inflammatory cytokines: Interleukin-1β (IL-1β), Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Tumor Necrosis Factor alpha (TNF-α), and Interferon gamma (IFN-γ) was pursued as follows.

An MSD (Meso Scale Diagnostics, Rockville, Maryland, USA) VPLEX 7-plex custom panel Human Inflammatory Cytokine Kit was used for the detection and quantification of the aforementioned cytokines. Protein extraction from the Periopaper® samples involved incubation in 70 µl of extraction solution (1X PBS, and 0.05% Tween-20) for one hour on ice, followed by a brief centrifugation at 4 ºC.[20, 21] Protein estimation in the samples was analyzed using BioRad DC protein assay and other methods as described previously. [22,23] Then, 50 µl of the supernatant containing equal amount of protein was added directly to the VPLEX 96 well 10 spot plate. The samples and the standard solution were incubated at room temperature for 2 hours on a plate shaker followed by three washes using MSD wash buffer. 25µl of detection antibody mixture was then added into each well and incubated at room temperature for 2 hours. 150µl of read buffer provided by the kit was added, then the data was read using an MSD SECTOR Imager 2400. The detection limits for each cytokine were as follows: IL-1β (0.646-375 pg/mL), IL-2 (0.09-938 pg/mL), IL-4 (0.02-158 pg/mL), IL-6 (0.06-488 pg/mL), IL-8 (0.07-375 pg/mL), IL-10 (0.04-233 pg/mL), and TNF-α (0.04-248 pg/mL), and IFN-γ (0.37-938 pg/mL).

Statistical Analysis

Descriptive analyses were performed to describe the means and the frequency of responses related to each item in the questionnaire. Fisher's Exact test was used to test the differences of categorical data between the groups, and Welch's t-test was performed for continuous data.

The Shannon Index was used for the alpha diversity analysis, using raw count data in phyloseq. Alpha diversity measures were compared between groups using Kruskal-Wallis tests. If a significant difference was observed, a post hoc Dunn's test with Benjamini-Hochberg correction for multiple comparisons was performed**.**

Beta diversity analysis was performed on rarefied ASV table, using weighted UniFrac distances and the permutational analysis of variance (PERMANOVA) test with 999 permutations in the R package "vegan" (adonis function; version 2.6.4). Experimental parameters included sex, diabetes, smoking status, and group (Group_P, Group_PM).[24] The results were visualized using principle coordinate analysis (PcoA) in the R package "ggplot2" (version 3.5.0).[25]

Differential abundance analysis was performed using the DESeq2 negative binomial Wald test, controlling the false discovery rate (FDR) for multiple comparison.[26] Sex, diabetes, and smoking status were also included in the model. FDR adjusted $p < 0.05$ was considered significant.

The missing cytokines values were imputed using the default options in R package "mice" (version 3.16.0). Cytokine analysis involved testing normality with the Shapiro-Wilk test. Since the cytokine data was not normally distributed for all the groups, the differences in concentrations of cytokines between the groups were assessed by the Kruskal-Wallis. The significance level of each analysis was set at 0.05.

Results

Patient Demographics

24 patients completed the study, 13 in group PM and 11 in group P (mean age 69.4, 5 males, 19 females, Table 1). An additional 9 samples were included in the peri-implantitis group from a previous cohort of this larger pilot study for the cytokine analysis.[27] Three patients indicated that they have been diagnosed with Type II Diabetes Mellitus, but all were well controlled (HbA1C < 6.5%). Six patients were former smokers, all of whom quit over 20 years ago. No current smokers were enrolled in the study. There were no statistically significant differences between the groups, except for sex, where there were significantly more females included in the sample.

Cytokine Analysis

No statistically significant differences were identified in the intra-crevicular concentrations of any of the cytokines analyzed between peri-implant mucositis and peri-implantitis groups in implants (Figure 1). When comparing teeth samples in patients between peri-implant mucositis and peri-implantitis, IL-10 exhibited statistically significantly greater concentrations in the peri-implant mucositis group, while there were no statistically significant differences were identified among the other cytokines analyzed (Figure 2).

Bacteriome Analysis

Alpha diversity analysis (Figure 3) indicated a statistically significant difference between subgingival plaque collected from sites with peri-implant disease (both peri-implant mucositis and peri-implantitis) compared with healthy teeth ($p<0.05$). No statistically significant differences were identified for alpha diversity between implants with peri-implant mucositis and peri-implantitis (Kruskal-Wallis $X^2(1) = 0.14$, *P*-value = 0.70). No statistically significant differences were identified for alpha diversity between healthy teeth in patients with peri-implant mucositis and peri-implantitis (Kruskal-Wallis $X^2(1) = 0.3$, *P*-value = 0.57). Beta diversity analysis (Figure 4) indicated no statistically significant difference between groups.

Differential abundance analysis (Figure 5A) indicated statistically significant differences in the abundance of 17 different bacterial taxa and species between implants with peri-implant mucositis and peri-implantitis. *Tannerella forsythia* was significantly more abundant at sites with peri-implantitis compared to peri-implant mucositis ($p<0.05$). When comparing subgingival plaque samples of healthy teeth in patients with peri-implant mucositis and peri-implantitis, relatively fewer bacterial taxa and species exhibited significant differences in their abundance (Figure 5B).

Mycobiome Analysis

After excluding samples with less than 100 reads, the final sample size was 37 for the mycobiome analysis. Alpha diversity analysis (Figure 6) indicated no statistically significant difference between sites with peri-implant mucositis and peri-implantitis (Kruskal-Wallis $X^2(1) = 3.1876$, P-value = 0.0742). Likewise, no statistically significant difference was identified between healthy teeth in patients with peri-implant mucositis and peri-implantitis (Kruskal-Wallis $X^2(1) = 1.3714$, P-value = 0.2416). Beta diversity analysis (Figure 7) indicated no statistically significant difference between groups.

Differential abundance analysis (Figure 8A) indicated a statistically significant relative reduction in the abundance of only *Malassezia sp.* in sites with peri-implant mucositis compared with peri-implantitis ($p<0.05$). When comparing subgingival plaque samples of healthy teeth in patients with peri-implant mucositis and peri-implantitis (Figure 8B), there were statistically significant differences in the relative abundance of *Verrucoconiothyrium prosopidis*, *Malessezia sp.,* and *Candida sp.* ($p<0.05$).

Discussion

The reversible nature of peri-implant mucositis and its eventual transition to peri-implantitis (when left unmanaged) makes early detection of indicators unique to peri-implantitis an attractive goal. This could be especially valuable in borderline clinical situations where the peri-implantitis lesion is not yet radiographically detectable. By comparing the microbiomes, mycobiomes, and cytokine profiles of these two diseases, we sought to elucidate not only the changes as the disease progresses, but also to identify a possible indicator that may be used to assess risk of worsening peri-implant disease.

Cytokines

The results of the cytokine analysis in the PICF of the two diseased groups showed no statistically significant differences in the levels of any cytokines tested. These findings are supported by several systematic reviews and meta-analyses that have included a comparison between these two disease entities; however, there is some evidence showing significantly higher levels of IL-6 in sites with peri-implantitis compared with peri-implant mucositis.[28-31]

Interleukin-6 is a mostly pro-inflammatory cytokine capable of inducing the inflammatory response, stimulating macrophages, increasing protease secretion, suppressing osteoblasts, and promoting osteoclast differentiation.[28,32,33] Interleukin-6 has also been described as one of the links between the innate and the acquired immune responses due to its role in inducing the differentiation of B cells and CD4+ T cells.[31] Based on its ability to promote bone destruction by manipulating the balance of osteoblasts and osteoclasts, it would be reasonable to expect that increased levels of IL-6 would be implicated in the bone destruction seen in peri-implantitis. Higher sample sizes for both diseased groups in the present study may have allowed for more accurate estimates of mean cytokine levels and potentially resulted in a significant difference being seen in IL-6 levels.

Bacteriome

The results of the bacteriome analysis found a significant difference in alpha diversity only when comparing the healthy control teeth to the diseased implants, with no significant difference noted when comparing peri-implant mucositis to peri-implantitis. Similarly, no significant difference was found for beta diversity between any groups. These findings suggest that diseased implants exhibit higher bacterial richness and diversity than healthy teeth, but the diversity between diseased implants are similar. This is in agreement with other studies which have performed similar analyses.[34,35]

Differential abundance analysis determined that sites with peri-implantitis were significantly enriched with various taxa and/or species compared to sites with peri-implant mucositis: *Capnocytophaga sp.*, *Tannerella forsythia*, *Veillonella rogosae*, *Leptotrichia sp., Actinomyces sp., Gemella bergeri, Treponema parvum, Pyramidobacter piscolens,* and *Filifactor alocis*.

Capnocytophaga, Veilonella, Actinomyces, and *Leptotrichia* are generally associated with more stable biofilms seen in healthy sites, but their roles as initial colonizers do not preclude their presence from diseased sites.[8,36] Their greater abundance in sites with peri-implantitis may also be attributed to sampling error or contamination with supragingival plaque.[8,35,36] *Filifactor alocis* is typically found in sites with periodontitis, but is considered to be an uncommon species in the peri-implant microbiome, which speaks to the complexity of the microbial community in peri-implant disease.[8,36] *Pyramidobacter piscolens* is seldom described in the literature, but has been found in association with diseased implants.[37] Although *Gemella bergeri* is considered a normal part of the oral flora and has been implicated by some reports as an opportunistic pathogen associated with endocarditis and brain infections, it is also seldom described in association with peri-implant disease.[38] *Tannerella forsythia* is a member of the red-complex bacteria, which are classically strongly associated with periodontitis. Studies assessing the peri-implant mucositis- and peri-implantitis-associated microbiota have shown that the red complex members are found in higher abundance with both diseases, but are especially higher in sites with peri-implantitis, which was confirmed in this study.[8]

The bacteriomes associated with peri-implant mucositis and peri-implantitis are both very different than the bacteriome associated with peri-implant health, but it has been shown that the bacterial profiles of the two diseased states are not often drastically different from each other.[8,34-36] It is most often reported that sites with peri-implantitis have greater diversity and amounts of the most pathogenic species (such as those in the red complex) that contribute to the degree of dysbiosis. Bacterial diversity and degree of dysbiosis have also been associated with increased probing depths at sites with peri-implantitis, which is supported by the fact that most of the pathogenic bacteria are also anaerobic.[34] In the present study, a range of different severities were included in the peri-implantitis samples and the data were not stratified by probing depth, which may explain why more of the red complex species were not identified as significantly more abundant. A larger sample size for both groups may have also provided a better estimation of the true bacteriome in each group and allowed for more meaningful comparisons.

Mycobiome

The results of the mycobiome analysis found no statistically significant differences in alpha or beta diversity between the diseased groups or when compared to healthy control teeth. Differential abundance analysis showed only one genus, *Malassezia,* was relatively depleted in peri-implant mucositis. Interestingly, *Malassezia* are not generally found intraorally, they are described as inhabiting the skin and generally cause skin-related infections; thus its presence may indicate contamination or suggest that it may act as an opportunistic pathogen.[39] It is difficult to compare these findings with the body of literature as this is an area that remains under-studied. Studies that have assessed the mycobiome of diseased implants have focused on sites with peri-implantitis and most often identified the presence of *Candida*

albicans.[8,40] The mycobiome is a potentially relevant consideration when treating peri-implant diseases due to the fact that *C. albicans* can alter the biofilm with a polymeric substance that may help shield the local community from antimicrobial treatments and host defenses.[8,41]

Limitations

Although the methodology of the present study aimed to reduce the risk of contamination by rinsing with clean water and removing supragingival plaque prior to collecting subgingival plaque samples, it is possible that trace amounts of bacterial and fungal species were displaced subgingivally during this process. The present study followed similar methodology to other studies therefore the risk of contamination should be similar.

Despite using similar methodology as other studies examining the cytokine profiles and microbiomes of peri-implant disease, some of the discrepancies in the results may be attributed to changes in diagnostic criteria since their publication. Previous similar studies that were published prior to the introduction of the AAP 2017 Classification of Periodontal and Peri-implant Diseases and Conditions would have used different criteria for diagnosing peri-implant mucositis and peri-implantitis.[42-44]

Finally, the sample size in the present study was relatively small. Although the present study is a part of a larger pilot study, a larger sample size with an even distribution of samples with peri-implant mucositis and peri-implantitis may have provided more meaningful results.

Future research

Future studies should be done to expand upon the findings of the present study and possibly consider examining the virome as an additional element that may be contributing to the overall complexity of peri-implant disease.

Conclusion

Peri-implant mucositis and peri-implantitis represent two different disease entities but can be thought of as a continuum with similar underlying etiopathogenesis. In marginal cases, it may be difficult to distinguish the two diseases clinically. We found that there were very similar cytokine profiles, bacteriomes, and mycobiomes in these two diseases. Although we were unable to identify any specific biomarkers that could help determine the transition from peri-implant mucositis to peri-implantitis, the findings of this study may still contribute to the overall body of evidence, especially in the under-studied area of the peri-implant mycobiome.

Funding

This study was supported by funding from the MMSF and University internal funds.

Acknowledgements

I would like to express my gratitude to my research committee members for their support and guidance throughout this project: Dr. Chrysi Stavropoulou, Dr. Prashen Chelikani, and Dr. Anastasia Kelekis-Cholakis. I would also like to thank the members of the Chelikani lab – Vivianne Cruz de Jesus, Nisha Singh, Wasif Khan, Ankita Vaishampayan – for their crucial role in analyzing the samples, compiling data, and performing statistical analyses. My coresidents and clinic staff also deserve recognition for assisting with patient recruitment, and operatory setup and takedown. A special thank you to my

colleague and former co-resident Setareh Enghiad, whose efforts laid down the groundwork for this project. Finally, I would like to thank my family for their continued support and editing abilities.

Appendix

Table 1. Characteristics of the study population

	Peri-implant mucositis (*N*=13)	**Peri-implantitis (*N*=11)**	**Total (*N*=24)**	**P-value**
Sex				0.04
F	8 (61.5%)	11 (100%)	19 (79.2%)	
M	5 (38.5%)	0 (0%)	5 (20.8%)	
Age				0.10
Mean (SD)	66.0 (11.99)	73.4 (9.21)	69.4	
Min - Max	41 - 87	64 - 90	41 – 90	
Diabetes				0.58
N	12 (92.3%)	9 (81.8%)	21 (87.5%)	
Y	1 (7.7%)	2 (18.2%)	3 (12.5%)	
Hypertension				1.00
N	7 (53.8%)	6 (54.5%)	13 (54.1%)	
Y	6 (46.2%)	5 (45.5%)	11 (45.9%)	
Arthritis				0.66
N	10 (76.9%)	7 (63.6%)	17 (70.8%)	
Y	3 (23.1%)	4 (36.4%)	7 (29.1%)	
Former smoker				1.00
N	10 (76.9%)	8 (72.7%)	18 (75.0%)	
Y	3 (23.1%)	3 (27.3%)	6 (25.0%)	
Brushing				0.63
Less than twice a day	2 (15.4%)	3 (27.3%)	5 (20.8%)	
Twice a day	11 (84.6%)	8 (72.7%)	19 (79.2%)	
Flossing				0.18
Less than once a day	2 (15.4%)	5 (45.5%)	7 (29.1%)	
At least once a day	11 (84.6%)	6 (54.5%)	17 (70.8%)	
Mouthwash				0.68
N	8 (61.5%)	5 (45.5%)	13 (54.1%)	
Y	5 (38.5%)	6 (54.5%)	11 (45.9%)	
Dry mouth				0.58
N	12 (92.3%)	9 (81.8%)	21 (87.5%)	

Y	1 (7.7%)	2 (18.2%)	3 (12.5%)	
Bleeding gums				0.14
N	12 (92.3%)	7 (63.6%)	19 (79.2%)	
Y	1 (7.7%)	4 (36.4%)	5 (20.8%)	
Burning sensation				1.00
N	12 (92.3%)	10 (90.9%)	22 (91.7%)	
Y	1 (7.7%)	1 (9.1%)	2 (8.3%)	
Bad breath				0.58
N	12 (92.3%)	9 (81.8%)	21 (87.5%)	
Y	1 (7.7%)	2 (18.2%)	3 (12.5%)	

Note: Values are presented as n (%) unless otherwise indicated.

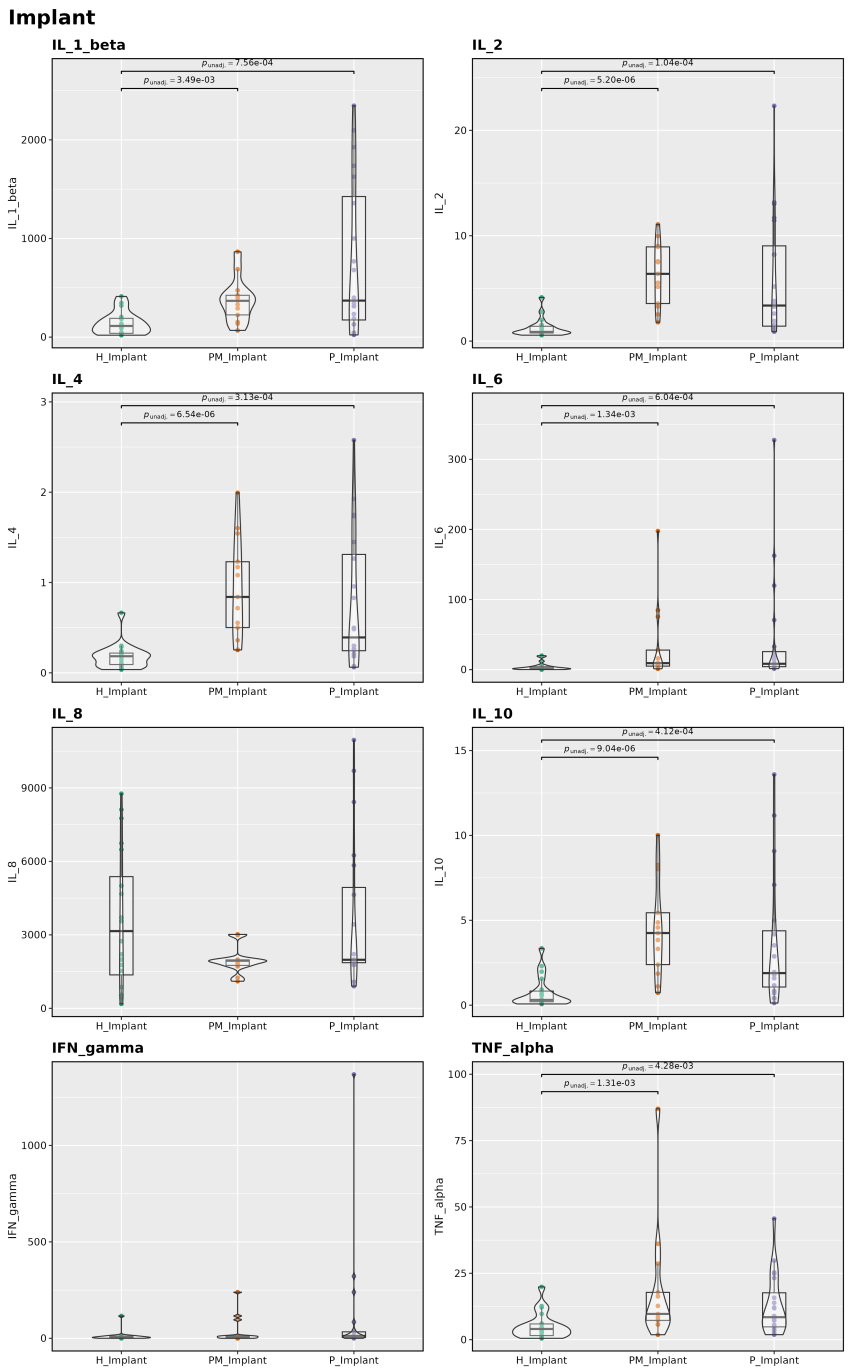

Figure 1 (Implant Samples): Kruskal Wallis test to compare the statistical differences between cytokines in Healthy (H), Peri-implantitis (P) and Peri-implant mucositis (PM). All pairwise tests were performed and p-values for only significant differences are illustrated on the plot.

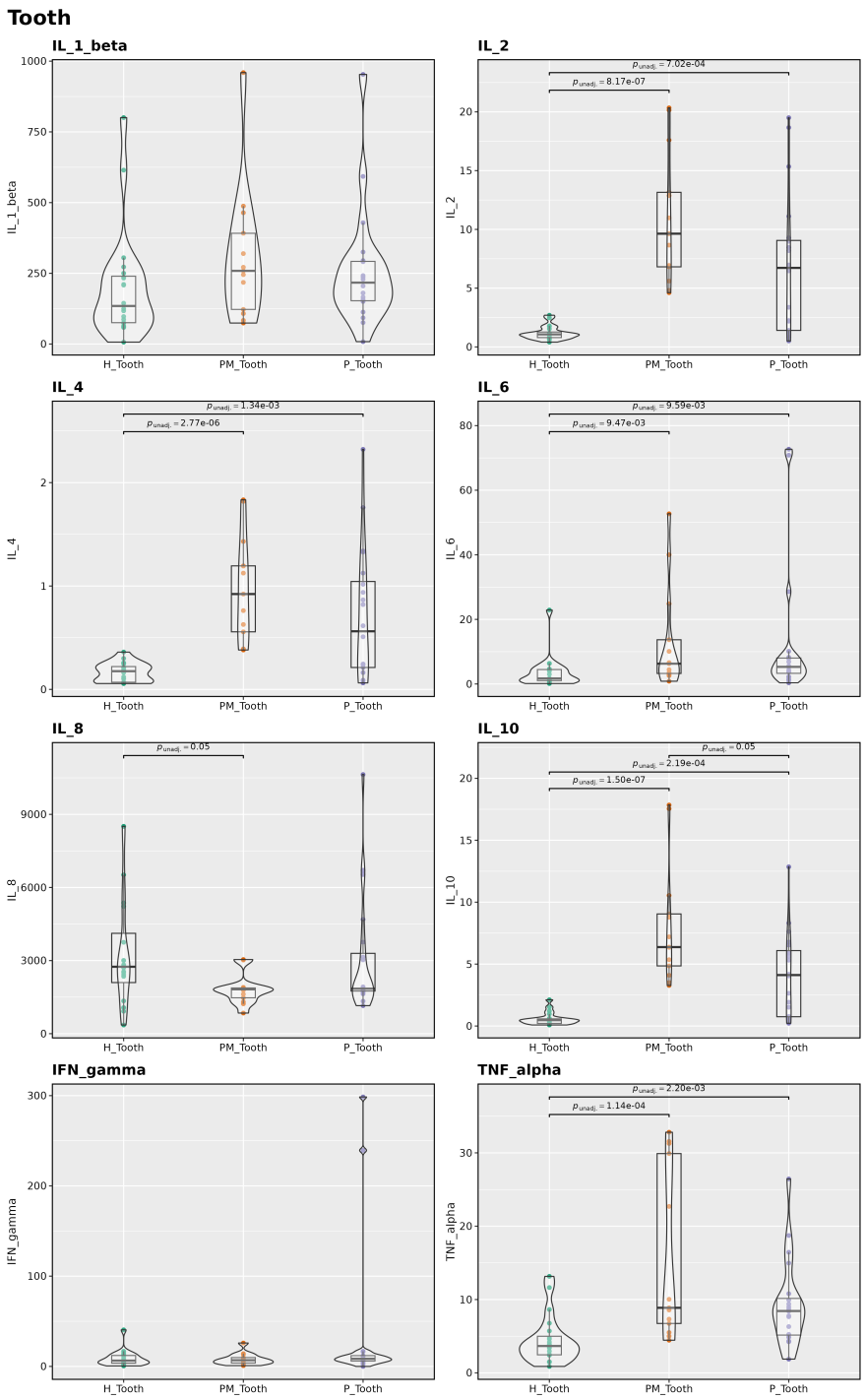

Figure 2 (Tooth Samples): Kruskal Wallis test to compare the statistical differences between cytokines in Healthy (H), Peri-implantitis (P) and Peri-implant mucositis (PM). All pairwise tests were performed and p-values for only significant differences are illustrated on the plot.

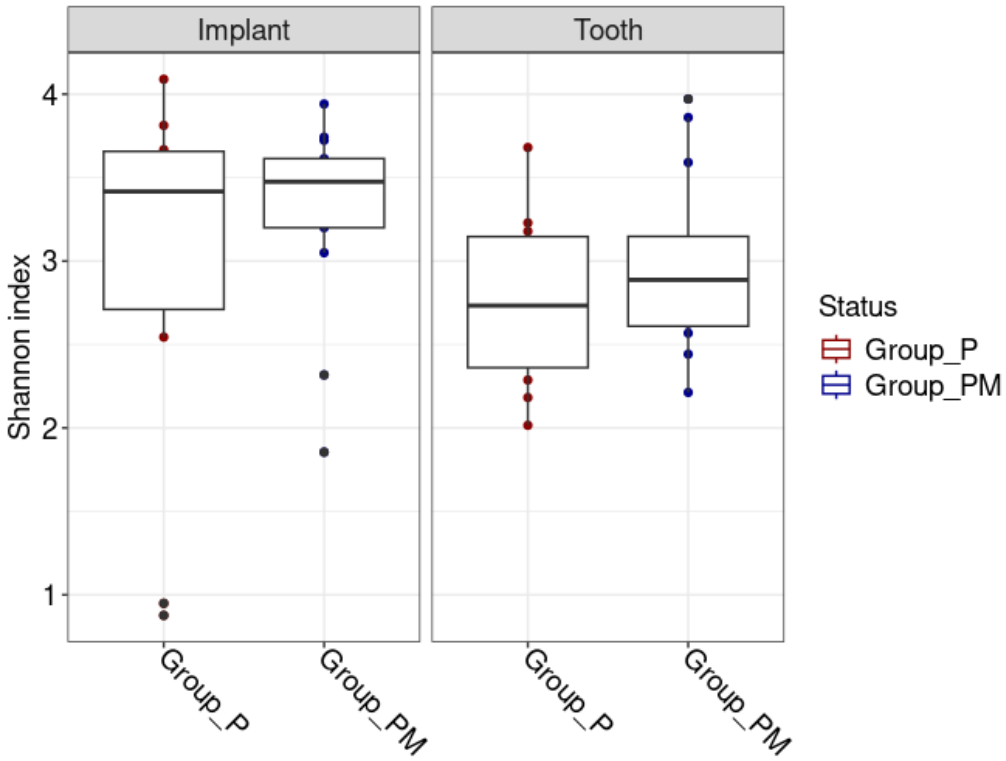

Figure 3: Alpha diversity analysis of subgingival bacteriome. The median Shannon index values were compared across groups using the Kruskal-Wallis test and Dunn's post hoc tests for multiple comparisons with *P*-values adjusted with the Benjamini-Hochberg method. The medians are represented by the lines, boxes represent the interquartile range, and whiskers indicate the range. Overall, a significant difference was observed between the alpha diversity of subgingival plaque from healthy implants and teeth adjacent to implants with peri-implantitis (Implant: Kruskal-Wallis $X^2(1) = 0.14$, *P*-value = 0.70; Tooth: Kruskal-Wallis $X^2(1) = 0.3$, *P*-value = 0.57).

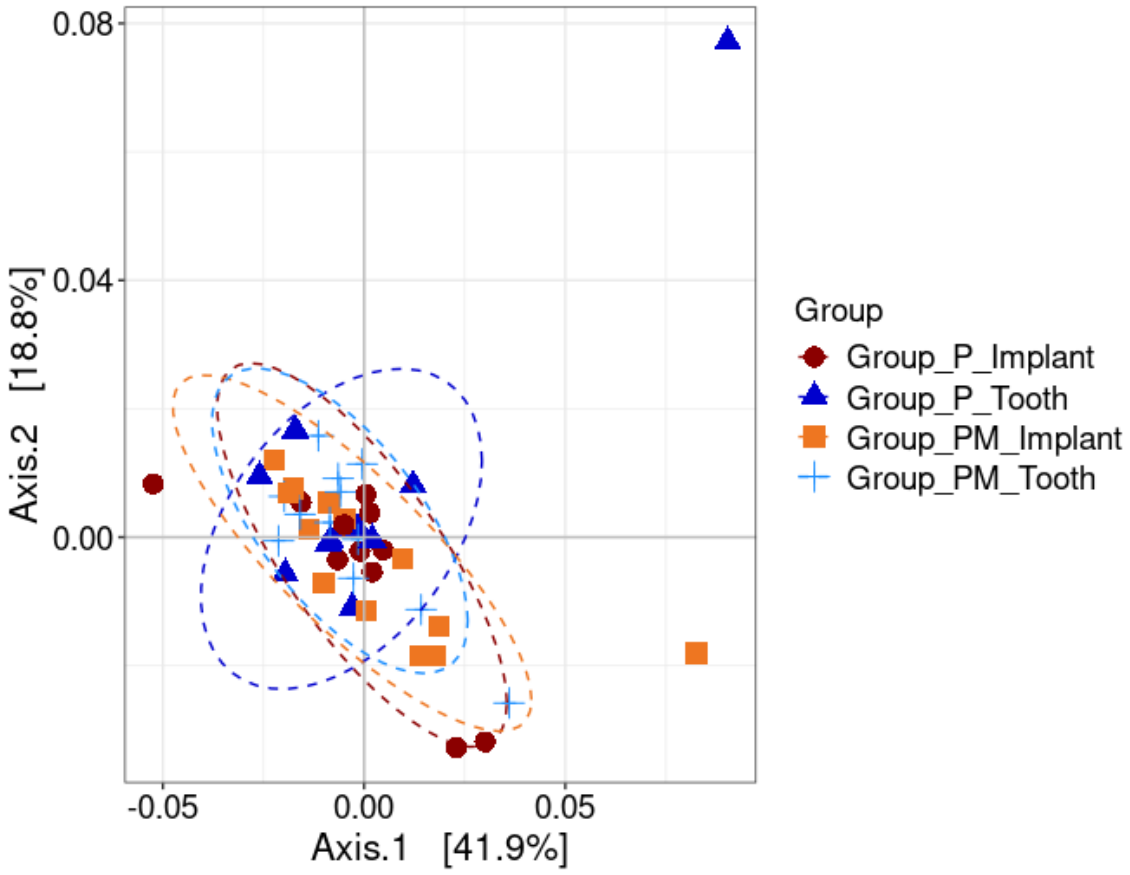

Figure 4: Beta diversity analysis of subgingival plaque. Principal coordinate analysis (PCoA) plot of weighted UniFrac distance. Each data point represents an individual sample. The ellipses represent a 95% confidence level and shape and color represent the groups. Overall, no significant difference was observed between the groups.

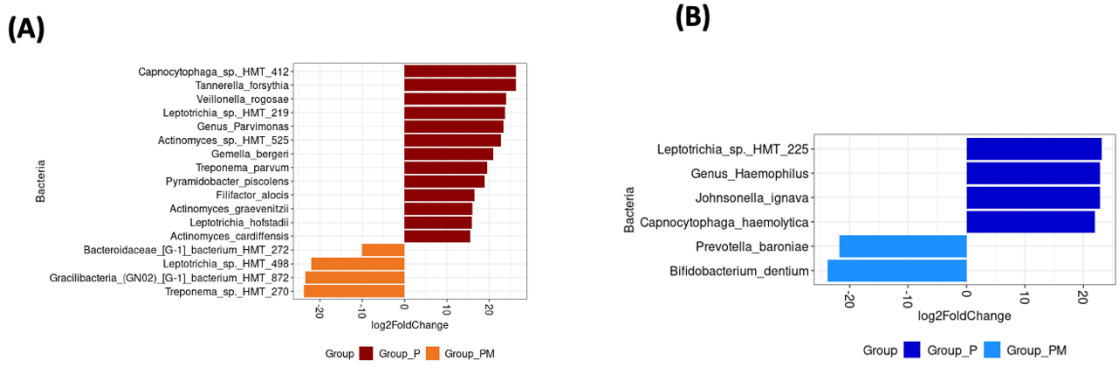

Figure 5: Differential abundance analysis of bacterial taxa. The plot shows the relative fold change in the abundance of bacteria in (A) implants and (B) tooth from the peri-implantitis and peri-implant mucositis groups. The differential abundance was tested using the DESeq2 negative binomial Wald test and only taxa with FDR adjusted $P < 0.05$ are shown.

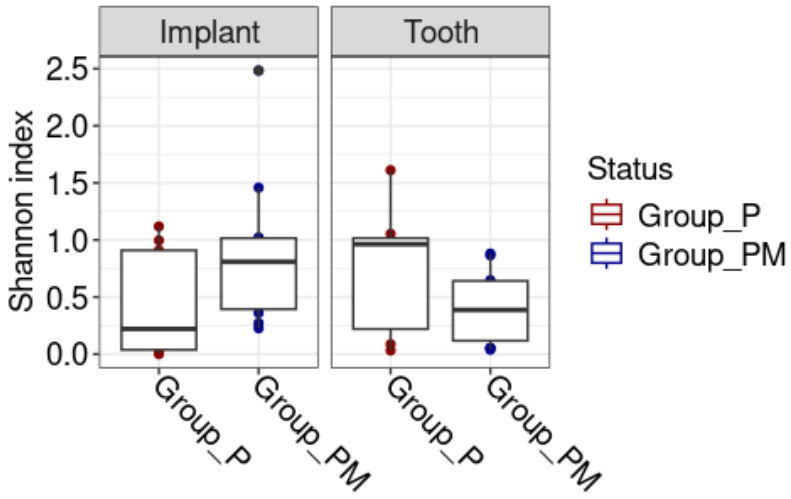

Figure 6: Alpha diversity analysis of subgingival mycobiome. The median Shannon index values were compared across groups using the Kruskal-Wallis test for multiple comparisons with *P*-values adjusted with the Benjamini-Hochberg method. The medians are represented by the lines, boxes represent the interquartile range, and whiskers indicate the range.

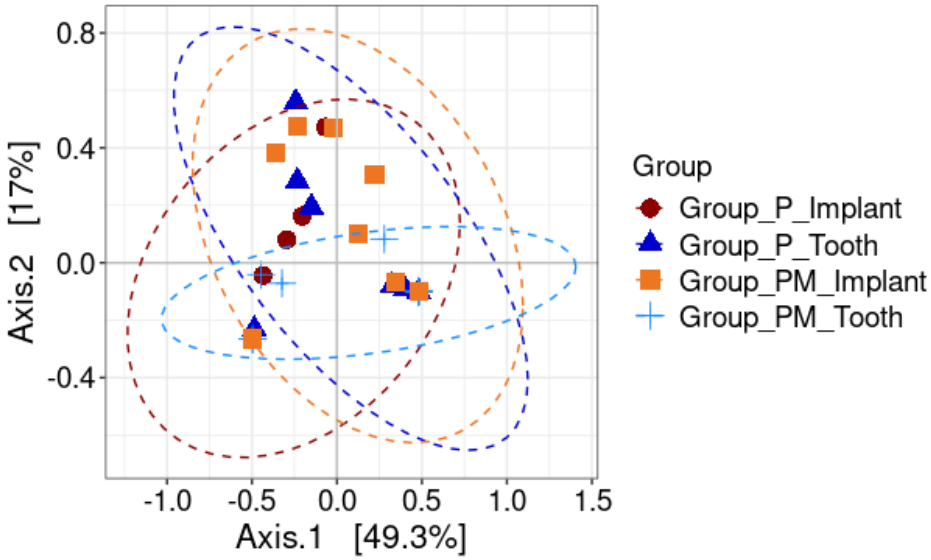

Figure 7: Beta diversity analysis of subgingival plaque. Principal coordinate analysis (PCoA) plot of weighted UniFrac distance. Each data point represents an individual sample. The ellipses represent a 95% confidence level and shape and color represent the groups.

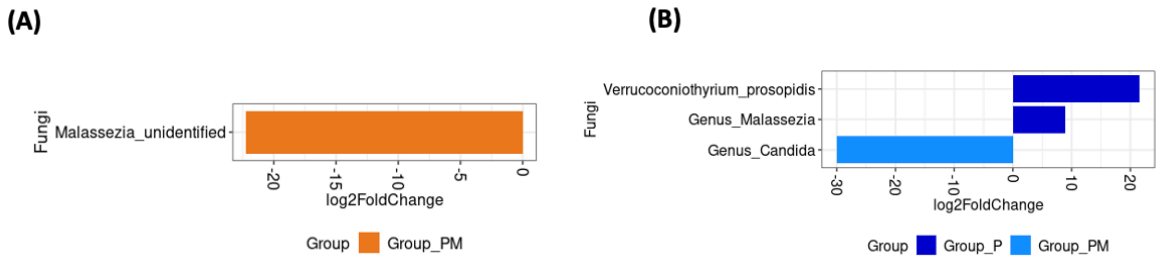

Figure 8: Differential abundance analysis of fungal taxa. The plot shows the relative fold change in the abundance of fungal taxa in (A) implants and (B) tooth of patients with peri-implant mucositis (PM) and peri-implantitis (P). The differential abundance was tested using the DESeq2 negative binomial Wald test and only taxa with FDR adjusted $p < 0.05$ are shown.

References

1. Buser D, Sennerby L, De Bruyn H. Modern implant dentistry based on oseeointegration: 50 years of progress, current trends and open questions. Periodontology 2000. 2017;73(1):7-21.

2. Elani H, et al. Trends in dental implant use in the U.S., 1999-2016, and projections to 2026. J Dent Res. 2018;97(13):1424-30.

3. Heitz-Mayfield L, Salvi G. Peri-implant mucositis. J Periodontol. 2018;89(S1):S257-S266.

4. Renvert S, et al. Peri-implant health, peri-implant mucositis, and peri-implantitis: case definitions and diagnostic considerations. J Periodontol. 2018;89(S1):S304-S312.

5. Berglundh T, et al. 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. 2018;89(S1):S313-S318.

6. Lee CT, et al., Prevalences of peri-implantitis and peri-implant mucositis: systematic review and meta-analysis. J Dent. 2017;62:1-12.

7. Schwarz F, et al. Peri-implantitis. J Periodontol. 2018;89(S1):S267-S290.

8. Di Spirito F, et al. Microbiota of peri-implant healthy tissues, peri-implant mucositis, and peri-implantitis: a comprehensive review. Microorganisms. 2024;12(6):1137.

9. Alqahtani F. Role of oral yeasts in the etiopathogenesis of peri-implantitis: An evidence-based literature review of clinical studies. Archives of Oral Biology. 2020:104650.

10. Kalsi AS, Moreno F, Petridis H. Biomarkers associated with periodontitis and peri implantitis: a systematic review. J Periodontal Impl Sci. 2021;51(1):3-17.

11. Duarte PM, et al. Could cytokine levels in the peri-implant crevicular fluid be used to distinguish between healthy implants and implants with peri-implantitis? A systematic review. J Periodontal Res. 2016;51(6):689-98.

12. American Dental Association, American Academy of Periodontology. Periodontal Screening & Recording: An early detection system. Training kit, 1992.

13. Bolyen E, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology. 2019;37(8):852-7.

14. de Jesus V, et al. Characterization of supragingival plaque and oral swab microbiomes in children with severe early childhood caries. Front Microbiol. 2021;12:683685.

15. Callahan B, et al. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods. 2016;13(7):581–3.

16. Abarenkov K, et al. UNITE QIIME release for Fungi. Version 04. 02. 2020. UNITE Community. 2020.

17. Dewhirst FE, et al. The Human Oral Microbiome. J Bacteriology. 2010;192(19):5002-17.

18. Bisanz J. qiime2R: importing QIIME2 artifacts and associated data into R sessions. Github. 2018. https://github.com/jbisanz/ qiime2R.

19. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE. 2013;8(4): e61217.

20. Sakai A, et al., Profiling the cytokines in gingival crevicular fluid using a cytokine antibody array. J Periodontol. 2006;77(5):856-64.

21. Lee HJ, et al. The subgingival microflora and gingival crevicular fluid cytokines in refractory periodontitis. J Clin Periodontol. 1995;22(11):885-90.

22. Curtis MA, et al. The total protein concentration of gingival crevicular fluid. Variation with sampling time and gingival inflammation. J Clin Periodontol. 1988;15(10):628-32.

23. Xie P, et al. Expression of HMGB1 and HMGN2 in gingival tissues, GCF and PICF of periodontitis patients and peri-implantitis. Braz J Microbiol. 2011;42(3):1213-9.

24. Anderson MJ. Permutation tests for univariate or multivariate analysis of variance and regression. Canadian Journal of Fisheries and Aquatic Sciences. 2001;58(3):626-39.

25. Wickham, H. Ggplot2. New York: Springer-Verlag; 2016.

26. Love, MI, et al. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology. 2014;15(12):550.

27. Enghiad S, et al. Role of oral microbiota and intrinsic host factors in peri-implantitis – a pilot study [Master's thesis]: University of Manitoba; 2022.

28. Chmielewski M., Pilloni A. Current molecular, cellular, and genetic aspects of peri-implantitis disease: a narrative review. Dent J (Basel). 2023;11(5):134.

29. Alassy H, et al. Peri-implantitis diagnosis and prognosis using biomarkers in peri-implant crevicular fluid: a narrative review. Diagnostics (Basel). 2019;9(4):214.

30. Faot F, et al. Can peri-implant crevicular fluid assist in the diagnosis of peri-implantitis? A systematic review and meta-analysis. J Periodontol. 2015;86(5):631-45.

31. Ghassib I, et al. Use of IL-1B, IL-6, TNF-a, and MMP-8 biomarkers to distinguish peri-implant diseases: a systematic review and meta-analysis. Clin Implant Dent Relat Res. 2019;21(1):190-207.

32. Naruishi K., Nagata T. Biological effects of interleukin-6 on gingival fibroblasts: cytokine regulation in periodontitis. J Cell Physiol. 2018;233(9):6393-400.

33. Hienz SA, et al. Mechanisms of bone resorption in periodontitis. J Immunol Res. 2015;2015:615486.

34. Shi Y, et al. Microbial profiles of peri-implant mucositis and peri-implantitis: Submucosal microbial dysbiosis correlates with disease severity. Clin Oral Implants Res. 2022;33(2):172-183.

35. Gazil V, et al. Current Data on Oral Peri-Implant and Periodontal Microbiota and Its Pathological Changes: A Systematic Review. Microorganisms. 2022;10(12):2466.

36. Butera A, et al. Oral Microbiota in Patients with Peri-Implant Disease: A Narrative Review. Appl Sci. 2022;12(7):3250.

37. Yu X, et al. Distributions of Synergistetes in clinically-healthy and diseased periodontal and peri-implant niches. Microb Pathog. 2016;94: 90-103.

38. Lopez EG, Martin-Galiano AJ. The Versatility of Opportunistic Infections Caused by Gemella Isolates Is Supported by the Carriage of Virulence Factors From Multiple Origins. Front Microbiol. 2020;11:524.

39. Saunte DML, et al. Malassezia-associated skin diseases, the use of diagnostics and treatment. Front Cell Infect Microbiol. 2020;10:112.

40. Schwarz F, et al. Real-time PCR analysis of fungal organisms and bacterial species at peri-implantitis sites. Int J Implant Dent. 2015;1(1): 9.

41. Pisano M, et al. Oral candidiasis in adult and pediatric patients with COVID-19. Biomedicines. 2023; 11(3):846.

42. Liu J, et al. High mobility group box 1 protein level as a novel biomarker for the development of peri-implant disease. Sci Rep. 2017;7: 7027.

43. Fonseca FJPO, et al. Cytokines expression in saliva and peri-implant crevicular fluid of patients with peri-implant disease. Clin Oral Implants Res. 2014;25(2):e68-e72.

44. Zhang Y, et al. Increased IL-6 levels in peri-implant crevicular fluid correlate with peri-implantitis. Oral Med Pathol. 2005;10:95-99.