Role of oral microbiota and intrinsic host factors in peri-implantitis – a pilot study

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Abstract word count: 290

Word count:3715

Number of tables/figures: 5

Number of references: 40

Keywords: peri-implantitis, cytokines, dental implants, fungi, bacteria, dental plaque, foreign-body reaction

Knowledge Transfer Statement: The results of our study challenge the concept that peri-implantitis is a bacterial disease with specific bacterial pathogens and strengthens the idea that bacterial invasion happens secondary to bone loss.

ABSTRACT

Introduction: Although many studies have evaluated microbial composition of peri-implant pockets, the role of mycobiome on peri-implantitis is not well established.

Objectives: The aim of this study is to characterize the role of oral microbiota in peri-implant health and disease by determining the composition of the oral bacteriome and mycobiome from subgingival dental plaque, cytokine profile in the crevicular fluid, and to determine if there are any relationships between the oral microbiota, cytokine profile and peri-implant health and disease.

Methods: Gingival crevicular fluid and subgingival plaque samples were collected from 20 periodontally healthy patients with healthy implants [Group H] and 9 periodontally healthy patients with peri-implantitis [Group P]. Eight IL-1β, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10, and TNF-α were analyzed in crevicular fluid samples isolated from both groups. 16S rRNA and ITS rRNA amplicon sequencing was performed to detect bacterial and fungal species.

Results: No statistically significant difference was found in concentrations of IL-2, IL-8, IL-10, and TNF-α. However, statistically significant differences were observed for IL-4 and IL-6. Alpha diversity analysis showed a significant difference between the groups H - Implant vs. P - Tooth and P - Implant vs. P - Tooth. Significant differences in species abundances were also observed between Group H and Group P. *Candida parapsilosis* and *Aspergillus restrictus* were significantly enriched in the Group P.

Conclusions: To the best of our knowledge, this is the first time *Aspergillus restrictus* is related to peri-implantitis. Moreover, we found that the relative abundance of *Candida parapsilosis* is significantly higher in peri-implantitis sites. Our findings further challenge the concept that peri-implantitis is a bacterial disease with specific bacterial pathogens and strengthens the idea that bacterial invasion happens secondary to bone loss because of not very well understood processes such as foreign body reaction.

Background

Peri-implantitis, an inflammatory disease resulting in loss of the supporting structures of dental implants, is the major cause for implant failure. Clinical signs of peri-implantitis include inflammation, bleeding on probing, suppuration, increased probing depth, bone loss, and mobility. A recent systematic review and meta-analysis estimated the prevalence of peri-implant mucositis and peri-implantitis to be 46% and 20% respectively (Lee et al., 2017). With more than 5 million people in North America receiving dental implant treatment annually, an insight into disease mechanisms is of primary importance. Furthermore, there is no standard treatment for peri-implantitis and most of the proposed treatments are challenging and expensive. Therefore, the prevention and early detection of susceptible patients are essential.

Studies have suggested a difference in bacterial composition in peri-implantitis pockets than healthy implant ones. Specifically, five bacterial species have been shown to have a stronger link to peri-implantitis: *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Prevotella intermedia*, and *Campylobacter rectus*. In addition, the cytokine profile of healthy implants is much different from diseased implants. Studies show that the levels of different cytokines such as Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-17 (IL-17), Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-8 (IL-8), Interleukin-10 (IL-10), and Tumor Necrosis Factor alpha (TNFα) increase in the peri-implant crevicular fluid (PICF) (Duarte et al., 2016). Thus far, no unique microbial and inflammatory markers have been identified with either peri-implant mucositis or peri-implantitis. Furthermore, most of the oral microbiome studies to date have focused on the bacteriome and ignored the mycobiome (fungi). A systematic review studying seven clinical studies concluded that although there is still no evidence of direct etiopathogenesis of fungi in peri-implant disease, fungi present more at subgingival microflora of peri-implantitis sites and might contribute to immunologic dysbiosis in peri-implant pocket (Alqahtani, 2020). Additionally, the role of host factors such as taste preferences and how they interact with the oral microbiome (bacteriome and mycobiome) to influence inflammatory responses is not yet characterized.

The aim of this study is to characterize the role of oral microbiota in peri-implant health and disease by determining the composition of the oral microbiome (bacteriome and mycobiome) from subgingival dental plaque, the cytokine profile in the crevicular fluid, and to determine if there are any relationships between the oral microbiota and cytokine profile with peri-implant health and disease. The null hypothesis is that the bacterial, fungal, and cytokine profile of implants and teeth in healthy patient and peri-implantitis patients do not differ.

MATERIALS AND METHODS

Patients of the Dr. Sam Borden Periodontology Clinic, University of Manitoba, Winnipeg, Manitoba were recruited between October 2020 to November 2021 to participate in this cross-sectional pilot study. Patients were included if: 1) they were ≥ 18 years old, 2) they had at least one implant with peri-implantitis **[Group P]** defined according to the 2017 classification (Schwarz et al., 2018) as "1) presence of peri-implant signs of inflammation, 2) radiographic

evidence of bone loss following initial healing, and 3) increasing probing depth as compared to probing depth values collected after placement of the prosthetic reconstruction" (in the absence of previous radiographs, radiographic bone level ≥3 mm in combination with bleeding on probing [BOP] and probing depths [PD] ≥6mm was considered peri-implantitis); or they had at least one implant with healthy peri-implant tissues **[Group H].**

Group H was defined as 1) absence of peri-implant signs of soft tissue inflammation (redness, swelling, profuse bleeding on probing), 2) the absence of further additional bone loss following initial healing; and 3) otherwise healthy periodontium or gingivitis defined as Periodontal Screening and Recording Score of 1 or 2. The patients were excluded from the study if they had used antibiotics within the preceding 3 months; had uncontrolled diabetes (HbA1C > 7%, 3); were on corticosteroids and/or daily non-steroidal anti-inflammatory drugs; had any periodontal scaling at least 30 days prior to sample collection. Patients were asked to sign an informed consent form. The study protocol was approved by the University of Manitoba's Health Research Ethics Board (HREB HS24035 –H2020:296).

Before sample collection, each participant was asked to fill out a questionnaire about their general and oral health and taste preferences. After that, the participant was instructed to rinse their mouth with clean water 5 minutes prior to sample collection. The PICF was collected by isolating the implant crown from saliva by cotton rolls and placing Periopaper® (OraFlow, PlainView, New York, NY, USA) into four different sites around the implant crown for 30 seconds. The strips were then placed in a sterile Eppendorf tube. The same procedure was done for a periodontally healthy control tooth in the same arch for each patient. Subgingival plaque collection was done by scaling the implant with a sharp titanium curette. The plaque was dislodged from the curette by agitation for 1 minute into tubes containing 1mL of DNeasy® PowerSoil® Solution (Qiagen, Cat. # 76506, Hilden, Germany). The same procedure was done for the control tooth in the same patient by a sharp stainless-steel curette. All samples were stored at -80°C until further analysis.

DNA Extraction for 16S and ITS1 rRNA Amplicon Sequencing

Total DNA from subgingival plaque was isolated using DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Isolated DNA was used for paired-end Illumina MiSeq PE250 sequencing of the V4 hypervariable region of the bacterial 16S rRNA gene and the fungal Internal Transcribed Spacer (ITS) rRNA gene at Génome Québec Innovation Center (Montreal, Canada). The 515F/806R primer pair (515F: 5' GTGCCAGCMGCCGCGGTAA 3' and 806R: 5'-GGACTACHVGGGTWTCTAAT 3') was used for the 16S rRNA amplicon sequencing. The ITS1-F/ITS2 primer pair (ITS1-F: 5′ CTTGGTCATTTAGAGGAAGTAA 3′ and ITS2: 5′-GCTGCGTTCTTCATCGATGC 3′) was used for the ITS rRNA amplicon sequencing.

Determination of cytokines from peri implant/ periodontal crevicular fluid (PICF/GCF)

The Periopaper samples were analyzed for the detection of the pro- or anti-inflammatory cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNFα, and Interferon gamma (IFNγ) as follows. A MDS (Rockville, Maryland, USA) VPLEX 7-plex custom panel Human Inflammatory Cytokine Kit was used for the detection and quantification of the above-mentioned cytokines in PICF/GCF samples. For protein extraction Periopaper® samples, they were incubated in 70 µl of extraction solution for one hour on ice, followed by a brief centrifugation at 4 ºC. Protein estimation in the samples was analyzed using BioRad DC protein assay and other methods as described previously (Xie et al., 2011). Then, 50 µl of the supernatant containing equal amount of protein was added to the VPLEX 96 well 10 spot plate. The solution used for the extraction was 1X PBS, and 0.05%Tween-20. The samples and the standard solution were incubated at room temperature for 2 hours on plate shaker followed by 3 washes using MSD wash buffer. Next, a detection antibodies mixture was added in 25 µl into each well and incubated at room temperature for 2 hours. Data were read using a MSD SECTOR Imager 2400 (MSD, USA) after adding 150 µl of read buffer as provided by Kit and the units of the readings was expressed in pg/ml. The detection limit for each of the eight cytokines are as follows: IL-1β (0.646-375 pg/mL), IFN-γ (0.37-938 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), respectively.

Statistical Analysis

The questionnaire data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Fisher's Exact test was used to test the differences of categorical data. T-tests (Aspin-Welch's t-test for unequal variance or Student's t-test) were performed for continuous data. For cytokine analysis, normality was tested with the Shapiro-Wilk test. For normally distributed groups, the differences in concentrations of cytokines between groups were assessed by One-way ANOVA and Tukey test. For groups that were not normally distributed, the Kruskal-Wallis test was used to compare differences. The significance level of each analysis was set at 0.05.

The 16S rRNA and ITS rRNA amplicon sequencing data were analyzed using QIIME2 2018.11 (Quantitative Insights into Microbial Ecology) as described previously (de Jesus et al., 2021). Briefly, DADA2 implemented in QIIME2 was used to filter and merge the demultiplexed pair-end sequences, and to obtain the table of amplicon sequence variants (ASVs) (Callahan et al., 2016). The Human Oral Microbiome Database (HOMD, version 15.1) and the UNITE database (version 8.2; QIIME developer release) were used for the taxonomic assignment of bacteria and fungi, respectively, with classify-sklearn naïve Bayes taxonomy classifier in QIIME2 (Abarenkov et al., 2018). The data were imported into R using the R package "qiime2R" (version 0.99.13) and additional filtering was performed using "phyloseq" (version 1.30.0) to remove ASVs present only in 1 sample and samples with low number of reads (<100) (J, 2018).

Alpha (within samples) diversity Shannon index was calculated using raw count data in "phyloseq". Alpha diversity measures were compared between groups using Kruskal-Wallis tests and post hoc Dunn's test with Benjamini-Hochberg correction for multiple comparisons. Beta (between samples) 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 2.5.6). Experimental parameters included sex and group (Group H - implant, Group H - tooth, Group P - implant, Group P - tooth). The results were visualized using principal coordinate analysis (PCoA) in the R package "ggplot2" (version 3.3.3). A DESeq2 negative binomial Wald test was used to detect differentially abundant taxa at species level between groups, controlling the false discovery rate (FDR) for multiple comparison and adjusting by sex (Love et al., 2014). FDR adjusted $P < 0.05$ was considered significant.

RESULTS

Twenty-nine patients, 20 patients in Group H and 9 patients in Group P, completed the study (mean age 60.48 ± 12.99) (Table 1). There was one smoker (Cannabis and Hukkah) in Group P, and no smokers in Group H. There was no statistically significant difference between the two groups in terms of demographics, medical history, dental history, oral hygiene practices, and taste preferences as measured by the means of a questionnaire (Table 1). Therefore, correlating these parameters to microbiome, mycobiome and cytokine concentrations was not pursued.

Cytokine analysis

Eight cytokines were analyzed in crevicular fluid samples isolated from both groups as shown in Table 2. All cytokines were detected except for IFN-γ. The result suggested that there is no statistically significant difference in concentrations of IL-2, IL-8, IL-10, and TNF-α (p-value < 0.05) between groups. Whereas statistically significant differences are observed for IL-4 and IL-6 (p-value < 0.001 and p-value < 0.04 respectively, Table 2). The difference in concentration of IL-1β between Group H Implant and Group P Implant approached significance (p-value = 0.051). The concentration of IL-4 and IL-6 were statistically higher in Group P implant than Group H tooth. The concentration of IL-4 was also statistically higher in Group P implant than Group P tooth.

Bacteriome (16S rRNA) analysis

A total of 2,405 bacterial ASVs were identified across all 58 samples, after quality control. Among those, 31.1% were from the phylum Firmicutes, 26.1% were Bacteroidetes, 13.9% were Proteobacteria, and the remaining taxa were from at least 10 other phyla. At least 114 genera and 317 species of bacteria were identified in this population.

Alpha diversity analysis showed a significant difference between at least two groups (H(3) = 8.88, $P = 0.03$, Figure 1A). The post hoc Dunn's test showed a significant difference between the groups H - Implant vs. P - Tooth and P - Implant vs. P - Tooth (P.unadj.< 0.05). Only differences between the groups H - Implant and P - Tooth remained significant after adjustment for multiple comparisons (P.adj. = 0.03). Beta diversity analysis showed no significant differences between the groups (pseudo-F = 1.6, $R^2 = 0.08$, $P = 0.15$, PERMANOVA) (Figure 1B). Differential abundance analysis showed that many bacterial species were significantly enriched or depleted in implants with peri-implantitis compared to healthy implants. Interestingly, *P. gingivalis* was more abundant in implants with peri-implantitis (Figure 2A). Significant differences in species abundances were also observed between the subgingival plaque of teeth from patients with peri-implantitis and from individuals with healthy peri-implant tissues (Figure 2B).

Mycobiome (ITS rRNA) analysis

A total of 19 samples did not pass quality control or had low number of reads and were excluded from the fungal analysis. The final sample size per group for the mycobiome analysis was Group H - Tooth: N= 11, Group H - Implant: N=15, Group P - Tooth: N= 6, Group P- Implant: N= 7. Overall, a total of 109 fungal ASVs were identified after quality filtering and 56% of them were from the phylum *Ascomycota*, 32.1% were from the phylum *Basidiomycota*, and 11.9% were unclassified fungi. Across all samples, at least 28 genera and 39 species were identified. No significant differences in fungal alpha diversity (H(3) = 1.48, $P = 0.69$) and beta diversity (pseudo-F = 0.97, $R^2 = 0.08$, $P = 0.49$, PERMANOVA) were observed between the groups (Figures 3A-B). Differential abundance analysis showed that *Candida parapsilosis* and *Aspergillus restrictus* were significantly enriched in the group of implants with peri-implantitis compared to healthy controls (Figure 3C). There were no significant differences between the subgingival plaque of teeth from the healthy and peri-implantitis groups ($P < 0.05$).

DISCUSSION

Finding biological markers of any disease can help targeting the treatment and designing more effective ways to monitor disease activation, progression, and remission. Hence, this study is aimed at finding the cytokine, bacteriome, and mycobiome profile of peri-implantitis versus healthy implants or healthy teeth. The results indicate that there is significantly more IL-4 and IL-6 in peri-implantitis PICF compared to healthy teeth or healthy implants (only IL-4). Also, the concentrations of IL-1β is higher in peri-implantitis PICF compared to healthy implant PICF. On the other hand, there is more diversity in peri-implantitis subgingival bacteriome, compared to healthy teeth in the same subject. The mycobiome diversity did not differ between different groups, although the following two species were seen more in Group P: *Candida parapsilosis* and *Aspergillus restrictus*.

The cytokine IL-4 is mainly produced by T-helper cells, that can cause fusion of macrophages, and differentiation of B cells to plasma cells. It is considered a major cytokine in allergic

inflammation and in inhibition of IFN-γ response. The findings of our study show that the concentration of this cytokine is higher in PICF of peri-implantitis sites compared to GCF of a healthy tooth in the same subject. This result is consistent with the fact that B cells are more numerous in established peri-implantitis lesions (Gualini & Berglundh, 2003) and the recent view that loss of osteointegration and peri-implantitis could be the result of a delayed foreign body reaction (Albrektsson et al., 2014).

IL-6 was found to have higher concentrations in peri-implantitis sites. This pro-inflammatory cytokine can cause collagen destruction in gingival tissues. Elevated levels of IL-6 in diseased PICF have been reported previously by others (Ata-Ali et al., 2015; Yaghobee et al., 2014), but not consistently (Fonseca et al., 2014; Severino et al., 2011). Levels of IL-1β did not reach significance in our study, perhaps due to a large standard deviation but was clearly elevated in peri-implantitis sites. IL-1β has long been studied for its role in periodontitis. This cytokine has a prominent role in bone resorption and has synergistic effects with TNF-α. TNF-α also showed increased levels in our study, but it fell short of clinical significance. These results are consistent with most of the previous research on the topic (Ata-Ali et al., 2015; Fonseca et al., 2014; Güncü et al., 2012; Luo et al., 2011; Yaghobee et al., 2014).

The cytokines IL-2, IL-8, IL-10 all showed increased levels in group P, both in implant and tooth sites; but this did not reach statistical significance. Others have reported similar results (Ata-Ali et al., 2015; Duarte et al., 2009; Fonseca et al., 2014; Güncü et al., 2012; Luo et al., 2011; Severino et al., 2011). IL-2 can activate natural killer (NK) cells, T- cells, and monocytes. It has been shown that IL-2 polymorphism can alter the risk of periodontitis and gastric cancer (Wu et al., 2009). The neutrophil chemokine IL-8 can elicit massive neutrophil accumulation and has been shown to induce bone resorption. Its elevated concentration has been linked to shift from health to disease both in peri-implant and periodontal tissues. (Eckert et al., 2018). IL-10 is an anti-inflammatory cytokine and its increased levels can hamper pathogen clearance. Excess IL-10 production can lead to dominance of T helper 2 cells and promote disease progression and upregulation of plasma B cell production.

In the microbiome analysis, a significant difference was observed between the bacterial alpha diversity ($P = 0.69$) but not beta diversity ($P = 0.15$) analysis of the subgingival plaque between the groups. The richness and variability of peri-implantitis sites were higher in Group P implant than Group P teeth or Group H implants. This phenomenon was also seen in other studies (Korsch et al., 2021). Korsch et al. showed that, in the implants that develop peri-implantitis at a later stage, bacterial diversity increases and higher densities of *Treponema*, *Fretibacterium*, *Pseudoramibacter* and *Desulfobulbus* are seen. Although differing to some study reports (Ghensi et al., 2020), our findings can further challenge the concept that peri-implantitis is a bacterial disease with specific bacterial pathogens, and strengthens the idea that bacterial invasion happens secondary to bone loss as a result of not very well understood processes such as foreign body reaction (Becker, 2012).

Peri-implant sites had higher abundances of *P. gingivalis*, *Fretibacterium sp*., *Treponema sp., Veillonellaceae sp., Johnsonella ignava, Desulfobulbus sp., and Bacteroidetes bacterium* compared to healthy implant sites. Other researchers have also seen similar elevations for

Treponema sp., P. gingivals, and Desulfobulbus (Ghensi et al., 2020; Korsch et al., 2021). *P. gingivalis* is a keystone periodontal pathogen that is capable of direct invasion to periodontal tissues. It dysregulates complement system and can induce production of IL-1β. This bacterium is also capable of upregulating IL-10, which is consistent with the findings of the present study. *Fretibacterium sp*. are strict anaerobic bacteria that are rarely found in periodontal health. But this bacterial taxon has been found in deep periodontal and peri-implant pockets. Higher proportions of phylum Bacteroidetes is reported previously in peri-implantitis sites (Sanz-Martin et al., 2017). Since all these bacteria are anaerobes, and pockets around peri-implantitis sites are deeper and more suitable for growth of these bacteria, this finding is expected. Though seen in association with gastric metaplasia, pancreatic cancer, and oral squamous cell carcinoma metaplasia (Pushalkar et al., 2012), little is known about the role of *Johansonella ignava* in periodontal and peri-implant disease. Our results show that this bacterium is not only more abundant in peri-implantitis sites, but also around healthy teeth of patients with peri-implantitis. This may suggest a cross-contamination in these individuals. Very little evidence exists for behavior of *Bacteroidetes bacterium* in periodontal or peri-implant pockets, since this bacterium is not usually detected by conventional methods, and need a biofilm to flourish.

No significant difference was observed between either the alpha diversity ($P = 0.69$) or beta diversity analysis of the fungal community of subgingival plaque ($P = 0.49$). Interestingly, our results suggest the relative abundance of *Candida parapsilosis* and *Aspergillus restrictus* are higher in peri-implantitis group, versus healthy implants. This is a significant finding, since *Candida parapsilosis* is a Candida species that is scarcely mentioned in connection with peri-implantitis, and most of the focus has been on *Candida albicans* so far, maybe because of its superior virulence (Schwarz et al., 2015). Recently, in an effort to improve the antibacterial effects of dental implant surfaces, a group of researchers found that *C. parapsilosis* has a unique property that *C. albicans* does not have. They found that after a short period of reduced survivability, *C. parapsilosis* shows increased survivability even beyond what was seen in control group on rough titanium surfaces (Roguska et al., 2018). Other studies also showed that this species is even more adherent to acrylic surfaces compared to Candida species, due to different attachment mechanisms (Critchley & Douglas, 1985). These unique properties can make *C. parapsilosis* more resistant in peri-implant deep pockets than other Candida species. *Aspergillus restrictus* is an airborne fungus normally associated with allergies, and is found in water, air, and soil. To the best of our knowledge, this is the first time *Aspergillus restrictus* is related to peri-implantitis. Interestingly, the role of these two fungi has been long known in complications associated with other prosthetic implant devices such as aortic valve prosthesis (Pospisil et al., 1984).

It is worth mentioning that there was no statistically significant difference between Group P and Group H in terms of self-reported oral hygiene practices including brushing, flossing, and the use of mouthwash. We showed that many bacteria that are higher in abundance in peri-implantitis sites, are anaerobic bacteria, nesting in deep pockets. Therefore, it is expected that routine oral hygiene measures could not remove these bacteria, since they are more effective against supragingival plaque biofilm (Waerhaug, 1981).

The generalizability of the results is limited by the small sample size, as recruitment was affected by the pandemic. Also, self-reported questionnaires are not accurate in measuring periodontal variables or taste preferences (Soter et al., 2008). Another limitation was that implant properties including length of service, type of prosthesis, implant system, location of the implant, etc. was not recorded and adjusted for. In addition, some data was missing in the questionnaire, and not all samples resulted in readable mycobiome results. It is recommended that studies with higher sample size and quality tests be performed in the future. The role of taste genetics could be investigated in future studies.

Whether initiated by bacterial colonization or foreign body reaction, peri-implantitis is a dysbiosis in immune response, fueled by bacterial, fungal, and viral challenge. The complicated interaction of host immune response with bacteriome and mycobiome, is yet to be fully uncovered. We have found a few species that were not previously shown to be associated with periimplantitis, which shows that the pockets around dental implants have their own unique characteristics including having a titanium wall. Further research in this field is necessary to introduce novel treatments, such as the ones seen in treatment of rheumatoid arthritis (Yuba et al., 2021).

In conclusion, this study shows that peri-implant pocket has its own unique micro-ecology. We found that certain bacterial and fungal species such as *Johansonella ignava* and *C. parapsilosis* might have more importance in peri-implant dysbiosis compared to periodontitis, due to their exceptional features.

Acknowledgement

This study was funded by internal funds of the University of Manitoba.

Conflict of Interest

The writers have no conflict of interest to report.

FIGURE LEGENDS:

Figure 1. Alpha and beta diversity analysis of subgingival bacteriome across the groups. (A) 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 groups H - Implant vs. group P - Tooth and P - Implant vs. P - Tooth (P.unadj.< 0.05). The differences between groups H - Implant and P - Tooth remained significant after adjustment for multiple comparisons (P.adj. = 0.03). (B) Principal coordinate analysis (PCoA) plot of weighted UniFrac distances. Each data point represents an individual sample. The ellipses represent a 95% confidence level and shape and color represent the groups. Overall, no significant differences were observed between the groups (pseudo-F = 1.6, R^2 = 0.08, *P* = 0.15, PERMANOVA).

Figure 2. 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 healthy and peri-implantitis 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 3. Diversity and differential abundance analysis of subgingival mycobiome. (A) Alpha diversity. 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. No significant difference was observed between the alpha diversity of subgingival plaque mycobiome (H(3) = 1.48, *P*-value = 0.69). (B) Beta diversity. Principal coordinate analysis (PCoA) plot of weighted UniFrac distances. Each data point represents an individual sample. The ellipses represent a 95% confidence level and shape and color represent the groups. Beta diversity analysis showed that disease status and site did not significantly affect the fungal community structure (pseudo-F = 0.97, R^2 = 0.08, *P* = 0.49, PERMANOVA). (C) Differential abundance analysis. The plot shows the relative fold change in the abundance of fungal taxa in implants from the healthy and periimplantitis groups. The differential abundance was tested using the DESeq2 negative binomial Wald test and only taxa with FDR adjusted *P* < 0.05 are shown.

Table 1. Characteristics of the study population.

	Health (*N*=20)	Peri-implantitis (*N*=9)	Total (*N*=29)	*P* value
Sex				0.42
F	13 (65.0%)	4 (44.4%)	17 (58.6%)	
M	7 (35.0%)	5 (55.6%)	12 (41.4%)	
Age				0.50
Mean (SD)	61.95 (8.95)	57.22 (19.58)	60.48 (12.99)	
Min - Max	40.00 - 76.00	23.00 - 88.00	23.00 - 88.00	
Diabetes				1
N	17 (85.0%)	8 (88.9%)	25 (86.2%)	
Y	3 (15.0%)	1 (11.1%)	4 (13.8%)	
Hypertension				0.69
N	13 (65.0%)	5 (55.6%)	18 (62.1%)	
Y	7 (35.0%)	4 (44.4%)	11 (37.9%)	
Arthritis				0.63
N	15 (75.0%)	8 (88.9%)	23 (79.3%)	
Y	5 (25.0%)	1 (11.1%)	6 (20.7%)	
Former smoker				1
N	11 (55.0%)	5 (55.6%)	16 (55.2%)	
Y	9 (45.0%)	4 (44.4%)	13 (44.8%)	
Brushing				1
Less than twice a day	4 (20.0%)	1 (11.1%)	5 (17.2%)	
Twice a day	16 (80.0%)	8 (88.9%)	24 (82.8%)	
Flossing				1
Less than once a day	7 (35.0%)	3 (33.3%)	10 (34.5%)	
At least once a day	13 (65.0%)	6 (66.7%)	19 (65.5%)	
Mouthwash				0.69
N	9 (47.4%)	3 (33.3%)	12 (42.9%)	
Y	10 (52.6%)	6 (66.7%)	16 (57.1%)	
Missing	1	0	1	

Dry mouth				1
N	16 (80.0%)	7 (87.5%)	23 (82.1%)	
Y	4 (20.0%)	1 (12.5%)	5 (17.9%)	
Missing	0	1	1	
Bleeding gums				0.53
N	19 (95.0%)	8 (88.9%)	27 (93.1%)	
Y	1 (5.0%)	1 (11.1%)	2 (6.9%)	
Burning sensation				0.31
N	20 (100.0%)	8 (88.9%)	28 (96.6%)	
Y	0 (0.0%)	1 (11.1%)	1 (3.4%)	
Bad breath				0.63
N	14 (73.7%)	8 (88.9%)	22 (78.6%)	
Y	5 (26.3%)	1 (11.1%)	6 (21.4%)	
Missing	1	0	1	
Like Sweets				0.72
Mean (SD)	0.8 (0.4)	0.7 (0.5)	0.8 (0.4)	
Min - Max	0.0 - 1.6	0.0 - 1.2	0.0 - 1.6	
Like Bitters				0.57
Mean (SD)	1.1 (0.4)	1.2 (0.5)	1.1 (0.4)	
Min - Max	0.4 - 1.7	0.4 - 2.0	0.4 - 2.0	

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

Table 2. Concentrations (pg/mL) of IL-1β, IL-2, IL-6, IL-4, IL-8, IL-10, and TNF-α

Cytokines	H Tooth (n= 20)	Group H Implant (n= 20)	Group P Tooth (n= 9)	Group P Implant (n= 9)	*P*-Value	Adjusted *p*-value					
						Group H Tooth vs. Group H Implant	Group H Tooth vs. Group P Tooth	Group H Tooth vs. Group P Implant	Group H Implant vs. Group P Tooth	Group H Implant vs. Group P Implant	Group P Implant vs. Group P Tooth
IL-1β†	202.0±194.6	137.3±114.8	159.2±88.64	340.1±343.7	0.07	0.71	0.94	0.28	0.99	**0.05**	0.20
IL-2‡	1.19±0.60	1.25±0.87	1.54±0.91	1.96±1.45	0.19	>0.99	>0.99	0.48	>0.99	0.26	>0.99
IL-4‡	0.051±0.08	0.13±0.15	0.04±0.08	0.24±0.24	**0.001**	0.1491	>0.99	**0.01**	0.11	>0.99	**0.01**
IL-6‡	2.68±5.11	3.27±4.63	5.30±9.19	7.74±7.15	**0.04**	>0.99	>0.99	**0.02**	>0.99	0.14	0.30
IL-8†	3340±2348	3627±2798	4553±3010	5836±3371	0.14	0.99	0.70	0.13	0.84	0.21	0.76
IL-10‡	0.58±0.51	0.73±0.89	1.02±0.83	1.41±1.35	0.11	>0.99	0.81	0.41	0.63	0.31	>0.99
TNF-α†	4.49±3.31	5.21±4.95	6.46±4.72	9.20±7.29	0.34	0.96	0.74	0.09	0.92	0.19	0.63

† ANOVA

‡ Kruskal-Wallis test

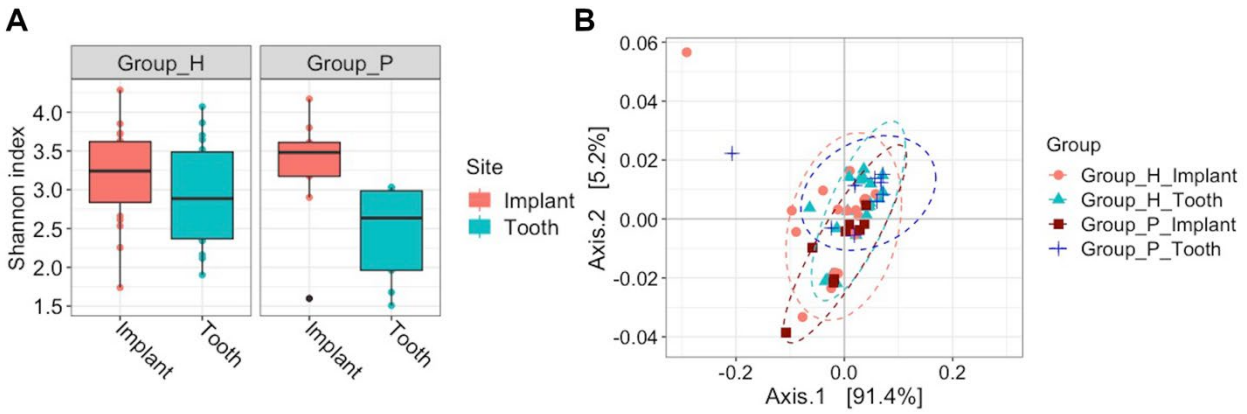

Figure 1. Alpha and beta diversity analysis of subgingival bacteriome across the groups. (A) The median Shannon index values were compared across groups using the Kruskal-Wallis test and Dunn's post hoc tests for multiple comparisons with *P*-values adjusted with the Benjamini-Hochberg method. The medians are represented by the lines, boxes represent the interquartile range, and whiskers indicate the range. Overall, a significant difference was observed between the groups H - Implant vs. group P - Tooth and P - Implant vs. P - Tooth (*P*.unadj.< 0.05). The differences between groups H - Implant and P - Tooth remained significant after adjustment for multiple comparisons (*P*.adj. = 0.03). (B) Principal coordinate analysis (PCoA) plot of weighted UniFrac distances. Each data point represents an individual sample. The ellipses represent a 95% confidence level and shape and color represent the groups. Overall, no significant differences were observed between the groups (pseudo-F = 1.6, R^2 = 0.08, *P* = 0.15, PERMANOVA).

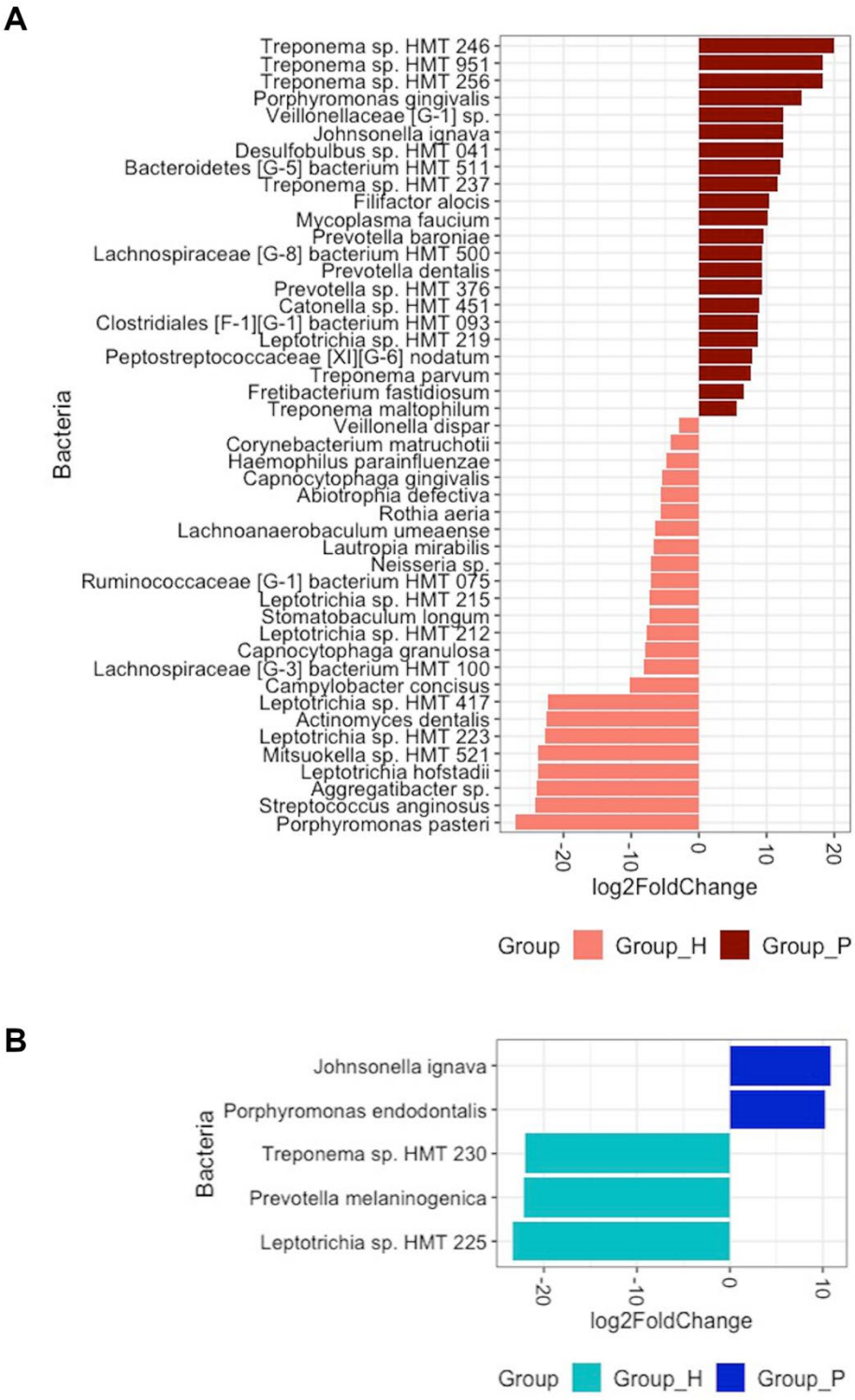

Figure 2. Differential abundance analysis of bacterial taxa. The plot shows the relative fold change in the abundance of bacteria in (A) implants and (B) tooth from the healthy and peri-implantitis groups. The differential abundance was tested using the DESeq2 negative binomial Wald test and only taxa with FDR adjusted $P < 0.05$ are shown.

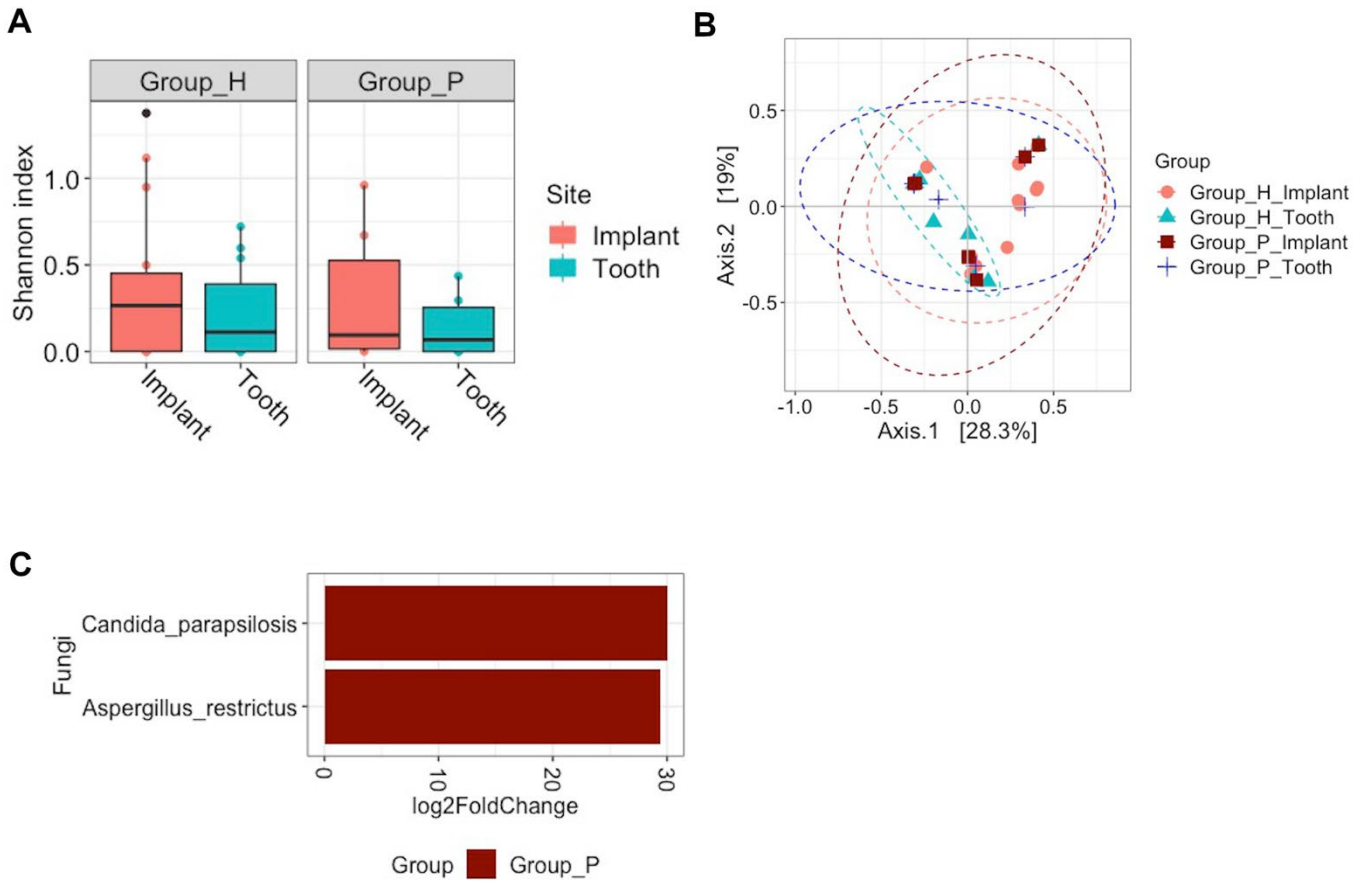

Figure 3. Diversity and differential abundance analysis of subgingival mycobiome. (A) Alpha diversity. The median Shannon index values were compared across groups using the Kruskal-Wallis test and Dunn's post hoc tests for multiple comparisons with *P*-values adjusted with the Benjamini-Hochberg method. The medians are represented by the lines, boxes represent the interquartile range, and whiskers indicate the range. No significant difference was observed between the alpha diversity of subgingival plaque mycobiome (H(3) = 1.48, *P*-value = 0.69). (B) Beta diversity. Principal coordinate analysis (PCoA) plot of weighted UniFrac distances. Each data point represents an individual sample. The ellipses represent a 95% confidence level and shape and color represent the groups. Beta diversity analysis showed that disease status and site did not significantly affect the fungal community structure (pseudo-F = 0.97, R^2 = 0.08, *P* = 0.49, PERMANOVA). (C) Differential abundance analysis. The plot shows the relative fold change in the abundance of fungal taxa in implants from the healthy and periimplantitis groups. The differential abundance was tested using the DESeq2 negative binomial Wald test and only taxa with FDR adjusted *P* < 0.05 are shown.

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