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Biofilm Accumulation and Immune Response During Experimental Peri-Implant Mucositis.

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Biofilm Accumulation and Immune Response During Experimental Peri-Implant Mucositis.

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1. Introduction

1.1 *Background*

Dental implants and implant-supported restorations are a predictable alternative to removable partial dentures and fixed tooth-supported prostheses. Dental implants are commonly used to replace missing teeth, with 4.5 million implants placed annually worldwide. The success rate of implant therapy is greater than 90% after the first year in function [1].

Bacterial biofilms colonize dental implants upon exposure to the oral cavity [2]. Undisrupted biofilms cause inflammation of the implant-mucosa unit, a process known as peri-implant mucositis. Peri-implant mucositis is defined as a reversible inflammatory condition of the mucosa without accompanying bone loss [3]. Evidence from animal studies suggests that persistent peri-implant mucositis may progress to peri-implantitis [4,5], which involves progressive bone loss. Loss of the affected implant may occur in patients with peri-implantitis. According to a recent review, 80% of patients and 50% of implants are affected with peri-implant mucositis whereas 28% of patients and 12% of implants are affected with peri-implantitis [6]. A more comprehensive understanding of peri-implant mucositis and the development of preventive strategies are needed.

The biology of peri-implant mucositis is not well understood. Several human and animal studies have demonstrated that bacterial plaque accumulation produces similar clinical responses, such as erythema, bleeding on probing and increased gingival crevicular fluid (GCF) flow, in the tissues surrounding teeth and implants [7-9]. However, differences in

the quality and quantity of the inflammatory infiltrate [8] and the quality of the microbial flora [10] between gingiva and peri-implant mucosa have been reported. For example, a study by Nowzari et al. [11] found that cytokines associated with the innate immune response (e.g., IL-8, TNF-alpha) are exaggerated in the peri-implant mucosa in comparison to the gingiva [11]. Thus several neutrophil (PMN) activation markers, including myeloperoxidase and lactoferrin, have been proposed as potential risk markers for peri-implant disease [12]. In addition, although the microbiota of peri-implant and periodontal infections is for the most part similar [13], implant sites have been shown to harbor organisms not traditionally associated with periodontitis, such as staphylococci and enterics [14,15]. To date, the temporal microbial succession and resultant host response in the peri-implant mucosa versus the periodontium, in response to plaque accumulation, has not been characterized.

1.2 *Gingivitis*

1.2.1 Microbiota changes in gingivitis

Gingivitis is defined by the presence of gingival inflammation without the loss of connective tissue attachment [16]. The etiology of gingivitis is bacterial plaque. A human experimental gingivitis model showed a significant shift in the bacterial biofilm composition from health to disease [17]. In this model, the microflora of healthy gingival sites was comprised of mainly aerobic Gram-positive cocci and rods. Two to four days after oral hygiene withdrawal, fusobacteria and filamentous bacteria proliferate. After four to nine days, the bacterial flora was characterized by the presence of curved rods and spirochetes. Gingivitis appeared 10 to 21 days following oral hygiene withdrawal and

was reversible with reinstitution of oral care procedures at both the clinical and microbiological levels. The range in the timing of the development of clinical gingivitis among subjects was thought to be the result of variability in the individual host response to bacterial plaque [17,18]. No study, however, has ultimately resolved the genetic, microbiological or immunological parameters underlying susceptibility to gingival inflammation.

Analyses of the subgingival microbiota in periodontal health and established gingivitis have shown differences in species predominance. *Actinomyces naeslundii, Campylobacter gracilis, Prevotella nigrescens* and *Tannerella forsythia* are increased in gingivitis samples. Health-associated species include *Streptococcus oralis*, *Streptococcus gordonii, A. naeslundii* and *Actinomyces gerencseriae* [19,20]. A three-year prospective study demonstrated elevated counts of *A. naeslundii*, *Capnocytophagia gingivalis* and *Prevotella intermedia* during baseline gingivitis and significant reductions in these species after one, two and three years of participation in a periodontal prevention regimen [21].

The above-cited studies have used techniques to characterize the microflora such as cultivation and microscopic observation [18] or DNA-DNA hybridization [21]. A more recent study used high-throughput 16S rRNA pyrosequencing to investigate the changes in the microbiome development during experimental gingivitis, comprehensively characterizing the bacterial communities in plaque during the health to gingivitis transition [22]. Analyses were carried out at baseline and after one and two weeks of

interrupted plaque control. Pyrosequencing yielded 344,267 sequences that were clustered into an average of 299 species-level Operational Taxonomic Units (OTUs) per sample. There was no significant difference in richness between baseline and the end of experimental gingivitis induction (week 2). There was a significant difference in diversity across time points, with diversity significantly higher in two-week communities compared to both baseline and one-week communities. The significant increase in community diversity after two weeks of experimental gingivitis in the absence of increased richness indicates that increased diversity was due to increasing evenness. Therefore, the dominant species in health decreased in relative abundance over time while previously minor constituent species increased in relative abundance, resulting in a more even distribution of species after two weeks of plaque accumulation. Comparison of bacterial community structure of plaque samples using Principal Coordinate Analysis (PCoA) showed spatial separation of one- and two-week communities from baseline communities. The relative abundance of *Actinobacteria* was significantly higher at baseline compared to one and two weeks while *Bacteroidetes* were significantly higher in one- and two-week samples compared to baseline. However, there was considerable variability among subjects regarding the shifts in phyla during experimental gingivitis. In addition, the OTUs identified as *Lautropia* sp. HOTA94, *Lachnospiraceae* sp. HOT100, *Prevotella oulorum* and *Fusobacterium nucleatum* subsp. *polymorphum* were most significantly positively correlated with bleeding on probing (BOP). An OTU identified as *Rothia dentocariosa* was most significantly negatively correlated with BOP. The results of this study demonstrate that in the absence of oral hygiene, the transition from periodontal health to gingivitis is accompanied by a shift in the bacterial community

structure of plaque and an increase in bacterial diversity. The OTUs that increased in relative abundance as gingivitis developed were often obligate anaerobes and mostly gram-negative taxa of the genera *Campylobacter, Fusobacterium, Lautropia, Leptotrichia, Porphyromonas, Selenomonas* and *Tannerella*. These findings are in accordance with the observations of Loe and coworkers (1966), who reported an increase in the proportion of gram-negative cocci and rods as well as filaments, spirilla and spirochetes as gingivitis developed [18]. In the classic experimental gingivitis model, however, subjects abstained from oral hygiene for 21 days and gingivitis did not appear for 21 days in some subjects. The pyrosequencing study described above involved cessation of oral hygiene practices for only 14 days. This may have resulted in the inclusion of healthy sites among gingivitis data, when analyzing data according to time points, which is a significant limitation. There was also great variability at the phylum- and species-level among the subjects at baseline as indicated by the PCoA plots, which may compromise the ability to observe statistically significant changes in community structure from baseline to the gingivitis time points with a limited sample size.

A study by Huang et al. (2014) examined the structure of the plaque microbiota in an experimental gingivitis model using pyrosequencing of the 16S rRNA gene in 50 subjects and developed a model to predict gingivitis severity and susceptibility based on plaque microbial structure. Five genera (e.g., *Streptococcus*) had elevated abundance at baseline whereas different 22 genera (e.g., *Leptotrichia*) had elevated abundance in both native and experimental gingivitis. All 150 species associated with health and gingivitis were clustered via PCoA based on relative abundance. The healthy and diseased taxa were

distinguished by a boundary separating baseline (healthy) samples from both native and experimental gingivitis, suggesting that microbiota structure is related to health or disease states. Native and experimental gingivitis microbial communities were largely consistent with respect to this boundary in the same subject. Therefore, community changes associated with gingivitis seem to recur similarly within subjects and each individual may have a personalized disease-associated configuration of the microbiota [23].

Huang et al. (2014) also observed that the projected coordinate of a given microbiota on the first principal component (PC1) of their PCoA plots demonstrated a gradient-like development of microbiota structure during experimental gingivitis and resolution, as changes in PC1 were consistent with the structural segregation between healthy and diseased microbiota. Thus, PC1 was used as a proxy for quantitatively measuring the development of the microbiota during the transition from native gingivitis to health and health to experimental gingivitis. The authors discovered that 15 genera were the drivers of heterogeneity among the microbiota along PC1. In health, genera traditionally associated with periodontitis (e.g., *Fusobacterium, Tannerella, Treponema*) were present in low abundance whereas their abundance increased along the PC1 axis. On the other hand, genera associated with health (e.g., *Rothia, Haemophilus*) were present in low abundance in the gingivitis samples and higher abundance in the healthy samples [23]. Additionally, gingivitis severity was correlated with PC1 values throughout the experimental gingivitis and resolution phases. Two distinct types of hosts emerged, with type I patients exhibiting less gingivitis sensitivity than type II patients. Type II patients demonstrated dramatic changes in PC1/microbial structure and gingivitis severity

compared to type I patients, who did not demonstrate such acute changes. For the average type II host, the rates of change in PC1 and gingivitis severity were 2.21-fold and 1.89-fold higher than in an average type I host. Genera including *Abiotrophia*, *Selenomonas* and uncultured *Lachnospiraceae* were enriched in type II hosts compared with type I hosts. Interestingly, compared with type I hosts, those genera associated with type II hosts during native and experimental gingivitis were also higher in relative abundance in type II hosts at baseline. The authors concluded that heterogeneity of plaque microbiota among hosts may explain phenotypic variations of gingivitis sensitivity [23].

Lastly, to determine whether PC1 could model disease progression and classify patients based on disease state, 41 additional subjects were sampled during native gingivitis and baseline for model validation. A microbial index of gingivitis (MiG) was derived based on the relative abundance of the 27 bacterial markers that distinguished health from native and experimental gingivitis. The predictive power of MiG27 was tested by determining the gingivitis statuses of the hosts using their native gingivitis microbiota. The accuracy of classification for a diseased versus healthy state was 94%. Therefore, plaque microbial structure is able to classify gingivitis susceptibility and severity in humans and the plaque microbial population during native gingivitis can predict the population structure during a later episode of experimentally-induced gingivitis in the same subject [23].

In summary, gingivitis is associated with a shift in the microbiome consisting of increased gram-negative anaerobes as well as increased diversity. Plaque microbial structure may predict susceptibility to gingivitis.

1.2.2 Host response in gingivitis

The host immune system responds dramatically to the increased bacterial burden and the shift in community structure as plaque accumulates. The inflammatory process in patients with gingivitis is characterized by a cellular infiltrate composed of PMNs, B- and T-lymphocytes, plasma cells, monocytes and macrophages [24,25].

The histopathology of gingivitis is characterized by four stages. The evidence for these stages described by Page and Schroeder [26] was based on prevailing information from animal biopsies and some human adolescent samples. The initial lesion forms within 2 to 4 days of plaque accumulation and involves vasculitis, increased GCF flow and migration of PMNs into the junctional epithelium. Loss of both epithelial cells in the coronal aspect of the junctional epithelium and perivascular collagen occurs. After 4 to 7 days, the early lesion forms. This stage in the evolution of gingivitis includes an accumulation of lymphocytes adjacent to the junctional epithelium and further loss of collagen. PMNs and lymphocytes are the predominant leukocytes in the infiltrate at this stage of gingivitis. The established lesion is the third stage of gingival inflammation. The distinguishing feature of this lesion is the predominance of plasma cells. Apical migration of the junctional epithelium and loss of connective tissue matrix elements are present. According to Page and Schroeder, the established lesion occurs after 7 days of plaque

accumulation. However, gingival biopsies from Brecx et al. [27] demonstrate that the lesion throughout 21-day abstention from oral hygiene during experimental gingivitis represents that of an early lesion, with numerous PMNs and lymphocytes but low numbers of plasma cells. Therefore, it may take longer than three weeks for the established lesion to develop. The established lesion may remain stable for a long period of time or progress to the advanced lesion, which is irreversible, involving pocket formation and bone loss [26].

The interleukin 1 (IL-1) family of cytokines, interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-alpha) and PGE2 are the cytokines and inflammatory mediators most consistently associated with gingivitis and periodontitis [28]. IL-1 alpha and beta are pro-inflammatory cytokines that stimulate PGE2 release from monocytes and fibroblasts. PGE2 then induces osteoclastic bone resorption and matrix metalloproteinase (MMP) secretion. IL-1 beta also enables ingress of inflammatory cells into sites of infection, promotes bone resorption and stimulates release of MMPs that degrade proteins of the extracellular matrix. IL-1 beta is a principal mediator of the gingival inflammatory response and is produced primarily by macrophages and PMNs. IL-6 stimulates plasma cell proliferation and thus antibody production. IL-6 is produced by lymphocytes, monocytes and fibroblasts. TNF-alpha shares many properties with IL-1. It is pro-inflammatory and stimulates MMP action, PGE2 production and bone resorption. It is secreted by monocytes and fibroblasts [28].

To determine the sequence of cytokine and lipid mediator activation within the periodontium during experimental gingivitis, Heasman et al. [29] collected GCF from healthy patients at baseline and weekly during four weeks of cessation of oral hygiene measures. GCF samples were assayed for several mediators, including PGE2, IL-1 beta and TNF-alpha. PGE-2 levels remained stable at baseline levels for the first three weeks and then increased by 2.5-fold at four weeks. IL-1 beta levels increased significantly at one week and remained at this heightened level for the duration of the study. These data suggest IL-1 beta is involved in the earliest phases of gingivitis whereas PGE2 is mediating the late response. TNF-alpha levels remained undetectable throughout this study. In another study of native gingivitis, however, both IL-1 beta and TNF-alpha levels in GCF were significantly higher in gingivitis subjects compared to periodontally healthy patients [30].

The application of suspension bead multiplexing methods for the simultaneous analysis of multiple inflammatory mediators has been used to characterize the levels of inflammatory mediators associated with naturally occurring as well as experimental gingivitis in humans [31, 32]. These studies have shown that chronic gingivitis is associated with a 1.35-fold increase in IL-1 beta and a 2.1-fold increase in IL-6 in GCF [31]. Gingivitis induction during an experimental gingivitis model was associated with a 2.6-fold increase in IL-1 beta and a 3.1-fold increase in IL-1 alpha. Interestingly, IL-8 decreased significantly in GCF during gingivitis induction and rebounded to baseline during resolution [32].

A study by Trombelli et al. [33] also evaluated IL-1 beta levels in GCF in naturally occurring as well as experimentally induced plaque-associated gingivitis. Experimental gingivitis was induced in one test quadrant while oral hygiene was continued in the contralateral control quadrant. In experimental gingivitis, IL-1 beta levels were significantly higher in test compared with control quadrants. Similar to the previously mentioned studies, GCF IL-1 beta was significantly higher in experimentally induced gingivitis sites compared to naturally occurring gingivitis sites. Higher IL-1 beta concentrations observed in experimentally induced gingivitis may reflect the increased microbial challenge to the gingival tissues associated with the experimental conditions of plaque accumulation.

Other inflammatory mediators, such as IL-1 receptor antagonist (IL-1 ra), chemokine (C-C motif) ligand 22 (CCL22) and growth-related oncogene (GRO), have been evaluated as potential markers for periodontitis, but not gingivitis. IL-1 ra is significantly increased in GCF from periodontitis patients compared to healthy controls, however this increased secretion of IL-1 ra in periodontitis does not inhibit IL-1 beta release [34]. CCL22, previously macrophage-derived chemokine, targets dendritic cells, NK cells and T cell subsets. CCL22 is a potent attractant for Th2 cells [35]. Gingival biopsies from periodontitis patients have greater CCL22 expression than biopsies from healthy patients [36]. GRO, a PMN chemoattractant, is significantly increased in GCF from periodontitis patients compared to healthy controls. However, no study has examined IL-1 ra, CCL22 and GRO in gingivitis.

Overall, gingivitis involves inflammation of the gingival unit, without accompanying bone destruction, as a result of the presence of pathogenic bacteria leading to increased levels of inflammatory cytokines and tissue-destructive molecules.

1.3 *Peri-implant mucositis*

1.3.1 Microbiota changes in peri-implant mucositis

The microbiota of periodontal and peri-implant infections are largely similar. Implants with clinical signs of peri-implantitis are associated with high counts and proportions of gram-negative anaerobic bacteria. Diseased implant sites have demonstrated a high prevalence of periodontal pathogens, including *Porphyromonas gingivalis, Treponema denticola, T. forsythia, Fusobacterium* species*, P. intermedia* and *Aggregatibacter actinomycetemcomitans*. However, some species not traditionally found in periodontitis sites have been isolated at implant sites. *Staphylococcus aureus*, enteric rods and *Candida albicans* have been isolated from sites with peri-implant disease [13].

Recently, pyrosequencing has been applied to the study of healthy and diseased peri-implant microbiomes. In a study by Kumar et al. [37], subgingival and submucosal plaque samples were collected from a total of 40 patients with periodontitis (n = 10), peri-implantitis (n=10) and periodontal (n = 10) and peri-implant (n = 10) health and analyzed using 16S pyrosequencing. Peri-implant sites demonstrated lower bacterial diversity than both healthy and diseased subgingival sites. The predominant phylotypes in both healthy and diseased peri-implant communities were members of the genera *Butyrivibrio*, *Campylobacter*, *Eubacterium*, *Prevotella*, *Selenomas*, *Streptococcus*, *Actinomyces*,

Leptotrichia, *Propionibacterium*, *Peptococcus*, *Lactococcus* and *Treponema*. Lower levels of *Prevotella* and *Leptotrichia* and higher levels of *Actinomyces*, *Peptococcus*, *Campylobacter*, *Streptococcus* and *Butyrivibrio* were associated with diseased implants compared to healthy implants. Peri-implant disease communities also demonstrated lower levels of *Prevotella*, non-mutans *Streptococcus*, *Lactobacillus*, *Selenomonas*, *Leptotrichia*, *Actinomyces* and higher levels of *Peptococcus*, *Mycoplasma*, *Eubacterium*, *Campylobacter*, *Butyrivibrio*, *Streptococcus mutans* and *Treponema* than biofilms associated with periodontitis. Therefore, it appears that discrepancies are present in the phylotypes that colonize implant surfaces compared to teeth and different phylotypes are implicated in native peri-implantitis versus periodontitis.

The majority of the limited research about the microbiota associated with peri-implant infection is related to peri-implantitis rather than peri-implant mucositis. Since peri-mucositis is a precursor to the establishment of peri-implantitis, it is imperative to elucidate the microbial profile present during this transitional step in the progression to implant bone loss. In a combined experimental gingivitis and mucositis study by Salvi et al. (2012), clinical, microbiological and host inflammatory markers were monitored around teeth and implants. Fifteen subjects with healthy, restored implants underwent three weeks of undisturbed plaque accumulation followed by three weeks of optimal plaque control. The presence or absence of plaque deposits and gingival/mucosal conditions were evaluated weekly. Microbial samples were analyzed using DNA-DNA hybridization for 40 species. No differences in the total microbial DNA counts or detection frequency for putative periodontal pathogens were found between implant and

tooth sites at any time point. However, this study also did not find significant intra-subject changes in levels of specific microorganisms during the experimental period for both implants and teeth [38]. In this design, sites were samples repeatedly over time, potentially disturbing the biofilm and preventing recolonization, which may explain the lack of difference between the groups. It should be noted that these results conflict with previously mentioned studies that found variations in the microflora around implants and teeth using different techniques.

In a split-mouth design study [39], the bacterial communities at implant and tooth sites with native peri-implant mucositis or gingivitis, respectively, were compared via broad-range PCR followed by DNA fingerprinting and sequencing of plaque samples. This technique does not provide information on abundance of taxa and is less comprehensive than high-throughput sequencing, which is a limitation of this study. Samples from the gingival sulci demonstrated significantly higher diversity than peri-implant samples. Twelve bacterial genera, including *Campylobacter* and *Neisseria*, were not found at implant sites but were frequently isolated at dental sites. In contrast, members of TM7 were detected solely at implant sites. Although the findings of the previously discussed study oppose the findings of this study in terms of presence or absence of *Campylobacter* at implant sites, the two studies are in agreement with respect to peri-implant microbial diversity. The diversity of the microflora in sites demonstrating established peri-implantitis or peri-implant mucositis seems reduced in comparison to that of periodontitis or gingivitis [39]. Different microbiomes surrounding implants and teeth may thus affect the mechanisms by which initial inflammatory lesions progress to irreversible changes.

1.3.2 Host response in peri-implant mucositis

It has been proposed that peri-implant soft tissues may develop a stronger inflammatory response to plaque accumulation than soft tissues around teeth. Biopsies harvested from one tooth and one implant site in partially edentulous patients in health and after three weeks of undisturbed plaque accumulation demonstrated a trend toward significantly increased B and T cells in the peri-implant mucosa compared to the gingiva [8]. Similar evidence was found in the Salvi et al. (2012) study. Weekly GCF samples were analyzed for matrix metalloproteinase-8 (MMP-8) and IL-1ß levels. GCF levels of MMP-8, but not IL-1ß, were significantly higher at implants compared with teeth over the six-week experimental period. Three weeks after the reinstitution of oral hygiene, reversibility of experimental gingivitis and mucositis was achieved at the biomarker level. Clinically, gingival and peri-implant mucosal health were not achieved after three weeks of resumed plaque control, suggesting that longer healing periods are required for both teeth and implants [38].

In addition to MMP-8, other indicators of PMN function, such as lactoferrin and elastase, have been identified as potential biomarkers for peri-implantitis. Lactoferrin, an iron-binding protein stored in secondary neutrophilic granules that are released during PMN chemotaxis, is associated with the number of PMNs present in a lesion. Elastase, a serine protease stored in primary neutrophilic granules, is released extracellularly during phagocytosis and activation of PMNs and degrades collagen, laminin and fibronectin. In a study that aimed to characterize the inflammatory host response around implants and

teeth in patients with peri-implantitis, GCF samples were collected from implants with peri-implantitis, stable implants in patients with both stable and peri-implantitis implants and control implants in patients with stable implants only [12]. In patients with peri-implantitis, lactoferrin concentration was higher at implants with peri-implantitis than at stable implants. Elastase activity was higher at implants with peri-implantitis compared to stable implants in controls. Therefore, lactoferrin concentration and elastase activity in crevicular fluid are markers of site-specific inflammation, specifically PMN activation, around dental implants.

The neutrophilic enzyme myeloperoxidase (MPO) is a heme-containing enzyme with non-specific peroxidase activity, enabling oxidation of a wide range of substrates. In the presence of hydrogen peroxide and chloride ions, MPO catalyzes the oxidation of Cl^- into hypochlorous acid, which kills many bacterial and fungal species *in vitro*. It has been demonstrated that total amounts of MPO are significantly higher in the peri-implant sulcular fluid surrounding implants with peri-implantitis or peri-implant mucositis compared to that of healthy implants. Also, MPO activity increases with increasing pocket probing depth (PPD), gingival index (GI) and bleeding on probing (BOP) scores. Therefore, MPO could be an important marker for peri-implant mucositis and peri-implantitis [40].

Since little is known about the host response during peri-implant mucositis, the host responses around teeth and implants in health and during periodontitis/peri-implantitis may provide a foundation for understanding peri-implant mucositis. Implant sites

demonstrate higher concentrations of inflammatory cytokines such as IL-8 and TNF-alpha [11]. A recent study compared the histology of periodontitis and peri-implantitis lesions in 40 patients with generalized severe chronic periodontitis and 40 patients with severe peri-implantitis. Peri-implantitis biopsies demonstrated significantly larger infiltrated connective tissue areas compared to periodontitis samples. In addition, peri-implantitis lesions contained significantly larger area proportions and densities of CD138-, CD68- and MPO-positive cells than periodontitis lesions [41].

In summary, the results of these studies demonstrate that there are dissimilarities in the host responses around implants evidenced as significant differences in histopathology and inflammatory markers measured in GCF. Most studies suggest an exacerbated response around implants in comparison to teeth.

1.4 *Rationale for this study*

Overall, the microbiome and host response associated with peri-implant mucositis are not fully understood. Although there are data suggesting that, compared to either periodontitis or gingivitis, peri-implant lesions demonstrate decreased microbial diversity, differences in the presence and prevalence of certain bacteria and an increased response from host inflammatory proteins, it is yet to be determined whether peri-implant mucositis processes closely mimic those of gingivitis.

To the extent that peri-implant mucositis and gingivitis differ in associated microbiomes and/or host response, clinical approaches to diagnosis, prevention and therapy may also

differ. As the gateway lesion to peri-implantitis, peri-implant mucositis represents a critical step in avoiding implant failure. Therefore, understanding characteristics of the microflora in health and initial disease, as well as destructive host inflammatory and immune response to bacterial pathogens, can guide research and the management of implants.

Most studies to date have examined peri-implantitis rather than its precursor, peri-implant mucositis. In the natural dentition, understanding the development of salient disease, periodontitis, derives from the characterization of its precursor, gingivitis. Similarly, our grasp of peri-implantitis must be based on a more detailed examination of peri-implant mucositis. The dramatic distinctions between implants and teeth, for example in molecular composition (titanium versus enamel), physical (especially surface and anatomical) qualities, histological attachment mechanisms, blood supply and mode of establishment in the mouth (eruption versus surgical insertion), raise the possibility that the bacterial species and communities associated with implants may have limited resemblance to those associated with teeth. These microbial differences, and such unique features as lack of periodontal ligament and reduced vascularity, could lead to variations in host response during initiation and progression of disease.

The present study aims to further characterize the evolution of the whole microbial profile and the associated changes in host response at teeth and implant sites in three phases: during a native condition, a 21-day period of oral hygiene cessation and after resumption of oral hygiene practices.

1.5 *Hypothesis*

For the above-mentioned reasons, we hypothesize that the microbial and immune responses differ between implants and teeth during native and experimental peri-implant mucositis/gingivitis and after peri-implant mucositis/gingivitis resolution.

2. Objectives

2.1. *Specific aims*

Aim 1. To cross-sectionally compare the microbial biofilms and GCF inflammatory markers between teeth and dental implants in their native state

Aim 2. To longitudinally evaluate the changes in the microbial flora and GCF inflammatory markers during 21 days of *de novo* plaque accumulation around dental implants in comparison to teeth

Aim 3. To compare the immunological and microbiological responses after oral hygiene measures are resumed around dental implants and teeth

3. Study design and methods

This is a controlled clinical trial to compare the responses to *de novo* plaque accumulation in peri-implant soft tissue and gingiva within subjects (n=15). We evaluated immunological and microbiological parameters at a baseline native state, over 21 days of experimental peri-implant mucositis/gingivitis and following reinstitution of oral hygiene practices. To achieve the previously mentioned objectives, we collected GCF and biofilm samples from two Straumann implants and two natural teeth in

systemically healthy patients. Host inflammatory markers were analyzed via ELISA and Luminex technology. The microbiome composition of the plaque samples was determined by high-throughput sequencing of the bacterial 16S rRNA gene.

3.1 *Study population*

Eighteen voluntary participants were recruited among the patient population formerly treated with implant therapy at the University of Connecticut, School of Dental Medicine. Table 1 shows the inclusion and exclusion criteria for this study.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	**Exclusion criteria**
• ≥21 years of age; • Presence of 2 implants in the maxillary or mandibular arch supporting fixed restorations in function for at least 1 year (Test); • Presence of 2 teeth in the corresponding position of the implant (Control); • Absence of active infections at both test and control sites • Absence of radiographic bone loss at both test and control sites • Pocket depth ≤4mm at both test and control sites • Must give written informed consent.	• Treatment with antibiotics for any dental or medical condition within 1 month before screening; • Systemic diseases that require chronic use of anti-inflammatory medications, antibiotics or anticoagulants; • Ongoing medications for systemic conditions initiated <3 months before the start of the study or the requirement to take prophylactic antibiotics for invasive dental procedures; • Significant organ disease including impaired kidney function, heart murmur, rheumatic fever, bleeding disorder; • Active infectious diseases such as hepatitis, tuberculosis and HIV; • Clinically detectable caries and/or periodontal disease; • Tobacco use of any kind; • Use of medications affecting periodontal status (diphenylhydantoin, cyclosporine,

	etc.) • No pregnancy or lactation or expectation to become pregnant within next 3 months.

3.2 *Clinical study design*

This is a controlled cross-arch study. The test site included two Straumann Tissue Level implants supporting fixed restorations in function for at least one year. The control site included two teeth in the corresponding position of the implants. Subjects were seen at a screening visit and at a "pre-experimental phase" visit to collect information on naturally occurring gingivitis and peri-implant mucositis if present. After prophylaxis to achieve optimal gingival and mucosal health, a customized stent was given to subjects to wear during toothbrushing and flossing on test and control sites to prevent daily plaque removal from these areas. Oral hygiene procedures were resumed after 21 days of plaque accumulation. Clinical parameters were measured and saliva, GCF and biofilm samples were obtained weekly for the duration of the study (Figure 1).

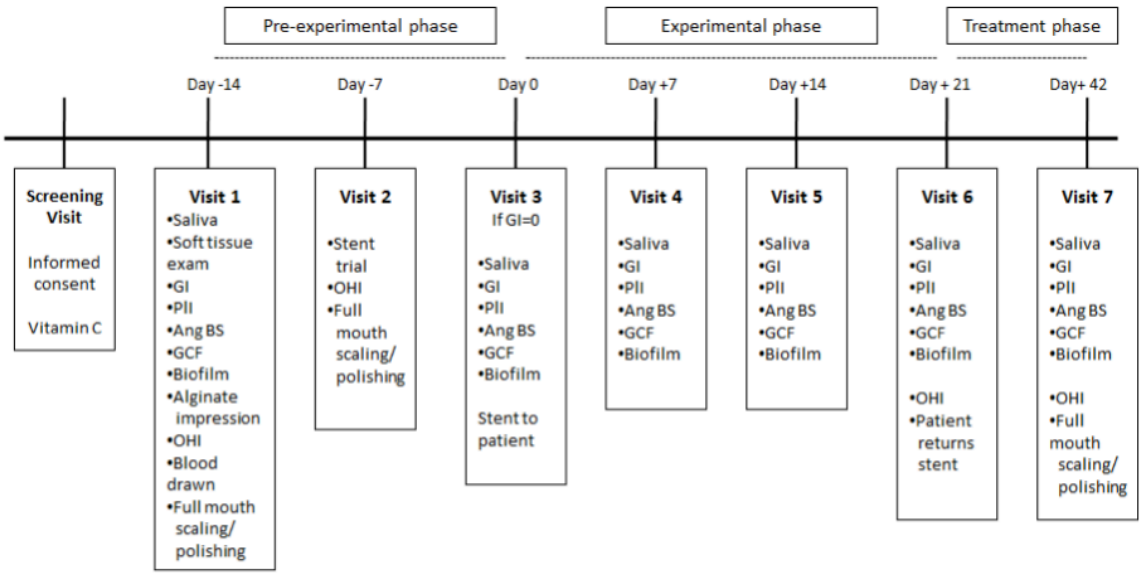


Figure 1. Experimental design and procedures. GI, modified plaque index (PlI) and angulated bleeding score (ABS) were recorded. GCF samples were collected for GCF flow determination and host marker evaluations. Microbial sampling was also performed Blood and saliva samples were stored at -80°C for future analysis.

3.3 *Clinical study procedures and sample collection*

3.3.1 Informed consent

During the screening visit, the experimental procedure was explained and a written informed consent was presented to the patient. Inclusion and exclusion criteria were verified.

3.3.2 Vitamin C administration

To prevent subclinical vitamin C deficiency that could affect susceptibility to inflammation, eligible subjects received a supply of vitamin C supplement (250 mg/day) to take for the 56-day pre-trial and trial period.

3.3.3 GCF flow rate

For determination of GCF flow rate, GCF was collected for 5 seconds from the mesial and buccal surfaces of the most posterior teeth and implants and measured according to Periotron 8,000 manufacturer's instructions (OraFlow Inc., Plainview, NY, USA). Following isolation of the site with cotton rolls, a Periopaper strip was inserted 1-2 mm subgingivally into the gingival sulcus.

3.3.4 Collection of GCF for host marker evaluation

GCF was also collected for host marker evaluation. GCF was collected for 30 seconds from the mesial and buccal surfaces of the most anterior teeth and implants. Samples were placed on ice, transported to the lab and stored at -80°C.

3.3.5 Clinical exam

GI was recorded on the mesial and buccal surfaces of teeth and implants using the Silness and Löe (1964) [42] method without the bleeding component, according to the modification described by Trombelli et al. (2004) [43]. Plaque Index (PlI) on mesial and buccal surfaces of teeth and implants was measured using the Silness and Löe (1964)[67] method and recorded according to Furuichi et al. (1992)[44]. ABS, according to the modification of the score by van der Weijden et al. (1994)[45], was recorded on the mesial and buccal surfaces of teeth and implants.

3.3.6 Biofilm collection

Biofilm samples were collected at the distal surfaces of the most posterior implant and tooth for visits 1, 3, 5 and 7. For visit 4, plaque was collected from the distal surfaces of the most anterior implant and tooth. A plastic curette was inserted into the sulcus and the biofilm was removed by a single stroke. The sample was placed into a 50µl Tris-EDTA (TE) buffer solution, which was stored at -80°C until further microbiological analysis.

3.3.7 Stent fabrication

An alginate impression was made for fabrication of the stent necessary to prevent disturbance of plaque accumulation during oral hygiene procedures.

3.3.8 Oral hygiene and prophylaxis during visits 1 and 2

Oral hygiene instruction (OHI) was provided to subjects. A toothbrush, floss and non-fluoridated toothpaste were distributed to each participant and participants were instructed to abstain for mouth rinse use during the study. Patients also received full mouth scaling and polishing during visit 1. If GI did not equal zero on visit 2, another session of scaling, polishing and OHI was provided prior to proceeding with the protocol. Lastly, patients were provided with the stent and instructed to wear it for 21 days during oral hygiene practices.

3.3.9 Colgate Total® toothpaste administration and reinstitution of oral hygiene

At the end of visit 6 (day +21), OHI was given and subjects returned the stent and reinstituted oral hygiene practices. Subjects were provided with Colgate Total® toothpaste to be used daily. In the final visit, subjects were given OHI and scaling and polishing were also provided.

3.3.10 Exam and sample collection sequence

During each visit, GI was recorded first. Second, GCF was collected for GCF flow rate determination and host marker evaluation. Paper strips contaminated with saliva and/or blood were discarded. Then biofilm samples were obtained. Following GCF and biofilm

sample collection, ABS was measured. Lastly, plaque was stained with disclosing solution, if require, and PlI was recorded.

3.4 *Microbiological laboratory procedures*

DNA was isolated from individual plaque samples and quantified. Total microbial load was determined via quantitative real time PCR. Microbiome composition of the samples was determined via high-throughput sequencing of the 16S rRNA gene using the Illumina MiSeq platform [46].

3.4.1 DNA isolation

Plaque samples were collected and placed in 50 µl of TE buffer. Samples were re-suspended in lysis buffer with lysozyme at 37°C for 30 minutes. Next, AL buffer and proteinase K (Qiagen DNA extraction kit) were added and the suspension was incubated at 56°C overnight. Samples were then incubated at 95°C for 5 minutes to inactivate proteinase K. DNA isolation was performed using a commercially available kit according to the instruction of the manufacturer (Qiagen DNeasy Blood and Tissue kit). DNA was eluted in 50 µl of MD5 solution (MoBio Laboratories) and its concentration was measured using a NanoDrop instrument (ThermoScientific). A positive control (180 µl of a known bacterial culture) was included to verify technique performance and a negative control (180 µl of lysis and TE buffers without any sample) was included for the assessment of sample contamination by foreign DNA.

3.4.2 Subgingival bacterial load quantification by real time PCR

The determination of the subgingival bacterial load of plaque samples was performed via real-time PCR using a TaqMan probe (Table 2) and universal 16S rRNA primers (Table 3) [47]. Samples were analyzed in singlicate in 96-well plates with a CFX96 Real-time system C1000 Thermocycler (BioRad). Standard curves were generated using DNA from serial dilutions of a pure culture of *F. nucleatum*.

Table 2. TaqMan probe [47].

(6-FAM)-5'-CGTATTACCGCGGCTGCTGGCAC-3'-(TAMRA)

Table 3. Universal primers used to determine subgingival bacterial load [47].

Forward: 5'-TCCTACGGGAGGCAGCAGT-3'
Reverse: 5'-GGACTACCAGGGTATCTAATCCTGTT-3'

Each reaction was performed in a total volume of 50 µl containing 25µl TaqMan Master Mix, 5 µl of each forward and reverse primer, PCR water (9 µl for plaque samples; 5 µl for standard curve), and 1 µl plaque DNA sample or 5µl of diluted DNA from *F. nucleatum* for standard curves. The qPCR cycle consisted of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The quantification of subgingival bacterial load was calculated using qPCR software (CFX Manager, Bio-Rad) and *F. nucleatum* standard curve with known 16S rRNA copy number.

3.4.3 Microbiome determination

Amplicon libraries were prepared using fusion primers with adaptor, indices, spacer and primers. Tables 4-6 list fusion primers used for MiSeq sequencing. Figures 2 and 3 provide schematic representations of the fusion primers and sequencing strategy,

respectively. These primers amplify a 423 bp region of the 16S rRNA gene spanning V1 and V2, the hypervariable regions that perform best in the assignment of species taxonomy to short sequence reads [48].

Table 4. Forward and reverse adaptor sequences used in fusion MiSeq primers.

Forward and reverse adaptor sequences
5'-CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'
5'- AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

Table 5. Sequences of universal primers used in fusion MiSeq primers.

Universal primers
Forward 5'-AGAGTTTGATCMTGGCTGAC-3'
Reverse 5'-CYIACTGCTGCCTCCCGTAG-3'

Table 6. Forward indices used in MiSeq primers.

Forward indices
GTGGTATGGGAG
ACTTTAAGGGTG
GAGCAACATCCT
TGTTGCGTTTCT
ATGTCCGACCAA
AGGTACGCAATT
ACAGCCACCCAT
TGTCTCGCAAGC
GAGGAGTAAAGC
GTTACGTGGTTG
TACCGCCTCGGA
CGTAAGATGCCT
TACCGGCTTGCA
ATCTAGTGGCAA
CCAGGGACTTCT
CACCTTACCTTA
ATAGTTAGGGCT
GCACTTCATTTC

Table 7. Reverse indices used in Miseq primers.

Reverse indices
TGCAGATCCAAC
CCATCACATAGG
GTGGTATGGGAG
ACTTTAAGGGTG
ATGTCCGACCAA
AGGTACGCAATT
ACAGCCACCCAT
TGTCTCGCAAGC
GAGGAGTAAAGC
GTTACGTGGTTG
TACCGCCTCGGA
CGTAAGATGCCT
TACCGGCTTGCA
ATCTAGTGGCAA
CCAGGGACTTCT
CACCTTACCTTA
ATAGTTAGGGCT

Figure 2. Fusion primers designed for bidirectional sequencing using the MiSeq Illumina platform. Primers included 5' and 3' adaptor sequences, index 1 and 2, heterogeneity spacers, forward and reverse 16S rRNA gene universal primers and bacterial template. Indices served to tag each sample, allowing for massive parallel sequencing. Heterogeneity spacers were 0 to 7 bp sequences implemented to introduce library diversity.

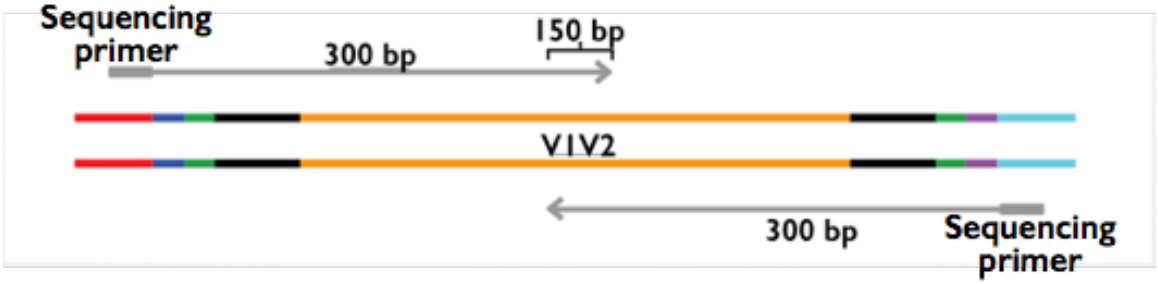


Figure 3. Sequencing strategy. A 528 bp amplicon was generated and sequenced bidirectionally with the MiSeq Reagent Kit v3 (300 cycles). A 150 bp overlap was expected after assembling the forward and reverse reads.

PCR was performed in thin-walled tubes with a C1000 Touch Thermocycler (BioRad, Hercules, CA). Each reaction was performed in triplicate using a total volume of 20 µl containing 10.5 µl water, 1.2 µl forward primer, 1.2 µl reverse primer, 0.4 µl 10mM dNTPs, 0.6 µl 50mM $MgSO_4$, 0.125 µl Platinum Taq polymerase and 2 µl 10X buffer. Thermal cycler conditions were as follows: pre-heating of samples at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and elongation at 72°C for 60 seconds. This was followed by a final elongation step at 72°C for 9 minutes. Reaction products were evaluated by gel electrophoresis (1.5% agarose gel run at 100V for 20 minutes), DNA was stained using ethidium bromide and visualized under short-wavelength UV-light (Gel-Doc system, BioRad). The AmpureXP bead was used to purify PCR products and select fragments of a size greater than 300 bp. Finally, amplicon library quantification analysis was conducted using Qubit (Life Technologies) and bacterial amplicon libraries were sequenced with Illumina MiSeq V3 chemistry (MiSeq Sequencer). For this, the sample library and 20% PhiX were denatured and loaded to the flow cell.

3.4.4 Processing and analysis of bacterial sequences

Sequences obtained from MiSeq were processed using the mothur software [49]. Forward and reverse sequence reads were trimmed using a sliding window strategy by quality score (Q30 > 35) and a window size of 50 bp. Forward and reverse reads were then assembled. Primers and indices were trimmed and sequences filtered to exclude sequences with ambiguous base calls and read lengths less than 200 bp and 400 bp. Unique sequences were aligned using the SILVA database as a reference [50] and ends

trimmed such that sequences only included a comparable anchor region. Sequences were further processed by a modification of the single linkage algorithm [51,53] to determine sequences with up to 2 bp difference from a more abundant sequence and merge their counts, which reduces variability thereby reducing errors caused by sequencing. Chimeric sequences were then removed by applying the UChime algorithm [53] as implemented in mothur. Next, a distance matrix was produced and sequences were clustered into OTUs based on a 3% difference [54]. Classification of sequences was performed using the Ribosome Database Project (RDP) classifier with the RDP reference trainset [55] or the Human Oral Microbiome Database (HOMD) trainset (www.homd.org) as templates. OTUs were classified according to the majority taxonomic assignments for reads in each OTU. If a taxonomic assignment at the species level was not possible, the most abundant sequence from each OTU was compared by Blast to the HOMD. In cases of more than 97% homology of the representative sequence to an HOMD reference sequence, the HOMD match was also included in parenthesis as part of the OTU taxonomy.

Alpha diversity analysis comprised richness, evenness and total diversity. Richness was assessed by evaluation of the number of observed OTUs. Evenness was obtained using the Shannon evenness index. Total diversity was evaluated by the inverse of the Simpson Index and the non-parametric Shannon index [56-58]. Beta diversity was measured with the Theta YC distance [59] for comparison of communities based on structure. PCoA was performed in mothur and graphs were visualized using rgl application in R (http://www.r-project.org).

3.5 *Host inflammatory marker analysis*

GCF volume was measured with the Periotron 8000 (OraFlow Inc., Plainview, NY), as it has been shown to correlate with peri-implant and gingival inflammation [43]. The site was first isolated with cotton rolls to prevent contamination with saliva. Then a Periopaper strip was gently inserted 1-2 mm into the gingival sulcus by a calibrated examiner for 5 seconds for GCF volume measurement or 30 seconds for host marker evaluation. Samples contaminated with blood were discarded. Functional activation of PMNs was assessed by MPO and lactoferrin GCF levels as described below. MPO was assayed as representative of neutrophilic oxidative killing mechanisms. Lactoferrin was measured as representative of non-oxidative killing mechanisms. GCF levels of IL-1 alpha, IL-1 beta, IL-1 ra, IL-8, TNF-alpha, CCL22 and GRO (alpha, beta and gamma) were quantified with a multiplex assay, allowing for simultaneous cytokine quantification (EMD Millipore, Billerica, MA).

3.5.1 Lactoferrin and MPO quantification in GCF

MPO and lactoferrin in GCF were quantified by enzyme-linked immunosorbent assay (ELISA). Elution buffer was prepared with 0.05% Tween 20, 1 mg/ml BSA in PBS with complete proteinase inhibitor (Roche Life Science, Indianapolis, IN) and filtered with 0.22 μm pore size syringe filter. Each paper strip was eluted with 60 μl of elution buffer, vortexed and stored overnight at 4°C. Then samples were vortexed, briefly centrifuged so that all elution buffer remained in the bottom of the eppendorf tube and both the eluted paper strips and GCF samples were transferred to new eppendorf tubes separated by a

basket. Samples were then centrifuged at 13,000 rpm for 10 minutes. Eluted samples were then diluted. Optimal dilutions for lactoferrin and MPO were determined after pilot trials.

Lactoferrin ELISA was performed with a commercially available kit according to the instructions of the manufacturer (Abcam, Cambridge, MA). Fifty µl of the GCF samples diluted by 1:500 were added to 96 wells that were precoated with a lactoferrin-specific antibody and incubated for two hours. Subsequently, a lactoferrin-specific biotinylated detection antibody was added, incubated for one hour and washed with wash buffer. Streptavidin-peroxidase conjugate was added, incubated for 30 minutes and unbound conjugates were washed away with wash buffer. Chromogen substrate was then used to visualize the streptavidin-peroxidase enzymatic reaction. This substrate produced a blue color product that changed to yellow after adding acidic stop solution. The absorbance was read on a microplate reader at a wavelength of 450 nm.

A similar assay was completed for MPO quantification (R & D, Minneapolis, MN). An MPO-specific mouse monoclonal antibody was precoated on 96-well plates. Standards and test samples diluted by 1:2000 were added to the wells and incubated. The biotinylated detection MPO-specific goat polyclonal antibody was added followed by washing. Avidin-Biotin-Peroxidase Complex was added and incubated. Subsequently, TMB was used to visualize the enzymatic reaction and absorbance was read on a microplate reader. Target concentrations for MPO and lactoferrin were calculated with a four-parameter logistic equation using SOFTmax Pro software (Sunnyvale, CA).

3.5.2 IL-1 alpha, IL-1 beta, IL-1 ra, IL-8, TNF-alpha, GM-CSF, CCL22 and GRO quantification in GCF

IL-1 alpha, IL-1 beta and IL-1 ra, IL-8, TNF-alpha, GM-CSF, CCL22 and GRO (alpha, beta and gamma) in GCF were analyzed by multiplex assay, allowing for simultaneous quantification of multiple cytokines (EMD Millipore). After a pilot trial, it was determined that IL-1 ra required a 1:10 dilution. Samples for other cytokines were not diluted. For multiplex cytokine detection, microspheres coated with fluorescent dyes and specific capture antibodies were added to the sample. After the bead captured an analyte from the test sample, a biotinylated detection antibody was introduced and the reaction mixture was incubated with streptavidin conjugate (reporter molecule) to complete the reaction on the surface of each microsphere. Samples were passed through a primary laser, exciting the dyes to mark the microsphere, and then passed through a secondary laser, exciting the fluorescent dye on the reporter molecule. High-speed digital-signal processors identified each microsphere and quantified the result of its bioassay based on the fluorescent reporter signals (Luminex 200). Target concentrations were calculated with a four-parameter logistic equation using SOFTmax Pro software (Sunnyvale, CA).

3.6 *Statistical analysis*

Clinical microbiological and host inflammatory marker assessments were performed across groups for Aims 1 and 3. For Aims 2 and 3, both within group and across group comparisons were performed. Statistical significance was considered when p values were

<0.05 and below the threshold determined by multiple testing using the Benjamini-Hochberg false discovery rate method.

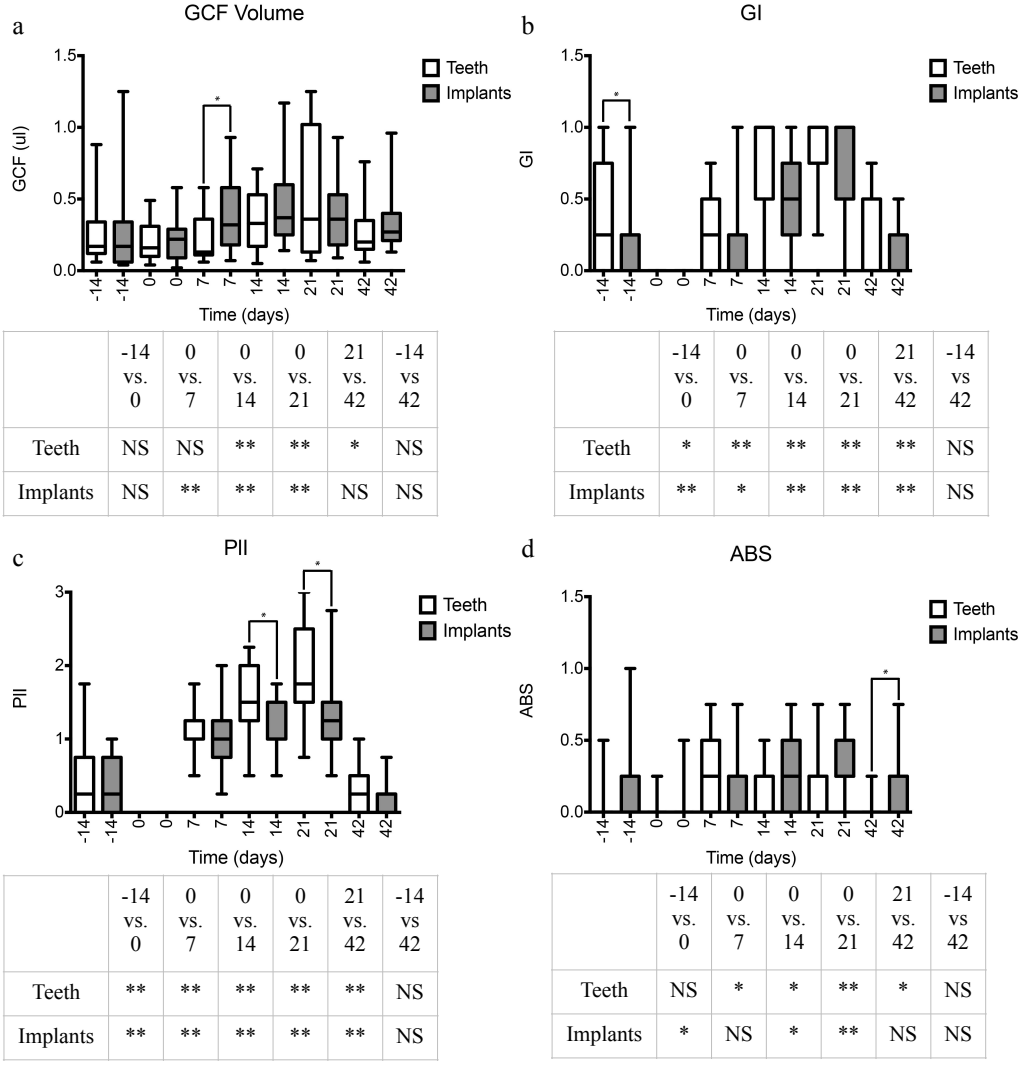
Paired-sample Wilcoxon Signed-Rank tests were employed to assess differences in clinical parameters, host inflammatory markers, subgingival bacterial load, alpha diversity and OTU abundance. For comparisons of OTU relative abundance, only those OTUs with at least 0.1% relative abundance in at least one sample were considered (533 OTUs). Significant separation of clusters after PCoA analysis was evaluated via analysis of molecular variance (AMOVA) [60]. Bivariate Spearman's Rank-Order Correlation tests were used to evaluate correlation of clinical, host and microbiological variables in implant and teeth sites (between group correlations), to evaluate the correlation among all variables within a group (within group correlations) and to evaluate the correlation of OTU relative abundance with Principal Component 1 in PCoA plots.

4. Results

4.1 General considerations

Clinical results were presented in a thesis by Dr. Jonah Barasz and have been updated for this thesis (Figure 4). Fifteen patients out of 18 enrolled completed the trial. One patient was excluded from the study when suppuration of the mucosal margin at a test site was observed at visit 5 (day 14). The site was treated with debridement, irrigation and 0.12% chlorhexidine rinse for seven days. The condition resolved completely after seven days. Two subjects exited the study due to changes in medical condition unrelated to the study proceedings prior to visit 1 (day -14) and visit 2 (day -7). Figures 5, 6, and 7 present

results of inflammatory mediator assays. GM-CSF was not included because it was non-detectable in most samples. Subgingival bacterial load measurement via qPCR is shown in Figure 8. Microbiome sequencing yielded 11,167,919 sequences after preprocessing, with an average of 27,674 sequences per sample. The range of sequences per samples was 13 to 53,374, however. Since comparison among samples requires the same number of reads, samples were subsampled to contain 5,298 sequences. This excluded 13 samples from the analysis (visit 1 for subjects 11 (control), 14 (control and test) and 17 (control); visit 3 for subjects 2 (test), 4 (test), 14 (test), 17 (control and test) and 18 (control); visit 4 for subject 7 (test); visit 7 for subjects 5 (test) and 16 (control)).



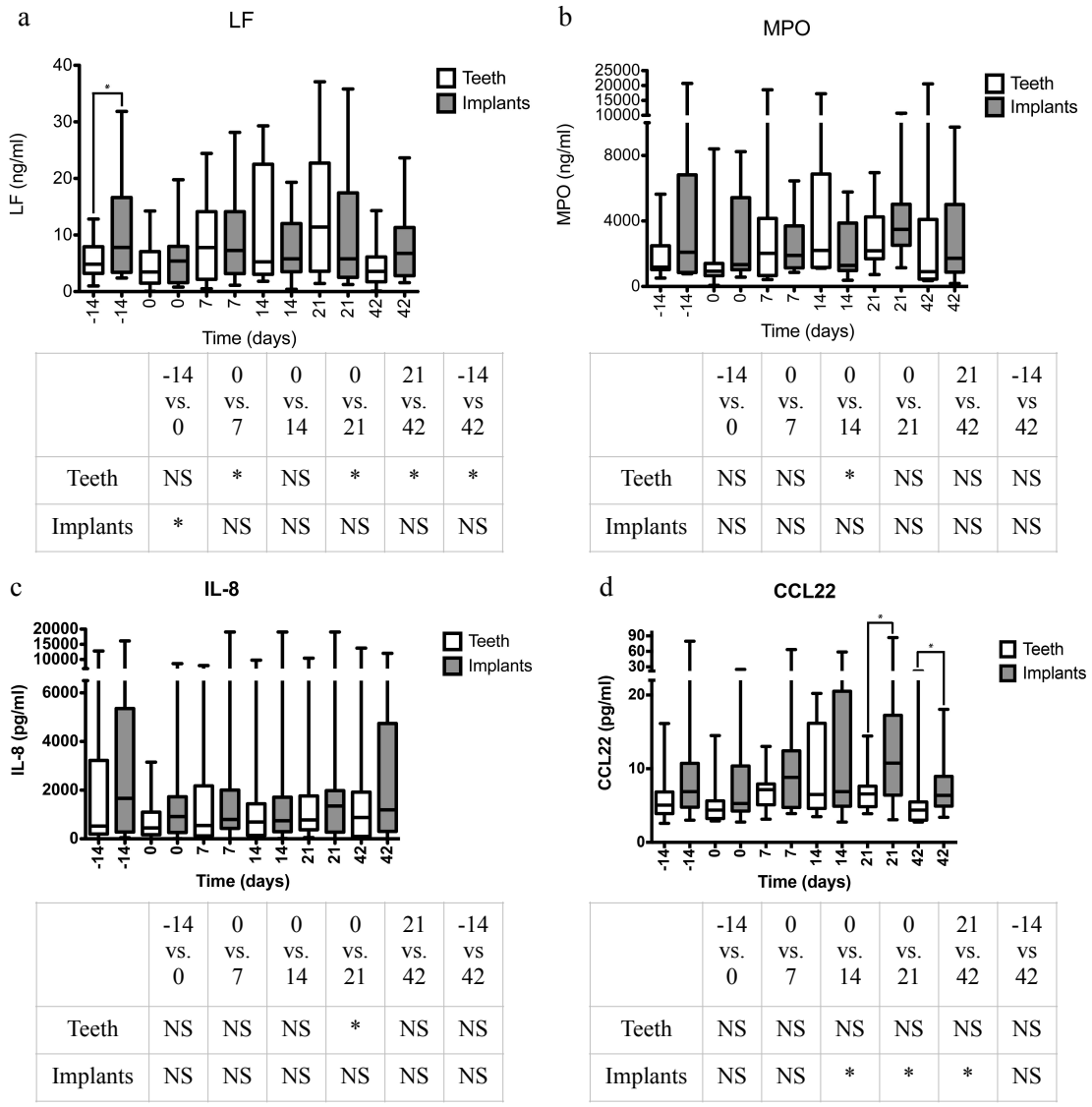
	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	NS	**	**	*	NS
Implants	NS	**	**	**	NS	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	*	**	**	**	**	NS
Implants	**	*	**	**	**	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	**	**	**	**	**	NS
Implants	**	**	**	**	**	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	*	*	**	*	NS
Implants	*	NS	*	**	NS	NS

Figure 4. Clinical response of teeth and implants during the trial. Box plots show GCF volume (a), GI (b), PlI (c) and ABS (d) in test (implants) and control (teeth) sites during study visits. Within group comparisons are shown in tables below the graphs. Across and within group differences at each time point are indicated by asterisks. * = $p<0.05$; ** = $p<0.01$



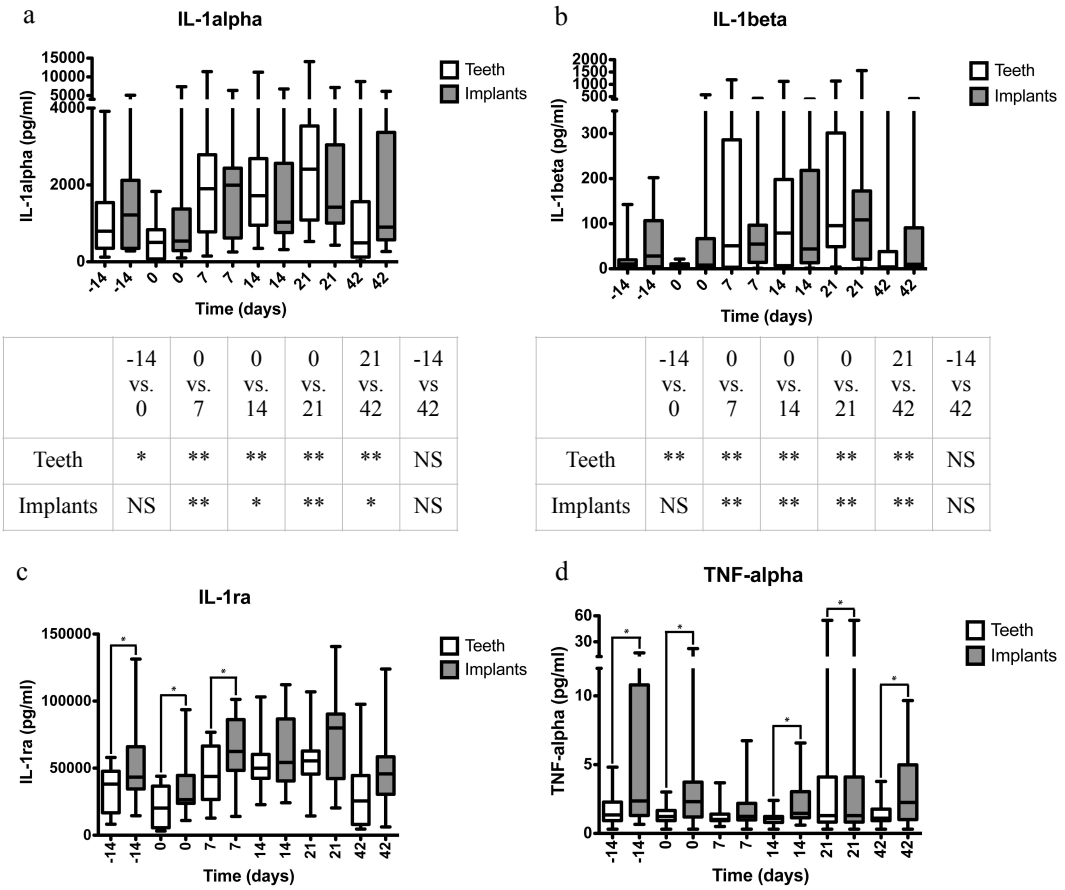
	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	*	NS	*	*	*
Implants	*	NS	NS	NS	NS	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	NS	*	NS	NS	NS
Implants	NS	NS	NS	NS	NS	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	NS	NS	*	NS	NS
Implants	NS	NS	NS	NS	NS	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	NS	NS	NS	NS	NS
Implants	NS	NS	*	*	*	NS

Figure 5. Inflammatory response of teeth and implants during the trial. Box plots show lactoferrin (LF; a), MPO (b), IL-8 (c) and CCL22 (d) concentrations in test (implants) and control (teeth) sites during study visits. Within group comparisons are shown in tables below the graphs. Across and within group differences at each time point are indicated by asterisks. * = $p<0.05$



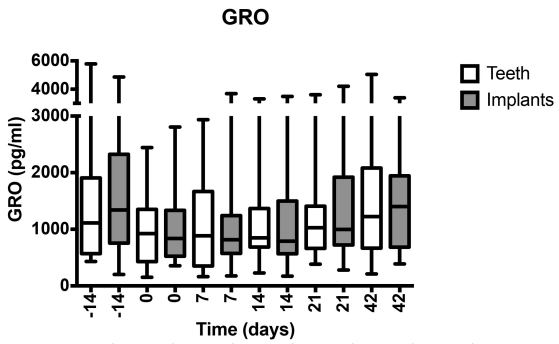
	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	*	**	**	**	**	NS
Implants	NS	**	*	**	*	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	**	**	**	**	**	NS
Implants	NS	**	**	**	**	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	**	**	**	**	**	NS
Implants	*	**	**	**	**	NS

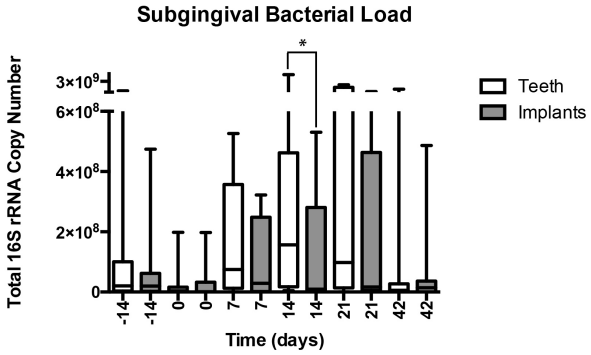
	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	NS	NS	NS	NS	NS
Implants	NS	NS	NS	NS	NS	NS

Figure 6. Inflammatory response of teeth and implants during the trial. Box plots show IL-1 alpha (a), IL-1 beta (b), IL-1 ra (c) and TNF-alpha (d) concentrations in test (implants) and control (teeth) sites during study visits. Within group comparisons are shown in tables below the graphs. Across and within group differences at each time point are indicated by asterisks. * = $p<0.05$; ** = $p<0.01$



	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	NS	NS	NS	NS	NS
Implants	*	NS	NS	NS	NS	NS

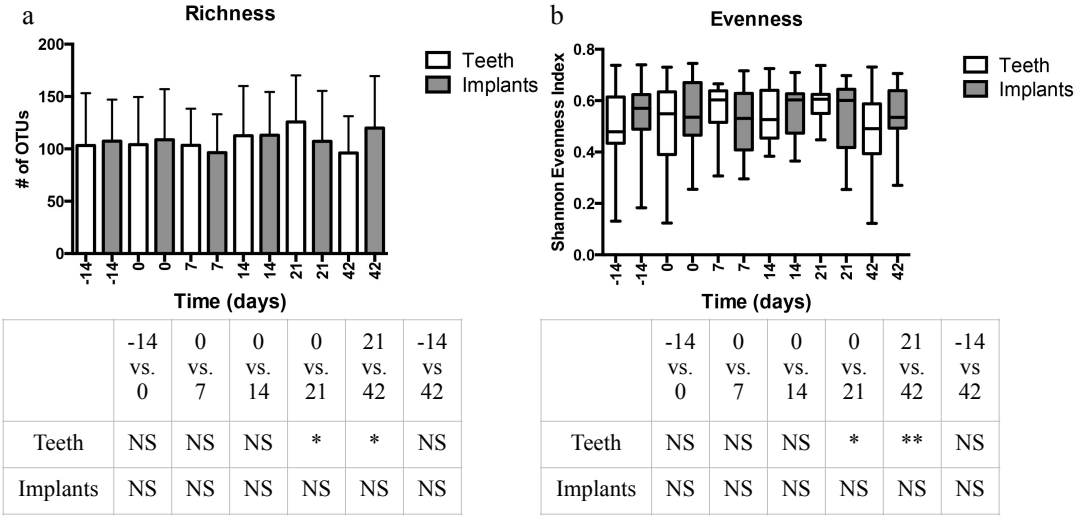
Figure 7. Inflammatory response of teeth and implants during the trial. Box plot shows GRO concentration test (implants) and control (teeth) sites during study visits. Within group comparisons are shown in tables below the graph. * = $p<0.05$



	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	**	**	**	**	**	NS
Implants	**	*	*	**	*	NS

Figure 8. Microbiological response of teeth and implants during the trial. Box plot shows subgingival bacterial load in test (implants) and control (teeth) sites during study visits.

Within group comparisons are shown in table below the graph. Across and within group differences at each time point are indicated by asterisks. * = p<0.05; ** = p<0.01



	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	NS	NS	*	*	NS
Implants	NS	NS	NS	NS	NS	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	NS	NS	*	**	NS
Implants	NS	NS	NS	NS	NS	NS

	-14 vs. 0	0 vs. 7	0 vs. 14	0 vs. 21	21 vs. 42	-14 vs 42
Teeth	NS	NS	NS	NS	*	NS
Implants	NS	NS	NS	NS	NS	NS

Figure 9. Microbiological response of teeth and implants during the trial. Box plots shows alpha diversity, as measured by richness (a), evenness (b), non-parametric Shannon Index (c) and Inverse Simpson Index (d), in test (implants) and control (teeth) sites during study visits. Within group comparisons are shown in tables below the graphs. Across and within group differences at each time point are indicated by asterisks. * = p<0.05; ** = p<0.01

4.2 *Results related to Aim 1*

The first aim of this thesis (Aim 1) was to cross-sectionally compare the microbial biofilms and GCF inflammatory markers between teeth and implants at the baseline visit (day -14), assuming this visit represents the native state ranging from health to gingivitis or peri-implant mucositis.

4.2.1 Clinical results

On day -14, GI was significantly higher at teeth compared to implants (Figure 4b). There were no significant differences in GCF volume, PlI or ABS, however (Figure 4a, c, d).

4.2.2 GCF inflammatory marker results

Although GI, a marker for clinical inflammation, was higher in teeth than implants on day -14 and ABS, GCF volume and PlI did not differ between groups, implants demonstrated significantly higher concentrations of lactoferrin (Figure 5a), IL-1 ra (Figure 6c) and TNF-alpha (Figure 6d) compared to teeth. There was also a trend for higher MPO (Figure 5b), CCL22 (Figure 5d), IL-1 alpha (Figure 6a), IL-1 beta (Figure 6b), IL-8 (Figure 5c) and GRO (Figure 7) concentration at implants compared to teeth at this time point. These results suggest that implants demonstrate higher inflammation at the molecular, but not clinical, level than teeth during the native state.

4.2.3 Microbiological results

There were no significant differences in subgingival bacterial load (Figure 8) or alpha diversity, as measured by richness (Figure 9a), evenness (Figure 9b), non-parametric

Shannon Index (Figure 9c) and Inverse Simpson Index (Figure 9d) between implants and teeth. Although implants and teeth had equal microbial load, as measured by PlI and 16S rRNA copy number, and teeth had increased clinical inflammation, as measured by GI, implants demonstrated higher concentrations of pro-inflammatory cytokines than teeth during the native state.

On day -14, PCoA of distance among samples based on an ecology metric that takes into account OTU presence and proportions (Theta YC) revealed no differences between teeth and implants on day -14, suggesting that community structure in teeth and implants is the same in the native condition (Figure 10).

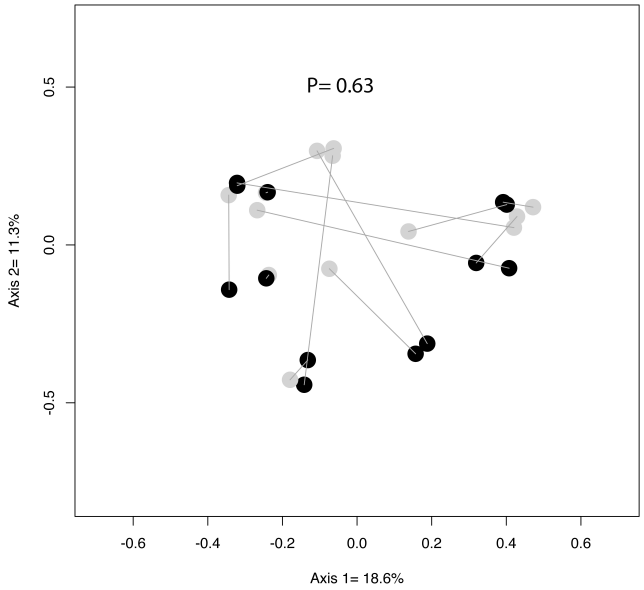


Figure 10. PCoA plot of community structure (Theta YC) in teeth and implants on day -14. Gray = teeth; black = implants.

Prior to adjusting for multiple comparisons, the relative abundances of *Propionibacterium propionicum* ($p<0.05$), *C. gracilis* ($p<0.05$), *Capnocytophaga* sp. (HOT336; $p<0.05$), *Selenomonas sputigena* ($p<0.05$) and *A. gerencseriae* ($p<0.05$) were significantly greater in teeth than implants (Figure 11). On the other hand, *Pasteurellaceae* sp. (*Terrahaemophilus aromaticivorans* HOT826; $p<0.05$), *Prevotella melaninogenica* ($p<0.05$) and *Prevotella salivae* ($p<0.05$), were significantly greater in implants than teeth (Figure 11). However, after adjusting for multiple comparisons, there were no significant differences in relative abundance of any of the OTUs between implants and teeth at this time point.

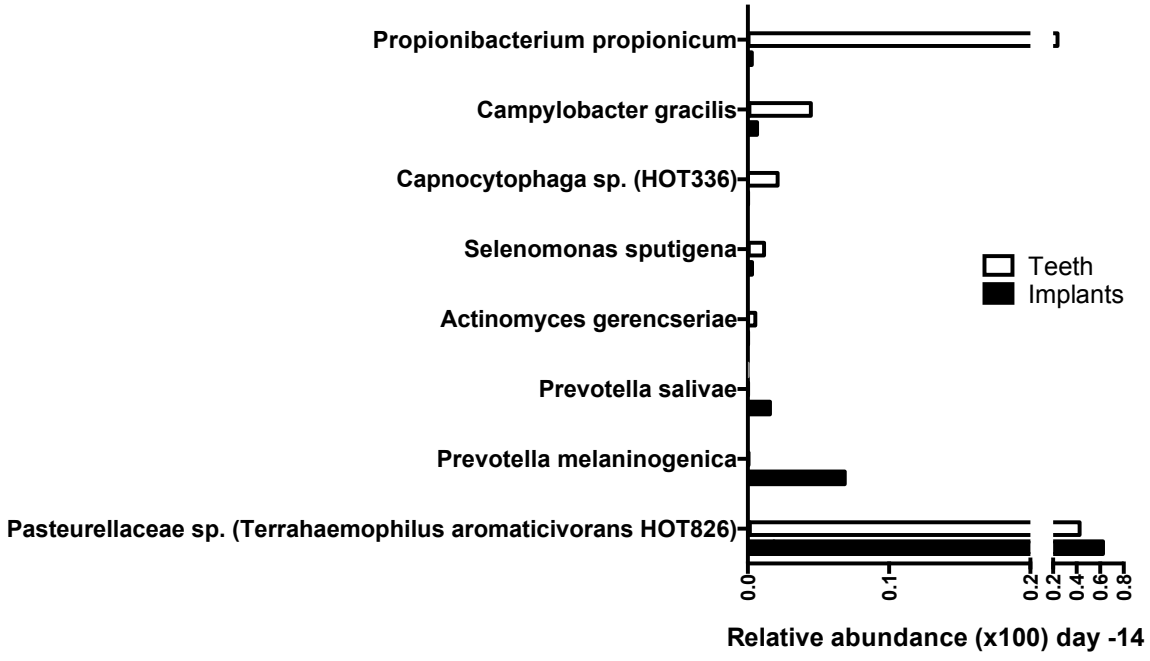


Figure 11. Relative abundances of OTUs that were statistically significantly different between teeth and implants on day -14 prior to adjusting for multiple comparisons. Bars represent range of relative abundance values.

4.3 *Results related to Aim 2*

The second aim of this thesis (Aim 2) was to longitudinally evaluate the changes in the microbial flora and GCF inflammatory markers during 21 days of *de novo* plaque accumulation around dental implants in comparison to teeth.

4.3.1 Clinical results

At baseline (day 0), GI (Figure 4b), PlI (Figure 4c) and ABS (Figure 4d) values were zero for both teeth and implants, indicating absence of clinical signs of inflammation. During the experimental phase, both teeth and implants accumulated plaque and developed clinical signs of inflammation, peaking at day 21 (Figure 4). GCF volume (Figure 4a), GI (Figure 4b), PlI (Figure 4c) and ABS (Figure 4d) were significantly higher on day 21 compared to day 0 for both teeth and implants. However, teeth and implants showed differences in terms of plaque accumulation, with implants accumulating less plaque during the experiment (Figure 4c). Consistent with this, GI tended to be lower in implants than in teeth at all time points during the experimental period, although these differences did not reach statistical significance. Interestingly, despite lower GI in implants, GCF volume was higher in implants when compared to teeth on day 7 of the experimental phase. This trend was also observed on day 14. ABS values did not differ statistically between teeth and implants but there was a trend for higher ABS in implants compared to teeth during experimental plaque accumulation.

4.3.2 GCF inflammatory marker results

The decrease in plaque accumulation and clinical inflammation from day -14 to day 0 corresponded with lower concentrations of several of the inflammatory markers. Only IL-1 ra (Figure 6c) was significantly lower on day 0 compared to day -14 for both teeth and implants. For implants only, the absence of plaque on day 0 resulted in decreased lactoferrin (Figure 5a) and GRO (Figure 7) compared to day -14. For teeth only, IL-1 alpha (Figure 6a) and IL-1 beta (Figure 6b) were significantly reduced on day 0 compared to day -14.

As plaque accumulation and clinical signs of inflammation increased over the experimental phase, many of the inflammatory markers also increased. Members of the IL-1 cytokine family seemed to be the most consistent biomarkers for plaque accumulation around both implants and teeth (Figure 6). IL-1 alpha (Figure 6a), IL-1 beta (Figure 6b) and IL-1 ra (Figure 6c) demonstrated significantly greater concentration at day 21 compared to day 0 for both implants and teeth. The PMN activity marker lactoferrin (Figure 5a), and to a lesser extent MPO (Figure 5b) and the chemokine IL-8 (Figure 5c), marked experimental gingivitis but not peri-implant mucositis; whereas CCL22 (Figure 5d) was a marker for experimental peri-implant mucositis only. TNF-alpha (Figure 6d) and GRO (Figure 7) concentrations did not change significantly throughout the experimental period for either teeth or implants.

Across group comparisons showed that on day 7, IL-1 ra (Figure 6c) was significantly higher at implants compared to teeth. On day 14, TNF-alpha (Figure 6d) was significantly higher at implants compared to teeth. On day 21, TNF-alpha (Figure 6d) and

CCL22 (Figure 5d) were significantly higher at implants compared to teeth. These results are consistent with the trend for implants to display higher levels of inflammatory markers than their teeth counterparts. However, there were no significant differences in lactoferrin (Figure 5a), MPO (Figure 5b), IL-8 (Figure 5c), IL-1 alpha (Figure 6a), IL-1 beta (Figure 6b) and GRO (Figure 7) between implants and teeth during the experimental phase.

The subject-specific magnitude of change in concentrations of inflammatory markers over the experimental phase was also evaluated. Deltas were calculated by subtracting the earlier visit value from the later visit value. The deltas from day 0 to 7 for IL-1 alpha, IL-1 beta and TNF-alpha were significantly greater for teeth compared to implants ($p<0.05$). The delta from day 0 to 14 for MPO was significantly greater for teeth compared to implants ($p<0.05$). These results suggest a greater early response for these biomarkers in teeth compared to implants.

In summary, IL-1 alpha, IL-1 beta and IL-1 ra were biomarkers for plaque accumulation around both teeth and implants. Lactoferrin was a marker of experimental gingivitis but not of peri-implant mucositis while CCL22 was a marker for experimental peri-implant mucositis only.

4.3.3 Microbiological results

As expected from the PlI results, subgingival bacterial load at teeth and implants was significantly lower on day 0 compared to day -14 (Figure 8). Subgingival bacterial load

increased significantly over the experimental phase, with a dramatic difference between day 0 and day 21 for both teeth and implants (Figure 8). None of the measures of alpha diversity were different on day 0 compared to day -14 for teeth or implants, however. Richness (Figure 9a), evenness (Figure 9b) and non-parametric Shannon Index (Figure 9c) increased from day 0 to day 21 for teeth but not implants. The Inverse Simpson Index did not differ throughout the experimental phase for teeth or implants. In accordance with the PlI results, subgingival bacterial load was significantly greater in teeth than implants on day 14 (Figure 8). There was no significant difference in alpha diversity (Figure 9) between implants and teeth during the experimental phase.

The magnitude of the change in subgingival bacterial load and alpha diversity over the experimental phase were also compared between teeth and implants. The delta from day 0 to 14 for subgingival bacterial load was significantly greater for teeth compared to implants ($p<0.05$).

PCoA revealed a significant difference in community structure on day 0 compared to day 7 ($p<0.01$) and day 0 compared to day 21 ($p<0.01$) for teeth, such that there was a shift along axis 1 as plaque accumulated. A similar trend, but not statistically significant, was observed from day 0 to 14. Since axis 1 captured most of the variability during experimental plaque accumulation in teeth, we then explored which OTUs correlated with PC1. Figures 12-14 show that *Veillonella parvula* and *R. dentocariosa* were correlated with PC1 from day 0 to 7, *R. dentocariosa* was correlated with PC1 from day 0 to 14 and *F. nucleatum* ss. *animalis* and *R. dentocariosa* were correlated with PC1 from

day 0 to 21. The shift along axis 1 comparing day 0 to 7 in teeth was positively correlated to *V. parvula* (r=0.76, p<0.01) and negatively correlated with *R. dentocariosa* (r=-0.78, p<0.01), suggesting that *V. parvula* may drive the shift in community structure during the first week of plaque accumulation while *R. dentocariosa* may drive community structure in health. The shift along axis 1 comparing day 0 to 14 in teeth was negatively correlated with *R. dentocariosa* (r=-0.86, p<0.01), indicating that *R. dentocariosa* may drive community structure in health. The shift along axis 1 comparing day 0 to 21 in teeth was positively correlated to *F. nucleatum* ss. *animalis* (r=0.78, p<0.01) and negatively correlated with *R. dentocariosa* (r=-0.89, p<0.01), suggesting *F. nucleatum* ss. *animalis* as the main driver of the shift in community structure after three weeks of plaque accumulation while, again, *R. dentocariosa* drives community structure during health. OTUs exhibiting a significant correlation with axis 1 in teeth prior to adjusting for multiple comparisons are displayed in Tables 8-13. These also point to expected trends, with species from the genera *Prevotella*, *Fusobacterium*, *Selenomonas*, *Mogibacterium*, *Atopobium*, *Treponema*, among others, positively correlating with the shift during plaque accumulation, while species from the genera *Actinomyces*, *Streptococcus*, *Rothia* and some beta-Proteobacteria exhibited a negative correlation. In contrast to the changes observed in community structure in teeth, which were expected and in accordance to previous studies [18,22], there was not a consistent shift in community structure over time for implants (Figures 15-17), suggesting that the microbiome response during experimental plaque accumulation differs in teeth and implants, with an unpredictable variable response in the latter. The Theta YC Index, which measures the distance between samples from one time point to another and thus differences in community

structure over time, for teeth and implants is displayed in Figure 18. This figure shows that the magnitude of the shift during the experimental period was the same for teeth and implants but implants did not shift in a predictable and consistent direction.

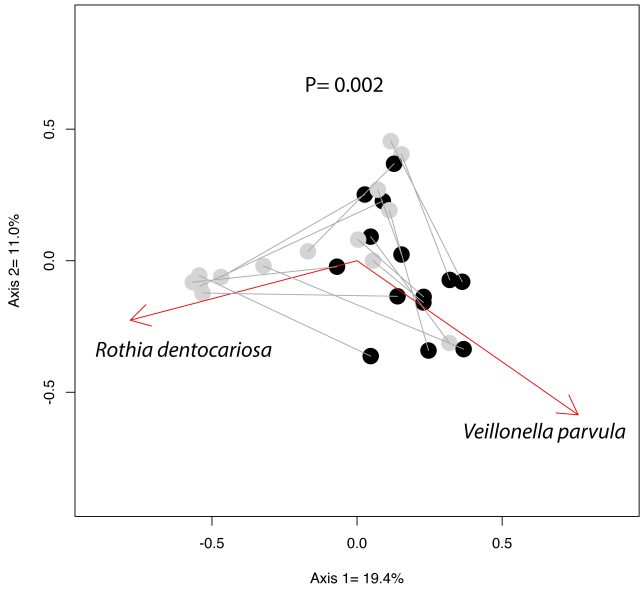


Figure 12. PCoA plot of community structure (Theta YC) in teeth from day 0 to day 7. Gray = day 0; black = day 7. Red arrows indicate OTUs whose relative abundance was significantly correlated with axis 1 and therefore drive variability along this axis.

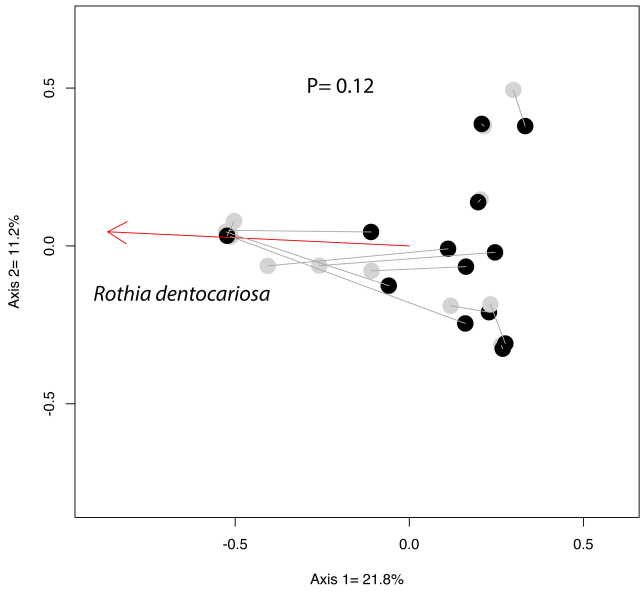


Figure 13. PCoA plot of community structure (Theta YC) in teeth from day 0 to day 14. Gray = day 0; black = day 14. Red arrow indicates an OTU whose relative abundance was significantly correlated with axis 1, driving variability along this axis.

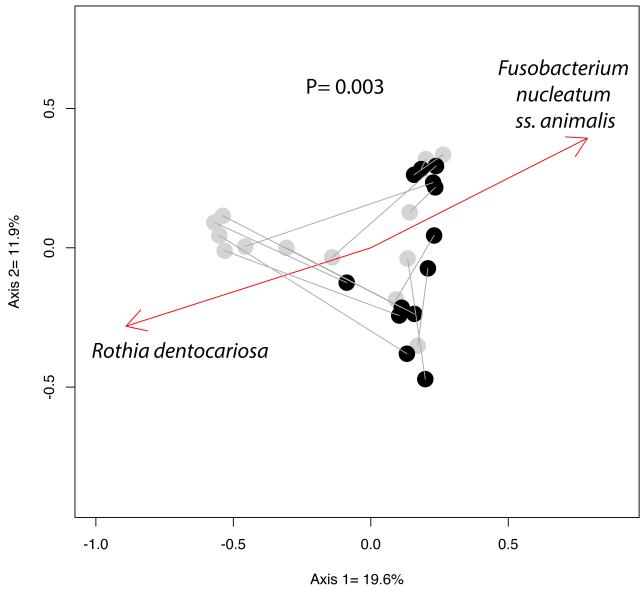


Figure 14. PCoA plot of community structure (Theta YC) in teeth from day 0 to day 21. Gray = day 0; black = day 21. Red arrows indicate OTUs whose relative abundance was significantly correlated with axis 1 and therefore drive variability along this axis.

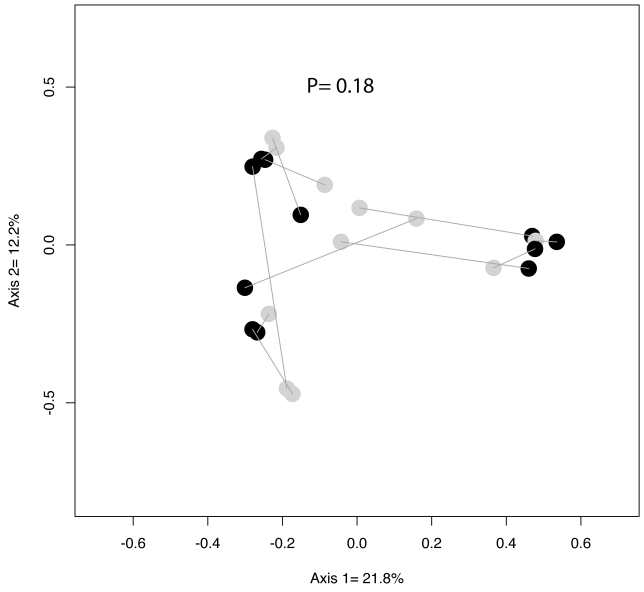


Figure 15. PCoA plot of community structure (Theta YC) in implants from day 0 to day 7. Gray = day 0; black = day 7.

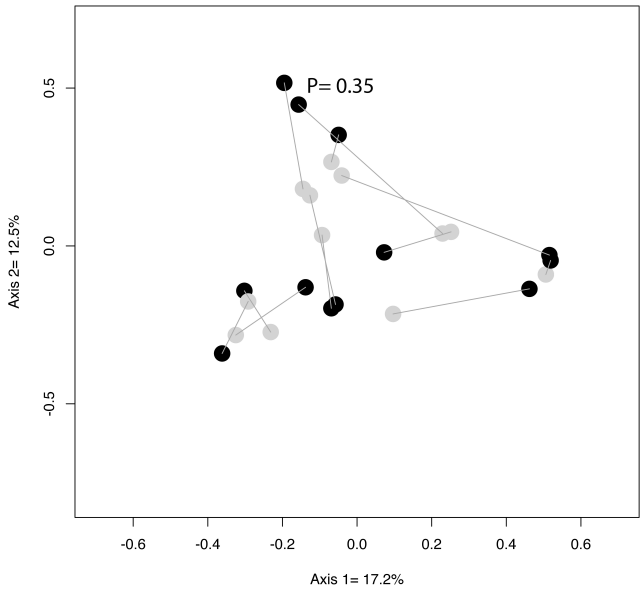


Figure 16. PCoA plot of community structure (Theta YC) in implants from day 0 to day 14. Gray = day 0; black = day 14.

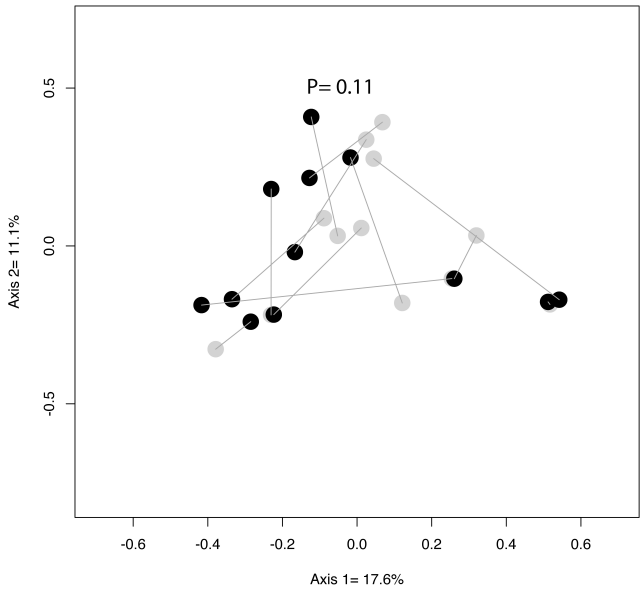


Figure 17. PCoA plot of community structure (Theta YC) in implants from day 0 to day 21. Gray = day 0; black = day 21.

Table 8. Positive Spearman's Rank-Order Correlations between OTUs and axis 1 for teeth from day 0 to day 7 prior to adjusting for multiple comparisons. * = $p<0.05$; ** = $p<0.01$

OTU	r
Veillonella parvula	0.76**
Actinomyces sp. (HOT170)	0.69**
Fusobacterium sp. (Fusobacterium nucleatum ss. animalis HOT420)	0.57**
Prevotella salivae	0.53**
Streptococcus sp. (HOT057)	0.51**
Atopobium rimae	0.47*
Actinomyces sp. HOT448	0.47*
Fusobacterium sp. (Fusobacterium nucleatum ss. nucleatum HOT698)	0.47*
Leptotrichia sp. HOT223	0.46*
Oribacterium sp. (HOT372)	0.43*
Selenomonas noxia	0.43*
Mogibacterium sp. (Mogibacterium diversum HOT593)	0.43*
Prevotella sp. HOT317	0.42*
Prevotella oris	0.42*
Neisseria bacilliformis HOT013	0.41*
Lachnoanaerobaculum sp. (Eubacterium [XIVa][G-1] saburreum HOT494)	0.40*
Prevotella denticola	0.39*
Dialister invisus	0.38*
Prevotella sp. HOT305	0.38*

Table 9. Negative Spearman's Rank-Order Correlations between OTUs and axis 1 for teeth from day 0 to day 7 prior to adjusting for multiple comparisons. * = $p<0.05$; ** = $p<0.01$

OTU	r
Rothia dentocariosa	-0.78**
Pseudomonas stutzeri	-0.49**
Acinetobacter baumannii	-0.44*
Delftia acidovorans HOT023	-0.41*
Halomonas sp.	-0.39*
Pseudoalteromonas sp.	-0.32*

Table 10. Positive Spearman's Rank-Order Correlations between OTUs and axis 1 for teeth from day 0 to day 14 prior to adjusting for multiple comparisons. * = $p<0.05$; ** = $p<0.01$

OTU	r
Veillonella parvula	0.59**
Prevotella nigrescens	0.58**
Campylobacter gracilis	0.58**
Mogibacterium sp. (Mogibacterium diversum HOT593)	0.57**
Fusobacterium sp. (Fusobacterium nucleatum ss. animalis)	0.52**
Pseudoramibacter alactolyticus	0.50*
Anaeroglobus geminatus	0.49*
Solobacterium moorei	0.48*
Veillonellaceae [G-1] HOT129	0.48*
Anaeroglobus geminatus	0.47*
Atopobium rimae	0.47*
Neisseria oralis	0.46*
Oribacterium sp. HOT102	0.46*
Anaeroglobus geminatus	0.46*
Atopobium rimae	0.46*
Shuttleworthia satelles	0.46*
Parvimonas micra	0.46*
Prevotella multisaccharivorax	0.46*
Comamonas testosteroni	0.46*
Aggregatibacter actinomycetemcomitans	0.46*
Alloprevotella rava	0.46*
Aggregatibacter paraphrophilus	0.43*
Parvimonas micra	0.41*
Leptotrichia goodfellowii	0.40*
Veillonella parvula	0.40*
Fusobacterium sp. (Fusobacterium nucleatum ss. nucleatum)	0.40*
Dialister invisus	0.40*
Prevotella denticola	0.39*
Prevotella oris	0.38*

Table 11. Negative Spearman's Rank-Order Correlations between OTUs and axis 1 for teeth from day 0 to day 14 prior to adjusting for multiple comparisons. * = $p<0.05$; ** = $p<0.01$

OTU	r
Rothia dentocariosa	-0.86**
Actinomyces sp. (HOT169)	-0.52**
Actinomyces sp. HOT171	-0.48*
Pseudomonas stutzeri	-0.45*
Actinomyces sp. HOT169	-0.44*
Acinetobacter baumannii	-0.42*
Streptococcus sp. (HOT068)	-0.42*
Leptotrichia sp. HOT221	-0.41*
Kingella oralis	-0.40*
Actinomyces sp. (Actinomyces naeslundii HOT176)	-0.39*

Table 12. Positive Spearman's Rank-Order Correlations between OTUs and axis 1 for teeth from day 0 to day 21 prior to adjusting for multiple comparisons. * = $p<0.05$; ** = $p<0.01$

OTU	r
Fusobacterium nucleatum ss. animalis	0.78**
Mogibacterium sp. (Mogibacterium diversum)	0.65**
Prevotella oris	0.65**
Prevotella nigrescens	0.63**
Prevotella sp. (HOT292)	0.56**
Selenomonas sp. (Selenomonas flueggei)	0.56**
Fusobacterium sp. (Fusobacterium nucleatum ss. nucleatum)	0.53**
Atopobium rimae	0.52**
Selenomonas sp. HOT126	0.51**
SR1 [G-1] sp. HOT874	0.50**
Dialister invisus	0.49*
Granulicatella elegans	0.48*
Porphyromonas sp.	0.48*
Treponema sp. (Treponema socranskii ss.paredis)	0.46*
Oribacterium sp. (HOT372)	0.46*
Atopobium rimae	0.46*
Prevotella sp. HOT309	0.45*
Eubacterium [XI][G-3] brachy	0.44*
Campylobacter gracilis	0.44*
Treponema sp. HOT237	0.43*
Alloprevotella tannerae	0.43*
Mitsuokella sp. HOT131	0.43*
Prevotella denticola	0.42*
Leptotrichia sp. (HOT392)	0.42*
Porphyromonas sp. HOT279	0.42*
Prevotella oris	0.42*
Aggregatibacter sp. HOT512	0.42*
Dialister pneumosintes	0.42*
Selenomonas sputigena	0.41*
Leptotrichia sp. HOT215	0.41*
Solobacterium moorei	0.41*
Treponema sp. (Treponema socranskii ss.socranskii)	0.41*
Peptococcus sp. (HOT167)	0.40*
Selenomonas sp. (HOT892)	0.39*
Alloprevotella rava	0.39*
Streptococcus mitis bv 2	0.38*
Prevotella oulorum	0.38*

Table 13. Negative Spearman's Rank-Order Correlations between OTUs and axis 1 for teeth from day 0 to day 21 prior to adjusting for multiple comparisons. * = $p<0.05$; ** = $p<0.01$

OTU	r
Rothia dentocariosa	-0.89**
Actinomyces sp. (HOT169)	-0.57**
Actinomyces sp. (Actinomyces naeslundii HOT176)	-0.50**
Acinetobacter baumannii	-0.49*
Pseudomonas stutzeri	-0.48*
unclassified Proteobacteria	-0.47*
Streptococcus sp. (HOT068)	-0.45*
Rothia mucilaginosa HOT681	-0.41*
Pseudoalteromonas sp.	-0.39*

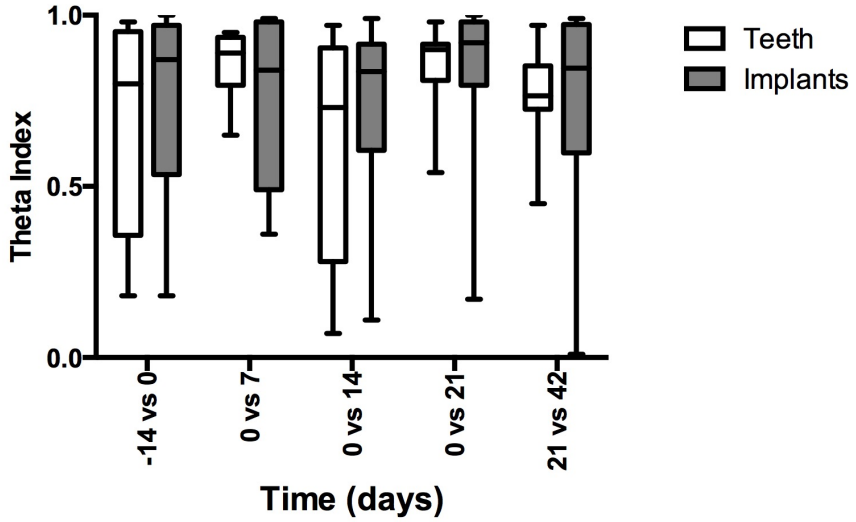


Figure 18. Theta Index for teeth and implants over time. Bars indicate median and range. No significant differences were found between groups.

OTUs that exhibited a significant difference in change in relative abundance from day 0 to day 21 prior to adjusting for multiple comparisons are displayed in Table 14 for teeth

and Table 15 for implants. Several of the OTUs that demonstrated significant differences in change in relative abundance from day 0 to day 21 were the same between implants and teeth, including unclassified *Proteobacteria*, *Pseudoalteromonas* sp., *Selenomonas* sp. (*S. flueggei*), unclassified *Bradyrhizobiaceae* (*Afipia broomeae*), *Vibrio* sp., *Aeromonas* sp., *Stenotrophomonas maltophilia*, *Pseudomonas* sp. (*P. pseudoalcaligenes*), *Prevotella oris* and *Capnocytophaga leadbetteri*. However, after adjusting for multiple comparisons, there were no significant differences in relative abundance of any of the OTUs from day 0 to day 21 for teeth or implants.

Table 14. OTUs that demonstrated significant differences in relative abundance from day 0 to day 21 in teeth prior to adjusting for multiple comparisons. * = $p<0.05$; ** = $p<0.01$

OTU	Δ Relative abundance day 0 to 21
Fusobacterium sp. (F. nucleatum ss. polymorphum)	0.0392*
Alloprevotella tannerae	0.0368*
Prevotella oris	0.0247*
Prevotella oulorum	0.0188*
Aggregatibacter sp. (HOT458)	0.0144*
Selenomonas sputigena	0.0085*
Leptotrichia wadei	0.0085*
Porphyromonas sp. (P. catoniae)	0.0066*
Prevotella sp. (HOT292)	0.0064*
Selenomonas sp. (S. flueggei)	0.0050*
Streptococcus sp. HOT071	0.0037*
Atopobium parvulum	0.0028*
Moryella sp. (Moryella sp. HOT419)	0.0027*
Prevotella salivae	0.0018*
Oribacterium sp. (HOT372)	0.0017*
Prevotella maculosa	0.0017*
Megasphaera micronuciformis	0.0015*
Gemella morbillorum	0.0015*
Capnocytophaga leadbetteri	0.0008*
Selenomonas artemidis	0.0006*
Oribacterium sinus	0.0006*
Alloprevotella rava	0.0005*
Porphyromonas sp. (HOT275)	0.0003*
Lachnoanaerobaculum umeaense	0.0003*
Bacillaceae 1 sp.	-0.0001*
unclassified Bradyrhizobiaceae (Afipia broomeae)	-0.0006*
Stenotrophomonas maltophilia	-0.0006*
Aeromonas sp.	-0.0007*
Corynebacterium durum	-0.0008*
Pseudomonas sp. (P. pseudoalcaligenes)	-0.0014*
Pseudoalteromonas sp.	-0.0021**
Vibrio sp.	-0.0024*
Streptococcus sp. (HOT064)	-0.0052*
unclassified Proteobacteria	-0.0061**
Rothia dentocariosa	-0.2432**

Table 15. OTUs that demonstrated significant differences in relative abundance from day 0 to day 21 in implants prior to adjusting for multiple comparisons. * = $p<0.05$; ** = $p<0.01$

OTU	Δ Relative abundance day 0 to 21
Prevotella oris	0.0251*
Tannerella forsythia	0.0065*
Selenomonas sp. (S. flueggei)	0.0039*
Capnocytophaga leadbetteri	0.0034*
Eikenella corrodens	0.0026*
Treponema sp. (T. socranskii ss. socranskii)	0.0004*
Actinomyces sp. HOT181	-0.0003*
Streptococcus sp. (HOT068)	-0.0003*
Streptococcus sp. (S. australis)	-0.0003*
Delftia acidovorans	-0.0004*
Agrobacterium tumefaciens	-0.0004*
Pseudomonas stutzeri	-0.0005*
Gemella sanguinis	-0.0006*
Actinomyces sp. (A. oris)	-0.0007*
Streptococcus sp. (Streptococcus infantis)	-0.0015*
Aeromonas sp.	-0.0018*
unclassified Bradyrhizobiaceae (Afipia broomeae)	-0.0021*
Actinomyces sp. (HOT170)	-0.0028*
Streptococcus sp. (HOT055)	-0.0037*
Rothia mucilagniosa	-0.0044*
Stenotrophomonas maltophilia	-0.0044*
Kingella oralis	-0.0048*
Pseudomonas sp. (P. pseudoalcaligenes)	-0.0061*
Pseudoalteromonas sp.	-0.0067*
Granulicatella sp. (G.adiacens)	-0.0071*
Vibrio sp.	-0.0099*
unclassified Proteobacteria	-0.0276**
Pasteurellaceae sp. (Terrahaemophilus aromaticivorans)	-0.1169*

To evaluate whether the change in OTU relative abundance occurred in the same manner in teeth and implants, we compared via paired-sample Wilcoxon Signed-Rank tests the changes (deltas) in relative abundance for all OTUs during the experimental phase. OTUs that exhibited a significant difference in change in relative abundance between implants

and teeth prior to adjusting for multiple comparisons are displayed in Figure 19 for day 0 to day 7, Figure 20 for day 0 to day 14 and Figure 21 for day 0 to day 21. In several instances, the change in relative abundance of a particular OTU was positive for teeth but negative for implants and vice versa (e.g., *Corynebacterium durum* from day 0 to day 7), which is in accordance with the inconsistent response to plaque accumulation in implants demonstrated in the PCoA plots. By day 21, several OTUs exhibited this behavior, with some of the most striking differences being those of *Streptococcus mitis* bv 2 and *Streptococcus* sp. HOT 071, and the periodontitis-associated OTUs *Treponema lecythinolyticum*, *Porphyromonas endodontalis*, *Tannerella forsythia* and *Bacteroidales* sp. HOT 272, which decreased in teeth during experimental plaque accumulation but increased in implants. However, after adjusting for multiple comparisons, there were no significant differences in the deltas (day 7-day 0; day 14-day 0; day 21-day 0), representing change in relative abundance, of any of the OTUs between implants and teeth.

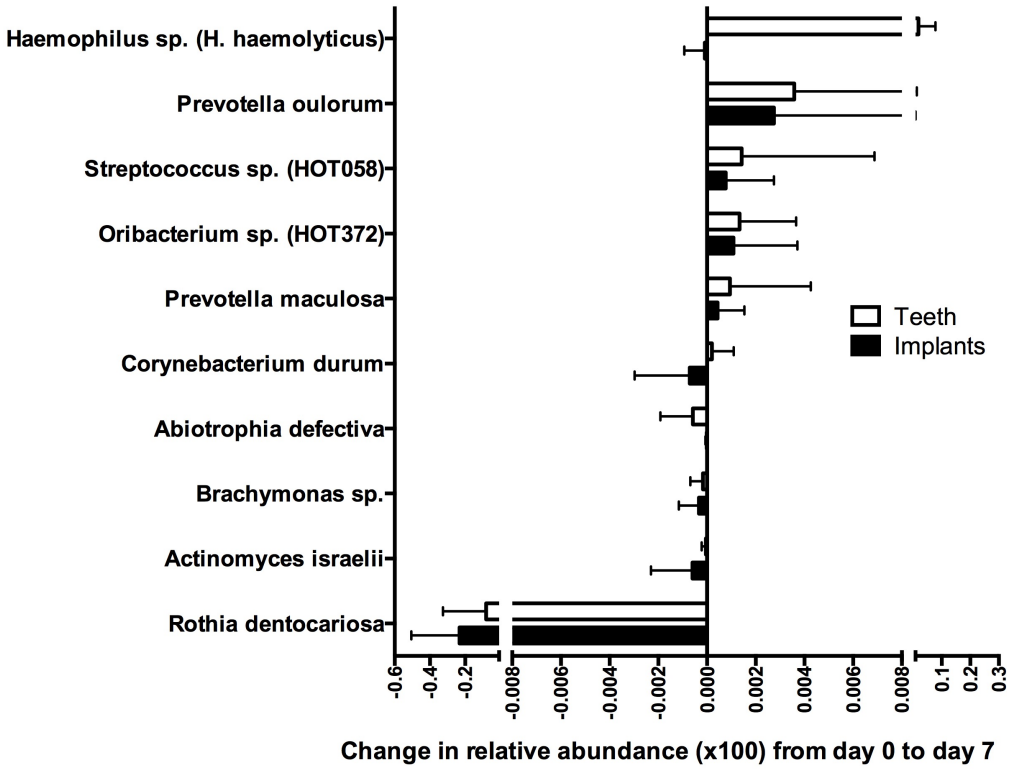


Figure 19. Change in relative abundance of OTUs that were statistically significantly different between teeth and implants from day 0 to day 7 prior to adjusting for multiple comparisons. Bars represent mean and standard deviation.

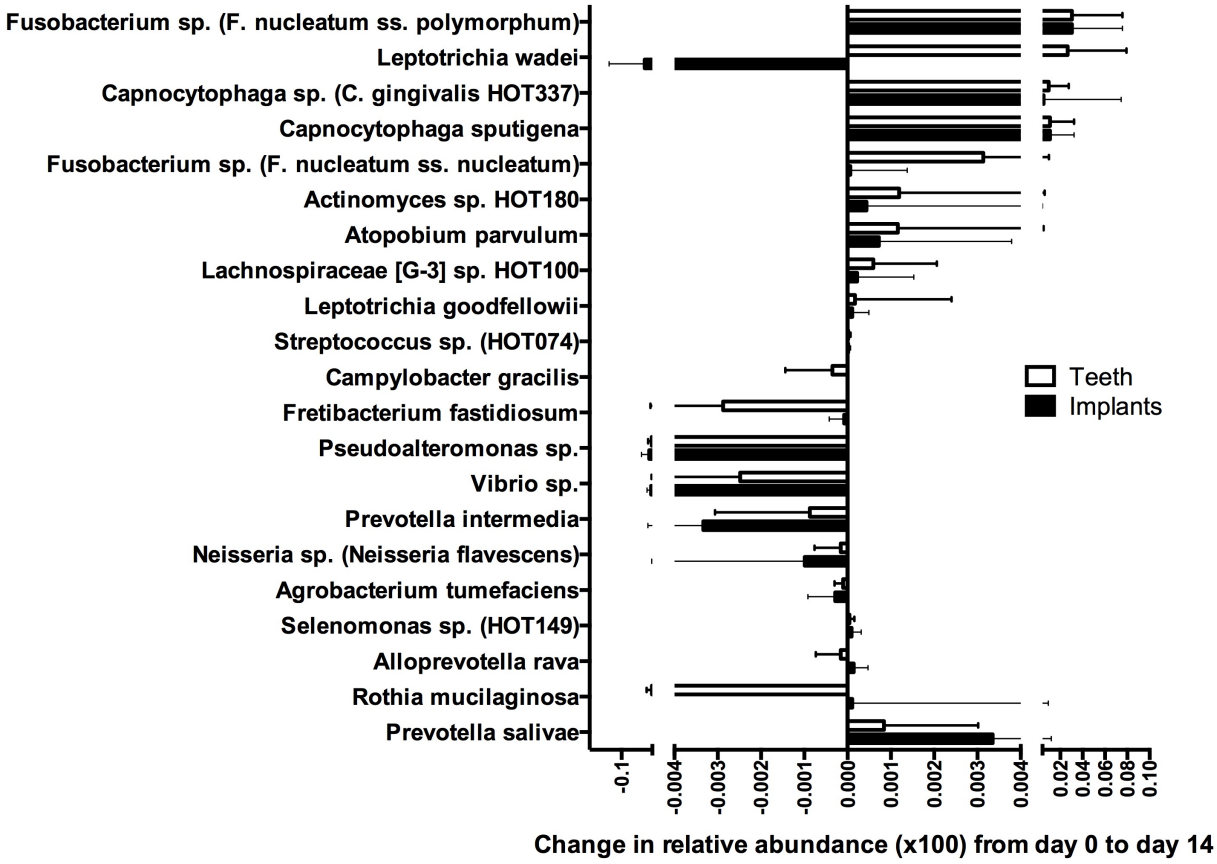


Figure 20. Change in relative abundance of OTUs that were statistically significantly different between teeth and implants from day 0 to day 14 prior to adjusting for multiple comparisons. Bars represent mean and standard deviation.

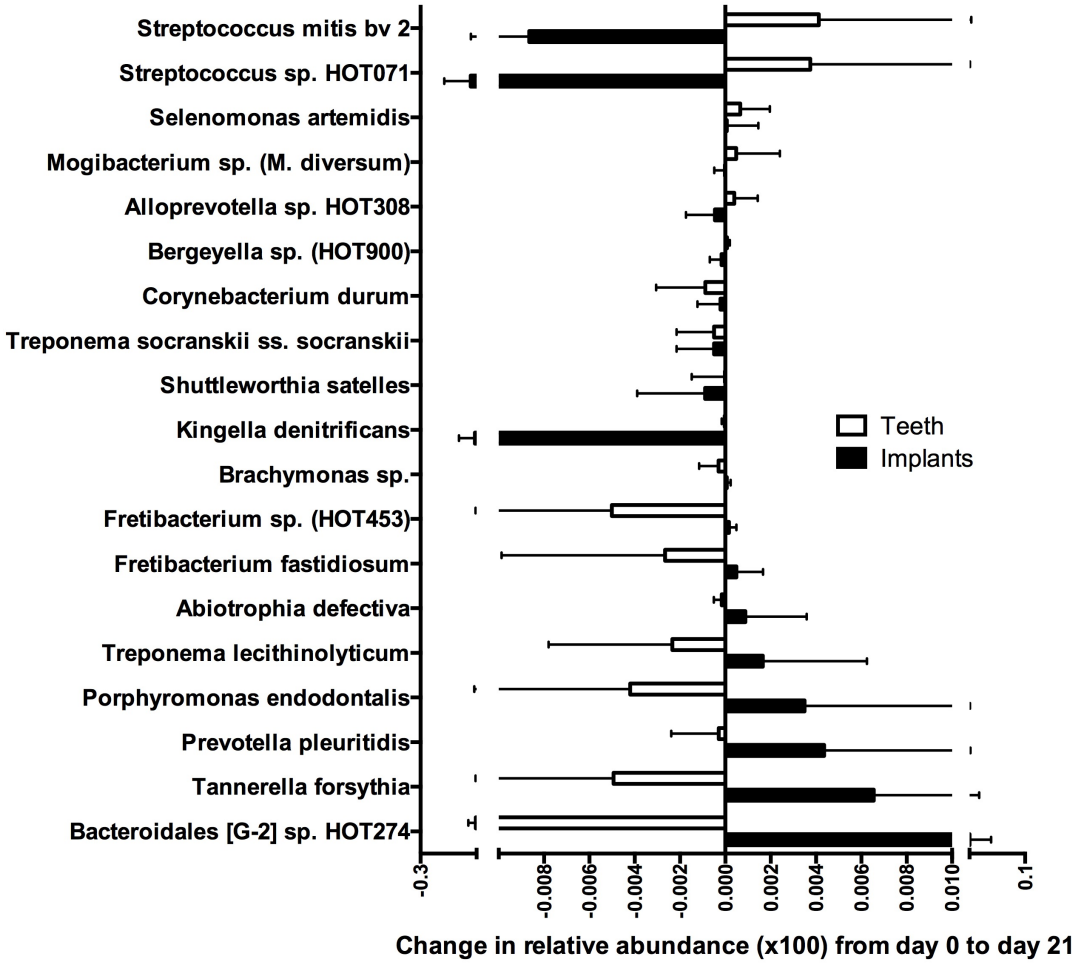


Figure 21. Change in relative abundance of OTUs that were statistically significantly different between teeth and implants from day 0 to day 21 prior to adjusting for multiple comparisons. Bars represent mean and standard deviation.

In summary, there was higher plaque accumulation as evidenced by higher subgingival bacterial load in teeth than implants. Diversity increased moderately during experimental plaque accumulation in teeth only. A shift in community structure consistent with previous studies was observed in teeth. In contrast, implants did not demonstrate an increase in diversity and the direction of the shift in microbiome community structure surrounding implants was inconsistent among subjects.

4.3 *Results related to Aim 3*

The third aim of this thesis (Aim 3) was to compare the immunological and microbiological responses after oral hygiene measures are resumed around dental implants and teeth.

4.4.1 Clinical results

Resumed oral hygiene resulted in a significant reduction in PlI from day 21 to day 42 for both teeth and implants (Figure 4c) and this corresponded to a statistically significant decrease in clinical inflammation, as measured by GI, in both teeth and implants (Figure 4b). GCF volume (Figure 4a) and ABS (Figure 4d) decreased significantly from day 21 to 42 in teeth but not in implants. However, there were no significant differences between day -14 and day 42 for any of the clinical parameters for both implants and teeth, indicating that implants and teeth return to their native state after three weeks of resumed oral hygiene. On day 42, ABS was significantly higher at implants than teeth, suggesting that clinical inflammation does not decrease as quickly at implants compared to teeth once oral hygiene is reinstated (Figure 4d).

4.4.2 GCF inflammatory marker results

IL-1 alpha (Figure 6a), IL-1 beta (Figure 6b) and IL-1 ra (Figure 6b) concentration decreased significantly from day 21 to day 42 for both teeth and implants. Lactoferrin concentration decreased significantly from day 21 to day 42 in teeth only (Figure 5a) while CCL22 concentration decreased significantly from day 21 to day 42 in implants only (Figure 5d). These results are consistent with the observations for these mediators as markers of gingivitis, peri-implant mucositis or both during the experimental phase. IL-1 alpha, IL-1 beta and IL-1 ra seem to be consistent markers for experimental gingivitis and peri-implant mucositis progression and resolution. Lactoferrin seems a marker for experimental gingivitis progression and resolution only, whereas CCL22 marks experimental peri-implant mucositis progression and resolution only. With the exception of lactoferrin concentration at teeth (Figure 5a), there were no significant differences between day -14 and day 42 for the inflammatory markers for both implants and teeth, again indicating that implants and teeth return to their native state after three weeks of resumed oral hygiene. Moreover, consistent with what was seen at the native state, on day 42, implants tended to have greater levels of all inflammatory markers, demonstrating statistically significantly higher concentrations of CCL22 (Figure 5d) and TNF-alpha (Figure 6d) compared to teeth.

4.4.3 Microbiological results

Subgingival bacterial load was significantly lower on day 42 than day 21 for both teeth and implants (Figure 8). Alpha diversity (Figure 9) was significantly lower on day 42

than day 21 for teeth only. Intergroup comparisons showed no differences in subgingival bacterial load and alpha diversity between teeth and implants at day 42. To evaluate whether the change in OTU relative abundance occurred in the same manner in teeth and implants, we compared the changes in relative abundance for all OTUs during resolution. OTUs that demonstrated a significant difference in change in relative abundance between implants and teeth prior to adjusting for multiple comparisons are displayed in Figure 22. The change in relative abundance of *R. dentocariosa* was greater in teeth than implants. The change in relative abundances of *Streptococcus* sp. HOT071 and *Streprococcus* sp. (*S. sanguinis*), OTUs associated with health, increased in implants but decreased in teeth. After adjusting for multiple comparisons, there were no significant differences in the deltas (day 42-day 21), representing change in relative abundance, of any of the OTUs between implants and teeth from day 21 to day 42.

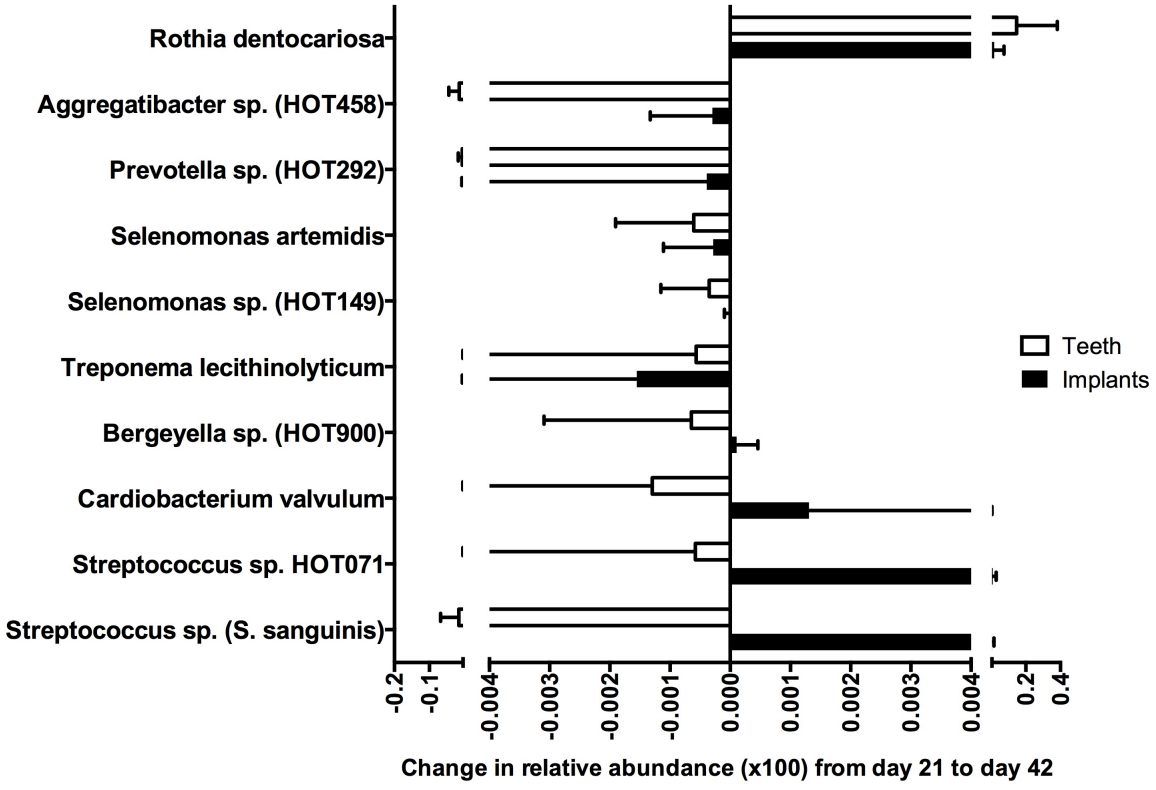


Figure 22. Change in relative abundance of OTUs that were statistically significantly different between teeth and implants from day 21 to day 42 prior to adjusting for multiple comparisons.

In summary, both teeth and implants returned to their native state as measured by clinical parameters and host inflammatory and microbiological markers. Across group comparisons showed that despite a lack of significant difference in PlI (Figure 4c), total microbial load (Figure 8) or alpha diversity (Figure 9) between teeth and implants, implants had more clinical inflammation, as measured by ABS, and higher concentrations of some inflammatory markers than teeth during the resolution phase. This is similar to the pre-experimental and experimental phase results.

4.5 *Correlations in clinical, host and microbiological markers between teeth and implants*

Spearman's Rank-Order correlations were calculated to determine whether clinical, inflammatory and microbiological markers in implants and teeth were correlated at each time point as well as at all time points taken together (Table 16). A positive correlation indicates that the marker behaved in a subject-dependent manner in implants and teeth, while negative correlation indicates that the marker behaved differently in implants compared to teeth.

Table 16. Spearman's Rank-Order Correlations between test (implants) and control (teeth) for each marker at each time point. Significant differences between groups are indicated in bold.

Marker	-14	0	7	14	21	42	All
GCF	0.01 (NS)	-0.26 (NS)	0.07 (NS)	0.08 (NS)	0.05 (NS)	-0.06 (NS)	0.14 (NS)
GI	**0.64 (p<0.01)**		0.37 (NS)	0.40 (NS)	0.48 (NS)	0.15 (NS)	**0.74 (p<0.01)**
PlI	0.48 (NS)		**0.71 (p<0.01)**	**0.54 (p<0.05)**	0.41 (NS)	0.38 (NS)	**0.86 (p<0.01)**
ABS	**0.51 (p<0.05)**	0.17 (NS)	0.34 (NS)	0.17 (NS)	0.47 (NS)	0.37 (NS)	**0.41 (p<0.01)**
LF	0.39 (NS)	0.01 (NS)	-0.12 (NS)	**0.54 (p<0.05)**	0.25 (NS)	0.29 (NS)	0.20 (NS)
MPO	0.18 (NS)	0.14 (NS)	0.00 (NS)	0.37 (NS)	0.06 (NS)	0.48 (NS)	**0.26 (p<0.05)**
IL-8	**0.71 (p<0.01)**	**0.70 (p<0.01)**	0.49 (NS)	**0.56 (p<0.05)**	**0.53 (p<0.05)**	**0.89 (p<0.01)**	**0.68 (p<0.01)**
CCL-22	0.19 (NS)	0.00 (NS)	0.10 (NS)	0.24 (NS)	-0.02 (NS)	**0.60 (p<0.05)**	**0.23 (p<0.05)**
IL-1 alpha	-0.13 (NS)	0.23 (NS)	0.10 (NS)	0.23 (NS)	0.16 (NS)	0.00 (NS)	**0.28 (p<0.01)**
IL-1 beta	0.13 (NS)	0.06 (NS)	0.25 (NS)	**0.68 (p<0.01)**	0.39 (NS)	0.27 (NS)	**0.37 (p<0.01)**
IL-1 ra	0.08 (NS)	0.12 (NS)	0.15 (NS)	-0.18 (NS)	0.01 (NS)	-0.16 (NS)	**0.23 (p<0.05)**
TNF-alpha	0.32 (NS)	**0.55 (p<0.05)**	0.35 (NS)	0.41 (NS)	**0.75 (p<0.01)**	0.18 (NS)	**0.46 (p<0.01)**
GRO	-0.31 (NS)	0.30 (NS)	0.44 (NS)	**0.60 (p<0.05)**	0.40 (NS)	0.50 (NS)	**0.45 (p<0.01)**
Bacterial Load	-0.01 (NS)	0.23 (NS)	**0.65 (p<0.01)**	0.24 (NS)	**0.81 (p<0.01)**	-0.01 (NS)	**0.46 (p<0.01)**
Richness	0.14 (NS)	-0.39 (NS)	0.53 (NS)	0.36 (NS)	0.41 (NS)	-0.03 (NS)	0.14 (NS)
Evenness	0.43 (NS)	-0.07 (NS)	0.24 (NS)	0.12 (NS)	0.37 (NS)	-0.06 (NS)	0.14 (NS)
Shannon Index	0.27 (NS)	-0.09 (NS)	0.34 (NS)	0.23 (NS)	**0.55 (p<0.05)**	0.00 (NS)	0.14 (NS)
Inverse Simpson Index	0.29 (NS)	-0.04 (NS)	0.43 (NS)	0.19 (NS)	0.36 (NS)	0.13 (NS)	0.15 (NS)

4.5.1 Native condition

On day -14, there were several statistically significant between-group correlations. GI ($r=0.64$, $p<0.01$) and ABS ($r=0.51$, $p<0.05$) and IL-8 ($r=0.71$, $p<0.01$) concentrations at implants and teeth were correlated, which suggests that implants and teeth have similar clinical inflammatory and PMN chemokine responses during the native condition (Table 16). There was also a trend for a positive correlation between PlI at implants and PlI at teeth, suggesting that plaque accumulation in the native condition occurs in a subject-specific manner. Interestingly, GCF volume and several inflammatory markers did not show a significant correlation, a result in accordance with differences observed between teeth and implants at day -14 (Figures 4-7), with implants showing a trend for higher levels than teeth.

4.5.2 Experimental phase

There were several statistically significant between-group correlations during the experimental phase (Table 16). On day 0, IL-8 ($r=0.70$, $p<0.01$), TNF-alpha ($r=0.55$, $p<0.05$) and bacterial load ($r=0.55$, $p<0.05$) in teeth and implants were significantly positively correlated. There were also significant positive correlations in teeth and implants for bacterial load on day 7 ($r=0.65$, $p<0.01$) and day 21 ($r=0.81$, $p<0.01$), suggesting that total 16S rRNA copy number varies in a subject-dependent manner in implants and teeth during experimental gingivitis/peri-implant mucositis. Consistent with this finding, PlI at teeth and implants was positively correlated on day 7 ($r=0.71$, $p<0.01$) and also on day 14 ($r=0.54$, $p<0.05$). In addition to day 0, IL-8 at teeth was positively correlated to IL-8 at implants on day 14 ($r=0.56$, $p<0.05$) and day 21 ($r=0.53$, $p<0.05$),

although IL-8 concentration does not vary greatly during the experimental phase for teeth or implants (Figure 5c). Lactoferrin ($r=0.54$, $p<0.05$), IL-1 beta ($r=0.68$, $p<0.01$) and GRO ($r=0.60$, $p<0.05$) in teeth and implants were significantly positively correlated on day 14. TNF-alpha ($r=0.75$, $p<0.01$) and non-parametric Shannon Index ($r=0.55$, $p<0.05$) in teeth and implants were significantly positively correlated on day 14. Since several clinical, inflammatory and microbiological markers in teeth and implants are positively correlated to each other during the experimental phase, it seems that teeth and implants respond in a subject-specific manner to plaque accumulation.

4.5.3 Resolution

On day 42, IL-8 ($r=0.89$, $p<0.01$) and CCL22 ($r=0.60$, $p<0.05$) concentrations at implants and teeth were correlated, which suggests that these cytokines respond in a subject-specific manner in implants and teeth during resolution (Table 16).

4.5.4 Correlations between teeth and implants at all visits

In order to evaluate if variation of biomarkers in teeth and implants was correlated across the trial in a subject-dependent manner, we tested intergroup correlations taking into account all time points. This analysis showed that regardless of time point, GI ($r=0.74$, $p<0.01$), PlI ($r=0.86$, $p<0.01$) and ABS ($r=0.41$, $p<0.01$), but not GCF, were correlated between implants and teeth (Table 16). These results suggest that the changes in these parameters during the trial follow a subject-specific pattern despite differences in total plaque accumulated by teeth and implants during the experimental phase (Figure 4).

With the exception of lactoferrin, the host inflammatory marker concentrations in teeth were correlated to those in implants overall (Table 16). This result may be due to the fact that, as seen in figure 5a, lactoferrin concentration increased significantly as plaque accumulation increased in teeth but it did not increase in implants.

Total 16S rRNA copy number in teeth was correlated to that in implants ($r=0.46$, $p<0.01$) while alpha diversity in teeth was not correlated to that in implants overall (Table 14). Subgingival bacterial load increased similarly in teeth and implants with increased plaque (Figure 8). On the other hand, alpha diversity increased as plaque accumulation increased in teeth but not in implants (Figure 9).

4.6 *Correlations among all variables evaluated*

In order to test which variables (clinical, host and microbiological) better correlated with inflammation as determined by GI and ABS and with plaque accumulation as determined by PlI and bacterial load, we determined Spearman's Rank-Order correlations taking all six visits together. All clinical and host marker variables were considered for this analyses while selected OTUs were tested. OTUs selected for correlations in the teeth group were those displaying the highest correlation coefficients with PC1 in PCoA analysis plots during experimental plaque accumulation for the three time points tested. OTUs selected for correlation analysis in the implant group were those displaying the highest correlation coefficients with both PC1 and PC2 at day 21 only, as this was the only day with more consistent trends in microbiome shifts in implants. The variables were organized into correlograms based on results from hierarchical clustering for teeth

(Figure 22) and implants (Figure 23). In teeth, GI was positively correlated with ABS, PlI, subgingival bacterial load, MPO, lactoferrin, IL-1 alpha, IL-1 beta, IL-1 ra and the OTUs *C. gracilis*, *Selenomonas* sp. (*S. fluggei)*, *Fusobacterium* sp. (*F. nucleatum* ss. *nucleatum*) and three OTUs in the genera *Prevotella* and negatively correlated with *R. dentocariosa* (Figure 22*)*. ABS in teeth was positively correlated with GI, PlI IL-1 alpha, IL-1 beta, IL-1 ra, lactoferrin *Fusobacterium* sp. (*F. nucleatum* ss. *animalis*), *Atopobium rimae* and two OTUs in the genera *Prevotella* (Figure 22*)*. PlI in teeth in turn was positively correlated with MPO, lactoferrin, IL-1 alpha, IL-1 beta, IL-1 ra, measures of alpha diversity, *C. gracilis*, *Selenomonas* sp. (*S. fluggei)*, *Fusobacterium* sp. (*F. nucleatum* ss. *nucleatum*) and OTUs in the genera *Prevotella* and negatively correlated with *R. dentocariosa* (Figure 22*)*. Lastly, subgingival bacterial load in teeth was positively correlated with *C. gracilis, Selenomonas* sp. (*S. fluggei),* OTUs in the genera *Prevotella*, IL-1 ra, IL-1 alpha and TNF-alpha and negatively correlated with *R. dentocariosa* (Figure 22*).* Since IL-1 alpha, IL-1 beta, IL-1 ra (Figure 6a-c) and lactoferrin (Figure 5a) were biomarkers of experimental plaque accumulation in teeth and *Fusobacterium* sp. (*F. nucleatum* ss. *animalis*) was positively correlated with the shift in axis 1 from day 0 to day 21 in teeth (Figure 14), these positive correlations were expected. Since *R. dentocariosa* was negatively correlated with the shift in axis 1 from day 0 to day 7 (Figure 12), day 0 to day 14 (Figure 14) and day 0 to day 21 (Figure 14) in teeth, this negative correlation was expected.

In implants, GI was positively correlated with MPO, lactoferrin, IL-1 alpha, IL-1 beta, IL-1 ra and OTUs of the genera *Treponema* and *Capnocytophaga* sp. (*C. gingivalis*) and

negatively correlated with *R. dentocariosa* and *Streptococcus* sp. (*S. mitis*; Figure 23). ABS in implants was positively correlated with GRO, MPO, lactoferrin, IL-1 alpha, IL-1 beta and IL-1 ra alpha and negatively correlated with *Streptococcus* sp. (*S. mitis*; Figure 23). PlI in implants was positively correlated with IL-1 alpha, IL-1 beta, IL-1 ra and *Capnocytophaga* sp. (*C. gingivalis*) and negatively correlated with *R. dentocariosa*. Lastly, bacterial load in implant was positively correlated with TNF-alpha, CCL22 and *Eubacterium* [XI][G-1]. Since IL-1 alpha, IL-1 beta, IL-1 ra (Figure 6a-c) and CCL22 (Figure 5d) were biomarkers of experimental plaque accumulation in implants, these positive correlations were expected.

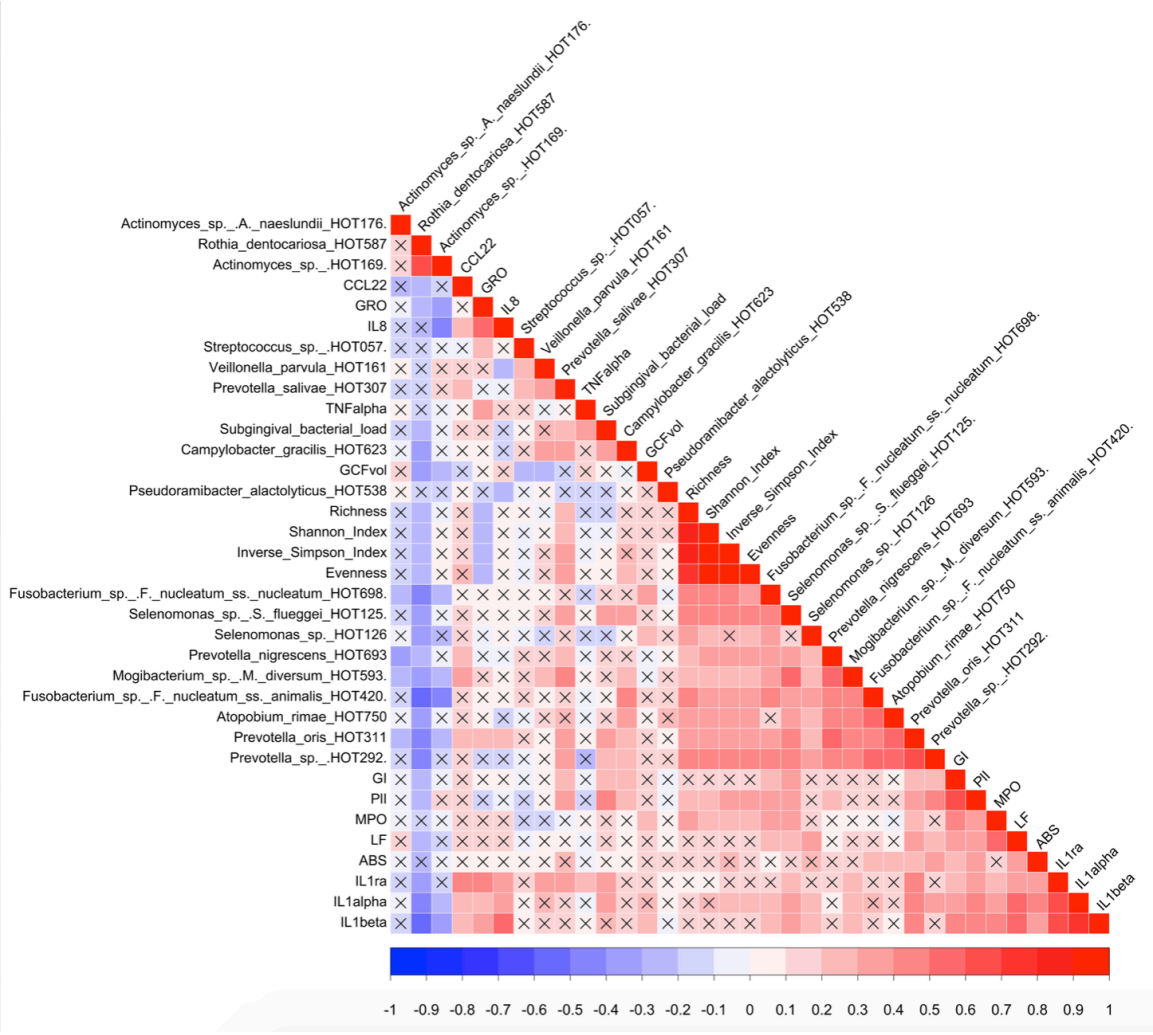


Figure 22. Within group Spearman's Rank-Order Correlations for teeth displayed as a correlogram based on hierarchical clustering. Colors indicate correlation coefficients. Non-significant correlations (more than 0.05) are marked with an x. Red = positive correlation; purple = negative correlation. Samples were organized based on hierarchical clustering results showing clusters of samples that correlate with each other.

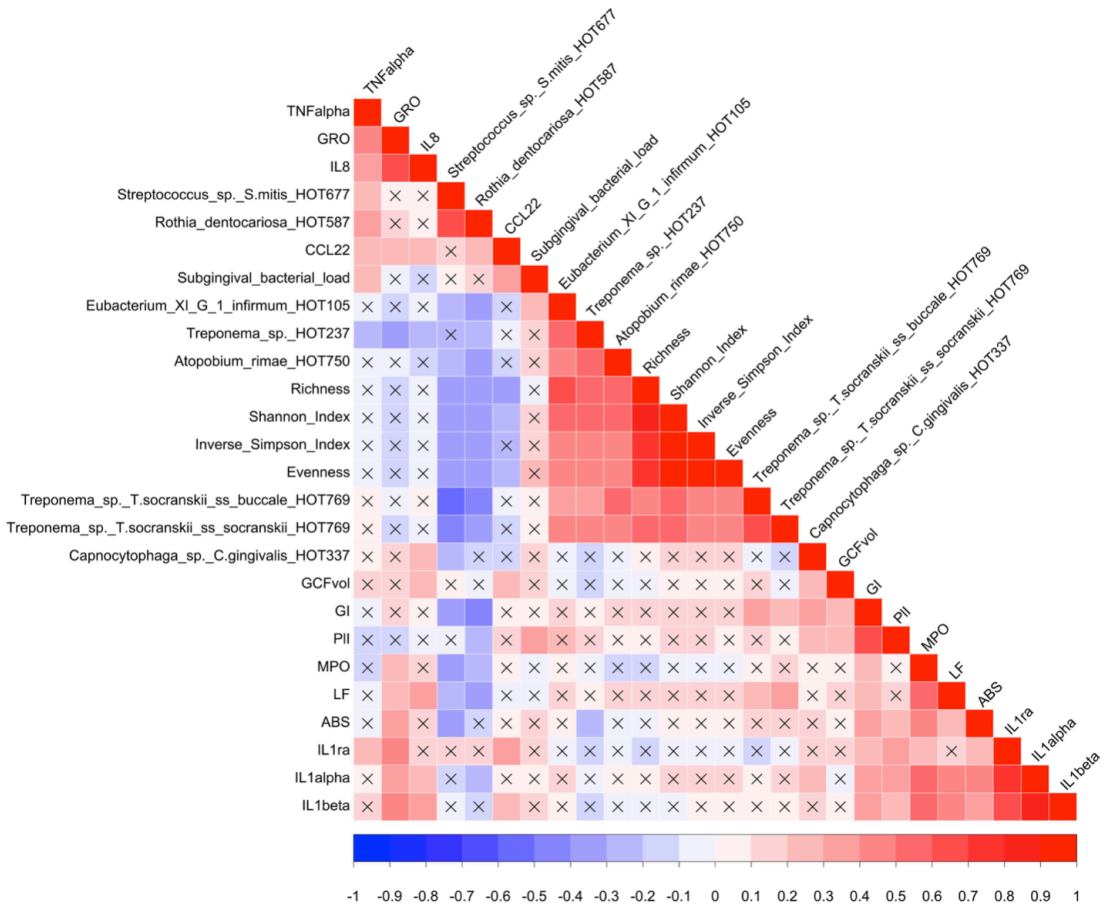


Figure 23. Within group Spearman's Rank-Order Correlations for implants displayed as a correlogram based on hierarchical clustering. Colors indicate correlation coefficients. Non-significant correlations (more than 0.05) are marked with an x. Red = positive correlation; purple = negative correlation. Samples were organized based on hierarchical clustering results showing clusters of samples that correlate with each other.

We also employed Spearman's Rank-Order Correlation test to examine whether presence or absence of crowns on teeth was related to plaque accumulation since we observed increased plaque accumulation in teeth in comparison to implants during the experimental phase. A within group correlation analysis between presence of crowns and PlI showed a significant negative correlation at day -14 and 42 and a trend toward a negative correlation between the presence of a crown and increased PlI on day 7 and day 14, indicating that plaque accumulation tended to be lower in the presence of crowns (Table 17).

Table 17. Spearman's Rank-Order Correlations between presence of crowns and teeth PlI. Day -14 and day 42 demonstrated significant negative correlations between presence of crowns and PlI. Day 7 and 14 demonstrated a trend toward negative correlations between presence of crowns and PlI.

	Day -14	Day 7	Day 14	Day 21	Day 42
r	-0.58	-0.51	-0.45	-0.32	-0.77
p-value	0.02	0.05	0.09	0.23	.001

5. Discussion

This study aimed to compare clinical parameters, GCF inflammatory markers and microbial biofilms between teeth and dental implants in their native state as well as to evaluate the changes in clinical parameters, inflammatory markers and microbial biofilms during 21 days of experimental plaque accumulation and after oral hygiene measures were resumed around dental implants in comparison to teeth. The microbiome and host

response associated with peri-implant mucositis is not fully understood. The prevention and treatment of peri-implant mucositis is critical, as this condition may progress to peri-implantitis and ultimately loss of the affected implant. Dissimilarities in the microbiomes and/or host response associated with peri-implant mucositis versus gingivitis may guide different clinical approaches to diagnosis, prevention and therapy. This is the first study to characterize the evolution of the whole microbial profile and changes in host response at teeth and implant sites during three phases: the native condition, experimental gingivitis/peri-implant mucositis and resolution. Although this was a pilot study, subject retention was high, with only three of 18 subjects lost. However, sample size was low and the data were not normally distributed, which required the use of non-parametric statistical tests, and adjustment for multiple comparisons was required as many variables were compared. These factors rendered our tests conservative and may have hindered our ability to discover true associations. Despite this, we observed several novel results and also confirmed results of previous work. Our study may serve as a basis for larger experiments of this nature.

During the native state (day -14), GI was significantly higher at teeth compared to implants despite equal levels of plaque. In contrast, ABS and GCF volume tended to be higher in implants than teeth during the native condition. Implants demonstrated significantly higher concentrations of lactoferrin, IL-1 ra and TNF-alpha compared to teeth. There was also a trend toward higher concentrations of MPO, CCL-22, IL-1 alpha, IL-1 beta, IL-8 and GRO at implants compared to teeth. A trend for lower GI and a statistically significant higher ABS in implants compared to teeth were also seen at the

resolution phase (day 42). Also in agreement with day -14, higher levels of inflammatory markers in implants and teeth were seen at the resolution phase. While all data point to greater inflammation in implants in comparison to teeth, GI does not seem to correlate well with molecular evidence of inflammation in implants. This may be due to differences in histology of implants and teeth. For example, implants lack a PDL and therefore the peri-implant mucosa has less vascularity than the gingiva. Since GI is a visual assessment based on color, a decreased blood supply will result in less mucosal color change. ABS, in contrast, seems to capture inflammation around implants. Moreover, reasons for increased inflammation around implants in comparison to teeth could not be explained by microbiome analysis, as implants did not have a unique or different microbiome than that present on teeth. Subgingival bacterial load was also similar between groups. These findings suggest the presence of a higher inflammatory response in implants compared to natural teeth despite a similar microbial challenge. In agreement with our results, Nowzari et al. (2012) reported significantly higher concentrations of pro-inflammatory cytokines IL-8 and TNF-alpha in the peri-implant sulcular fluid of healthy implants compared to the GCF of healthy teeth [11]. Differences between teeth and dental implants in surface characteristics and chemical composition may explain discrepancies in cytokine levels. When a foreign body, even commercially pure titanium, is placed in bone or soft tissue, an inflammatory reaction develops. Osseointegration of a dental implant is ultimately a foreign body response in the tissues. The majority of implants experience a steady state foreign body reaction and maintain osseointegration and healthy supporting tissues [61]. However, implants may

demonstrate elevated local inflammatory cytokines compared to natural teeth due to this foreign body response.

While PCoA revealed that community structure in teeth and implants is the same during the native condition, there was a trend for differences in the relative abundances of a few OTUs in teeth versus implants. There was a trend for the relative abundances of OTUs *A. gerencseriae* and *Capnocytophaga* sp. (HOT336) to be greater in teeth compared to implants whereas there was a trend for the relative abundances of *P. melaninogenica* and *P. salivae* to be greater in implants compared to teeth. This is in agreement with Kumar et al. (2012), who found higher levels of the genera *Actinomyces* and *Capnocytophaga* in healthy teeth than healthy implants and higher levels of the genera *Prevotella* in healthy implants than healthy teeth [37].

During the experimental phase, PlI increased significantly for both implants and teeth, demonstrating that the methodology to induce plaque accumulation was effective. However, teeth had higher PlI compared to implants over 21 days of oral hygiene abstention. Increased plaque accumulation around unrestored teeth compared to ceramo-metal restorations as a result of differences in surface roughness has been previously reported [62]. In our study, there was a trend toward a negative correlation between the presence of crowns and PlI in teeth. Accordingly, implants demonstrated less plaque accumulation than teeth. Consistent with higher PlI in teeth, GI tended to be higher in teeth compared to implants, although this could be due to GI not being an accurate marker of inflammation around implants. Salvi et al. (2012), on the other hand, found

higher GI at implants compared to teeth despite higher PlI in teeth compared to implants during experimental plaque accumulation [38]. Differences in methodology used to measure GI between our study and the Salvi et al. (2012) study may explain the disparity in these results. We measured GI using the Silness and Loe (1964) [42] method without the bleeding component, according to the modification described by Trombelli et al. (2004) [43]. Although this method was selected to allow plaque to accumulate in an undisturbed manner throughout the experimental phase, the ability to distinguish between a GI score of 1 and 2 is limited. This may have resulted in a less sensitive assessment of GI. While the mGI method described by Mombelli et al. (1997) [63] used in the Salvi et al. (2012) study may have allowed more accurate GI measurement, this technique disturbed the biofilm weekly during experimental plaque accumulation. As explained before, inflammation around implants may be better measured when there is a bleeding component in the scale used, and therefore in this study ABS seemed to better reflect the inflammatory status of implants than GI.

As plaque accumulation and clinical signs of inflammation increased over the experimental phase, many of the inflammatory markers also increased. IL-1 alpha, IL-1 beta and IL-1 ra were the most consistent markers for plaque accumulation around both teeth and implants. Lactoferrin was a marker of experimental gingivitis but not of peri-implant mucositis while CCL22 was a marker for experimental peri-implant mucositis only. Each of these mediators is characteristic of acute inflammation. IL-1 beta, produced primarily by macrophages and PMNs, is a principal mediator of the gingival inflammatory response and both IL-1 alpha and IL-1 beta stimulate PGE2 release.

Elevated concentrations of IL-1 alpha and IL-1 beta have been associated with both naturally occurring and experimental gingivitis [30-33]. In accordance with Salvi et al. (2012)[38], IL-1 beta concentration increased as plaque accumulated in both implants and teeth. While increased IL-1 ra concentration in periodontitis samples compared to healthy samples has been reported, Il-1 ra has not been evaluated in gingivitis or peri-implant mucositis. Consistent with previous periodontitis studies [34, 35], the concentration of IL-1 ra increased in teeth during the experimental phase in our study. Implants also demonstrated an increase in IL-1 ra during experimental peri-implant mucositis. Elevated concentration of IL-1ra, the IL-1 receptor antagonist, during experimental plaque accumulation may be explained by increased IL-1 ra secretion in response to IL-1 alpha and IL-1 beta release in an attempt to dampen the inflammatory response. Since IL-1 ra only binds the IL-1 beta receptor and does not affect IL-1 beta production, IL-1 beta concentration increased despite increased IL-1 ra concentration during the experimental phase. Binding of lipopolysaccharide (LPS) to Toll-like receptor 4 (TLR-4) is essential for IL-1 alpha and IL-1 beta secretion in gingival tissue. Increased LPS, due to higher proportions of gram-negative organisms as the plaque biofilm matures, stimulates secretion of these cytokines [64]. Th17 cells, which require IL-1 for differentiation, induce the production of osteoclastogenic mediators RANKL and TNF-alpha [65]. Therefore, IL-1 alpha and IL-1 beta, the central mediators in both experimental gingivitis and peri-implant mucositis in our study, may be responsible for progression of inflammation to an irreversible state (i.e., bone loss).

The PMN marker lactoferrin was a biomarker for experimental gingivitis but not peri-implant mucositis. The observation that lactoferrin concentration increased during experimental plaque accumulation around teeth confirms the findings of Ozdemir et al. (2009) [66]. Although higher concentrations of lactoferrin in the peri-implant sulcular fluid of implants with peri-implantitis compared that of healthy implants have been reported [12], lactoferrin was not a reliable marker of peri-implant mucositis in our study. Perhaps lactoferrin is a better biomarker for more severe peri-implant lesions.

Produced by macrophages, CCL22 is a chemoattractant for Th2 cells and acted as a biomarker for experimental peri-implant mucositis but not gingivitis. Recently, the cytokine microenvironment in aseptic loosening of total hip replacements was described in the orthopedic literature [67]. Compared to healthy synovial tissues of patients undergoing primary hip replacement, interface tissues of failed hip implants demonstrated upregulation of CCL22 and presence of foreign body giant cells. Similarly, Albrektsson and colleagues introduced the theory that successful osseointegration of dental implants relies on foreign body equilibrium [61,68]. This concept suggests that marginal bone resorption around dental implants is ultimately the result of a provoked foreign body response and is only secondarily related to biofilm-mediated processes. Perhaps elevated CCL22 in implants, but not teeth, during experimental plaque accumulation indicates a foreign body response to the titanium implant, exacerbated by the bacterial biofilm. Finally, the fact that a macrophage-activity marker, but no neutrophil marker, was significantly associated with peri-implant mucositis may indicate

that inflammation in the peri-implant mucosa is more of a chronic than acute type response.

Across group comparisons revealed significantly higher IL-1 ra, TNF-alpha and CCL22 concentrations at implants compared to teeth at various time points during the experimental phase. These results are consistent with the trend for implants to display higher levels of inflammatory markers than their teeth counterparts. Salvi et al. (2012) also found higher MMP-8 concentration at implants compared to teeth during the experimental phase, suggesting that implants develop a stronger inflammatory response to experimental plaque accumulation than teeth [38].

The microbiological results from the experimental phase were largely consistent with previous work from Löe et al. (1966)[18] and Kistler et al. (2013)[22] that demonstrated a shift toward gram-negative, obligate anaerobes over the course of experimental gingivitis. In our study, PCoA revealed a significant shift to the right along axis 1 as plaque accumulated in teeth. This shift was positively correlated with *V. parvula* after one week and *F. nucleatum* ss. *animalis* after three weeks and negatively correlated with *R. dentocariosa,* indicating that *V. parvula* and *F. nucleatum* ss. *animalis* are drivers of community structure during experimental plaque accumulation while *R. dentocariosa* drives community structure in health. There was a trend for the relative abundances of OTUs *Selenomonas* sp. (*S. flueggei*), *S. sputigena*, *S. artemidis, Fusobacterium* sp. (*F. nucleatum* ss. *polymorphum*), *Porphyromonas* sp. (HOT275), *Porphyromonas* sp. (*P. catoniae*) and *L. wadei* to increase from day 0 to day 21 in teeth. This is in agreement

with Kistler et al. (2013), who found higher levels of the genera *Selenomonas*, *Fusobacterium, Porphyromonas* and *Leptotrichia* during experimental gingivitis [22]. In contrast to Kistler et al. (2013), who took plaque samples from the same sites each week, we obtained biofilm samples from different sites throughout the study, allowing plaque to accumulate in an undisturbed fashion. While this method was advantageous in that it allowed the biofilm to mature over time, it also introduced variability, as the microbiome could have been different at different sites.

This is the first study to characterize the whole microbiome during experimental peri-implant mucositis. The magnitude of the shift in community structure over time, as measured by Theta Index, was the same for teeth and implants. Moreover, the relative abundances of many gram-negative OTUs, such as *T. forsythia* and *Eikenella corrodens*, tended to increase, while gram-positive organisms, such as *R. dentocariosa*, tended to decrease during experimental peri-implant mucositis, indicating a trend similar to teeth. However, the shift in the microbiome structure was less predictable in implants compared to teeth as evidenced by the different directions in which the microbiome community structure shifted in PCoA analyses. Since the shift in community structure was consistent in experimental gingivitis but inconsistent in experimental peri-implant mucositis, the microbiome response during experimental plaque accumulation seems to differ between teeth and implants. Perhaps surface characteristics determine different biofilm development patterns in implants. It is also possible that since plaque did not accumulate as much at implants, the peri-implant biofilm may not have had as much time to mature

as the biofilm surrounding teeth, which may also explain why diversity increased from day 0 to day 21 for teeth but not implants.

During resolution, all clinical parameters returned to their native state values for both implants and teeth. However, ABS was significantly higher at implants than teeth on day 42, indicating that clinical inflammation does not decrease as quickly at implants compared to teeth once oral hygiene practices are resumed. Salvi et al. (2012)[38] also found greater clinical inflammation, as measured by GI, at implants compared to teeth on day 42. Resolution of peri-implant mucositis may require a longer healing period than gingivitis. Consistent with the experimental phase observations, IL-1 alpha, IL-1 beta and IL-1 ra seem to be consistent markers for experimental gingivitis and peri-implant mucositis resolution since the concentrations of these mediators decreased significantly from day 21 to day 42 in both teeth and implants. Lactoferrin, which significantly from day 21 to day 42 in teeth only, may act as a marker for experimental gingivitis resolution only, whereas CCL22, which decreased significantly from day 21 to day 42 in implants only, may act as a marker for experimental peri-implant mucositis resolution only. Similar to the Salvi et al. (2012) host marker results, which demonstrated reversibility of experimental gingivitis and peri-implant mucositis at the biomarker level [38], there were no significant differences in inflammatory mediator concentration between the native state and resolution for both implants and teeth. Although there were no differences in subgingival bacterial load or diversity between implants and teeth on day 42, implants tended to have greater levels of all inflammatory markers compared to teeth during resolution. This observation is similar to the host marker findings during the native state.

The within group correlations demonstrate that the host markers MPO, lactoferrin, IL-1 alpha, IL-1 beta and IL-1 ra best define gingivitis while IL-1 alpha, IL-1 beta, IL-1 ra, CCL22 and TNF-alpha best define peri-implant mucositis. OTUs such as *C. gracilis*, *Selenomonas* sp. (*S. fluggei)*, *Fusobacterium* sp. (*F. nucleatum* ss. *nucleatum*) and *Fusobacterium* sp. (*F. nucleatum* ss. *animalis*) and the genera *Prevotella* were correlated with gingivitis. *C. gracilis*, *Fusobacterium* sp. (*F. nucleatum* ss. *nucleatum*) and *Fusobacterium* sp. (*F. nucleatum* ss. *animalis*) have been previously defined as core species present in at least 50% of subjects in both healthy and periodontitis groups [69]. In contrast, OTUs including *Capnocytophaga* sp. (*C. gingivalis*) and *Eubacterium* [XI][G-1] and the genera *Treponema* were associated peri-implant mucositis. *Eubacterium* [XI][G-1] and OTUs of the genera *Treponema* were identified as members of the core microbiome in periodontitis [69], suggesting that the microbial profile associated with peri-implant mucositis may have pathogenic consequences.

As expected, *R. dentocariosa*, a member of the core microbiome in periodontal health, was negatively correlated with both gingivitis and peri-implant mucositis. Genera correlated with gingivitis, such as *Fusobacterium* and *Selenomonas*, and peri-implant mucositis, such as *Eubacterium* and *Treponema,* in our study predicted gingivitis in the study by Huang et al. (2014)[23]. The key host markers for gingivitis and peri-implant mucositis described above could be used to develop assays for the presence and severity of inflammatory disease around teeth and implants. Similarly, tests related to identification of organisms associated with gingivitis or peri-implant mucositis in this

pilot study may be useful for clinical diagnosis. However, such cytokine and microbial tests would require validation in larger cohort studies.

6. Conclusions

From the work reported herein, it can be concluded that:

- In the native condition, dental implants have higher concentrations of host inflammatory mediators than their teeth counterparts despite equal level of bacterial plaque and similar microbiome structure.
- IL-1 alpha, IL-1 beta and IL-ra are consistent biomarkers of both experimental gingivitis and peri-implant mucositis, which suggests that both lesions may advance in a similar manner. Lactoferrin, however, is a biomarker for experimental gingivitis but not peri-implant mucositis while CCL22 is a biomarker for experimental peri-implant mucositis but not gingivitis.
- During experimental plaque accumulation, the structure of the microbiome surrounding teeth evolves in a consistent and predictable direction. On the other hand, the shift in microbiome community structure during experimental plaque accumulation was less predictable for implants. Teeth accumulate more plaque than implants and also demonstrate an increase in diversity of the biofilm as plaque accumulates whereas implants do not show changes in diversity.
- The main drivers of shifts in microbiome structure are *V. parvula, Fusobacterium* sp. (*F. nucleatum* ss. *animalis*) and *R. dentocariosa* around teeth and *R. dentocariosa* and OTUs of the genera *Actinomyces* around implants.

- After 21 days of oral hygiene reinstitution, the gingiva and peri-implant mucosa return to their native clinical condition. However, implants have higher ABS compared to teeth during resolution. Implants may require longer healing periods than teeth after experimental plaque accumulation.
- Similar to the native state, implants demonstrate higher levels of inflammatory mediators than teeth during resolution.

6. References

1. Esposito M, Hirsch JM, Lekholm U, Thomsen P. **Biological factors contributing to failures of osseointegrated oral implants. (I). Success criteria and epidemiology.** *Eur J Oral Sci* 1998;**106**:527-551.
2. Quirynen M, Vogels R, Pauwels M, et al. **Initial subgingival colonization of 'pristine' pockets.** *J Dent Res* 2005;**84**:340-344.
3. Albrektsson T, F I. **Consensus report: implant therapy**. In: Lang NP KT, ed. *Proceeding of the First European Workshop in Periodontology*: Berlin, Quintessence, 1994:365-369.
4. Lindhe J, Berglundh T, Ericsson I, Liljenberg B, Marinello C. **Experimental breakdown of peri-implant and periodontal tissues. A study in the beagle dog.** *Clin Oral Implants Res* 1992;**3**:9-16.
5. Lang NP, Bragger U, Walther D, Beamer B, Kornman KS. **Ligature-induced peri-implant infection in cynomolgus monkeys. I. Clinical and radiographic** findings. *Clin Oral Implants Res* 1993;**4**:2-11.
6. Zitzmann NU, Berglundh T. **Definition and prevalence of peri-implant diseases.** *J Clin Periodontol* 2008;**35**:286-291.
7. Pontoriero R, Tonelli MP, Carnevale G, Mombelli A, Nyman SR, Lang NP. **Experimentally induced peri-implant mucositis. A clinical study in humans.** *Clin Oral Implants Res* 1994;**5**:254-259.
8. Zitzmann NU, Berglundh T, Marinello CP, Lindhe J. **Experimental peri-implant mucositis in man**. *J Clin Periodontol* 2001;**28**:517-523.

9. Berglundh T, Lindhe J, Marinello C, Ericsson I, Liljenberg B. **Soft tissue reaction to de novo plaque formation on implants and teeth. An experimental study in the dog.** *Clin Oral Implants Res* 1992;**3**:1-8.

10. Renvert S, Lindahl C, Renvert H, Persson GR. **Clinical and microbiological analysis of subjects treated with Branemark or AstraTech implants: A 7-year follow-up study.** *Clin Oral Implants Res* 2008;**19**:342-347.

11. Nowzari H, Phamduong S, Botero JE, Villacres MC, Rich SK. **The profile of inflammatory cytokines in gingival crevicular fluid around healthy osseointegrated implants.** *Clin Implant Dent Relat Res* 2010;**14**:546-552.

12. Hultin M, Gustafsson A, Hallstrom H, Johansson LA, Ekfeldt A, Klinge B. **Microbiological findings and host response in patients with peri-implantitis.** *Clin Oral Implants Res* 2002;**13**:349-358.

13. Heitz-Mayfield LJ, Lang NP. **Comparative biology of chronic and aggressive periodontitis vs. peri-implantitis.** *Periodontol 2000* 2010;**53**:167-181.

14. Rams TE, Roberts TW, Feik D, Molzan AK, Slots J. **Clinical and microbiological findings on newly inserted hydroxyapatite-coated and pure titanium human dental implants.** *Clin Oral Implants Res* 1991;**2**:121-127.

15. Leonhardt A, Renvert S, Dahlen G. **Microbial findings at failing implants.** *Clin Oral Implants Res* 1999;**10**:339-345.

16. Armitage GC. **Clinical evaluation of periodontal disease.** *Periodontology 2000* 1995;**7**:39-53.

17. Löe H, Theilade E, Jensen SB. **Experimental gingivitis in man.** *J Periodontol* 1965;**36**:177-187.

18. Theilade E, Wright WH, Jensen SB, Löe H. **Experimental gingivitis in man. II. A longitudinal clinical and bacteriological investigation.** *J Periodontal Res* 1966;**1**:1-13.

19. Tanner A, Kent R, Maiden MF, Taubman MA. **Clinical, microbiological and immunological profile of healthy, gingivitis and putative active periodontal subjects.** *J Periodontal Res* 1996;**31**:195-204.

20. Tanner A, Maiden MF, Macuch PJ, Murray LL, Kent R. **Microbiota of health, gingivitis, and initial periodontitis.** *J Clin Periodontol* 1998;**25**:85-98.

21. Teles R, Bogren A, Patel M, Wennstrom J, Socransky S, Haffajee A. **A three-year prospective study of adjult subjects with gingivitis II: microbiological parameters.** *J Clin Periodontol* 2007;**34**:7-17.

22. Kistler J, Booth V, Bradshaw D, Wade W. **Bacterial community development in experimental gingivitis.** *PLoS ONE* 2013;**8**:e71227.

23. Huang S, Li R, Zeng X, He T, Zhao H, Chang A, Bo C, Chen J, Yang F, Knight R, Lui J, Davis C, Xu J. **Predictive modeling of gingivitis severity and susceptibility via oral microbiota.** *ISME J* 2014;8**;1768-1780.**

24. Ebersole JL, Cappelli D. **Acute-phase reactants in infections and inflammatory diseases**. *Periodontol 2000* 2000;**23**:19-49.

25. Berglundh T, Donati M:.**Aspects of adaptive host response in periodontitis**. *J Clin Periodontol* 2005;**32 Suppl 6**:87-107.

26. Page RC, Schroeder HE. **Pathogenesis of inflammatory periodontal disease. A summary of current work.** *Lab Invest* 1976;**34**:235-249.

27. Brecx M, Schlegel K, Gher P, Lang N. **Comparison between histological and clinical parameters during experimental gingivitis.** *J Periodontol Res* 1987;**22**:50-57.

28. **Informational paper: the pathogenesis of periodontal diseases**. *J Periodontol* 1999;**70**:457-470.

29. Heasman P, Collins S, Offenbacher S. **Changes in crevicular fluid levels of interleukin-1 beta, leukotriene B4, prostaglandin E2, thromboxane B2 and tumor necrosis factor alpha in experimental gingivitis in humans.** *J Periodontol Res* 2006;**28**:241-247.

30. Ertugrul A, Sahin H, Dikilitas A, Alpaslan N, Bozoglan A. **Comparison of CCL28, interleukin-8, interleukin-1 beta and tumor necrosis factor alpha in subjects with gingivitis, chronic periodontitis and generalized aggressive periodontitis** . *J Periodontol Res* 2013;**48**:44-51.

31. Offenbacher S, Barros S, Singer R, Moss K, Williams R, Beck J. **Periodontal disease at the biofilm-gingival interface.** *J Periodontol* 2007;**78**:1911-1925.

32. Offenbacher S, Barros S, Mendoza S, Mauriello S, Preisser J, Moss K, de Jager M, Aspiras M. **Changes in gingival crevicular fluid inflammatory mediator levels during the induction and resolution of experimental gingivitis in humans**. *J Clin Periodontol* 2010;**37**:324-333.

33. Trombelli L, Scapoli C, Carrieri A, Giovannini G, Calura G, Farina R. **Interleukin-1 beta levels in gingival crevicular fluid and serum under naturally occuring and experimentally induced gingivitis**. *J Clin Periodontol* 2010;**37**:697-704.

34. Gilowski L, Wiench R, Plocica I, Krzeminski T. **Amount of interleukin-1beta and interleukin-1 receptor antagonist in periodontitis and healthy patients**. *Arch Oral Biol* 2014;**59**:729-734.

35. Mantovani A, Gray P, Van Damme J, Sozzani S. **Macrophage-derived chemokine (MDC).** *J Leukoc Biol* 2000;**68**:400-404.

36. Cardoso C, Garlet G, Moreira A, Junior W, Rossi M, Silva J. **Characterization of CD4+CD25+ natrual regulatory T cells in the inflammatory infiltrate of human chronic periodontitis**. *J Leukoc Biol* 2008;**84**:311-318.

37. Kumar P, Mason M, Booker M, O'Brien K. **Pyrosequencing reveals unique microbial signatures associated with healthy and failing dental implants.** *J Clin Periodontol* 2012;**39**:425-433.

38. Salvi GE, Aglietta M, Eick S, Sculean A, Lang NP, Ramseier CA. **Reversibility of experimental per-implant mucositis compared with experimental gingivitis in humans.** *Clin Oral Implants Res* 2012;**23**:182-190.

39. Heuer W, Kettenring A, Stumpp S, Eberhard J, Gellermann E, Winkel A, Stiesch M. **Metagenomic analysis of the peri-implant and periodontal microflora in patients with clinical signs of gingivitis or mucositis.** *Clin Oral Invest* 2012;**16**:843-850.

40. Liskmann S, Zilmer M, Vihalemm T, Salum O, Fischer K. **Correlation of peri-implant health and myeloperoxidase levels: A cross-sectional clinical study.** *Clin Oral Implants Res* 2004;**15**:546-552.

41. Carcuac O, Berglundh T. **Composition of human peri-implantitis and periodontitis lesions**. *J Dent Res* 2014;**11**:1083-1088.

42. Silness J, Löe H. **Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition.** *Acta Odontol Scand* 1964;**22**:121-135.

43. Trombelli L, Tatakis D, Scapoli C, Bottega S, Orlandini E, Tosi M. **Modulation of clinical expression of plaque-induced gingivitis. II. Identification of "high-responder" and "low-responder" subjects.** *J Clin Periodontol* 2004;**31**:239-252.

44. Furuichi Y, Lindhe J, Ramberg P, Volpe A. **Patterns of de novo plaque formation in the human dentition.** *J Clin Periodontol* 1992;**19**:423-433.

45. Van der Weijden GA, Timmerman MF, Nijboer A, Reijerse E, Van der Velden U. **Comparison of different approaches to assess bleeding on probing as indicators of gingivitis**. *J Clin Periodontol* 1994;**21**:589-594.

46. Fadrosh D, Ma B, Gajer P, Sengamalay N, Ott S, Brotman R, Ravel J. **An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform**. *Microbiome I2014;***2**:6.

47. Nadkarni M, Martin F, Jacques N, Hunter N. **Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set**. *Microbiology* 2002;**148**:257-266.

48. Sundquist A, Bigdeli S, Jalili R, *et al*. **Bacterial flora-typing with targeted, chip-based pyrosequencing**. *BMC Microbiol* 2007;**7**:108.

49. Schloss P, Westcott S, Ryabin T, Hall J, Hartmann M, Hollister E. **Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities**. *Appl Environ Microbiol* 2009;**75**:7537-7541.

50. Schloss P. **The effects of alignment quality, distance calculation method, sequence filtering and region on the analysis of t16S rRNA gene-based studies.** *PLoS Comput Biol* 2010;**6**:e1000844.

51. Huse S, Welch D, Morrison H, Sogin M. **Ironing out the wrinkles in the rare biosphere through improved OTU clustering.** *Environ Microbiol*;**12**:1889-1898.

52. Schloss P, Westcott S. **Assessing and improving methods used in operational taxonomic unit-based appraches for 16S rRNA gene sequence analysis.** *Appl Environ Microbiol*;**77**:3219-3326.

53. Edgar R, Haas B, Clemente J, Quine C, Knight R. **UCHIME improves sensitivity and speed of chimera detection.** *Bioinformatics*;**27**:2194-2200.

54. Martin A. **Phylogenetic approaches for describing and comparing the diversity of microbial communities**. *Appl Environ Microbiol* 2002;**68**:3673-3682.

55. Cole J, Wang Q, Cardenas E, Fish J, Chai B, Farris R, Kulam-Syed-Mohideen A, McGarrell D, Marsh T, Garrity G *et al*. **The Ribosomal Database Project: Improved alignments and new tools for rRNA analysis**. *Nucleic Acids Res* 2009;**37**:D141-D145.

56. Lozupone C, Knight R. **Species divergence and the measurement of microbial diversity**. *FEMS Microbiol Rev* 2008;**32**:557-578.

57. Shannon C. **The mathematical theory of communication**. *MD Comput* 1948; **14**:306-317.

58. Simpson E. **Measurement of diversity**. *Nature* 1949;**163**(4148):688.

59. Yue J, Clayton M. **A similarity measure based on species proportions**. *Commun Stat Theo M* 2005;**34**:2123-2131.

60. Excoffier L, Smouse P, Quattro J. **Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondial DNA restriction data**. *Genetics* 1992;**131**:479-491.

61. Albrektsson T, Dahlin C, Jemt T, Sennerby L, Turri A, Wennerberg A. **Is marginal bone loss around oral implants the result of a provoked foreign body reaction?** *Clin Implant Dent Relat Res* 2014;**14**:155-165.

62. Chan C, Weber H. **Plaque retention on teeth restored with full-ceramic crowns: a comparative study**. *J Prosthet Dent* 1986;**56**:666-671.

63. Mombelli A, van Oosten M, Schurch E, Lang N. **The microbiota associated with successful or failing osseointegrated titanium implants**. *Oral Microbiol and Immunol* 1987;**2**:145-151.

64. Preshaw P, Taylor J. **How has research into cytokine interactions and their role in driving immune responses impacted our understanding of periodontitis?** *J Clin Periodontol* 2011;**38**:60-84.

65. Cheng, W, Hughes F, Taams L. **The presence, function and regulation of IL-17 and Th17 cells in periodontitis**. *J Clin Periodontol* 2014;**41**:541-549.

66. Ozdemir B, Ozcan G, Karaduman B, Teoman A, Ayhan E, Ozer N, Us D. **Lactoferrin in gingival crevicular fluid and peripheral blood during experimental gingivitis**. *Eur J Dent* 2009;**3**:16-23.

67. Jamsen E, Kouri V, Olkkonen J, Cor A, Goodman S, Konttinen Y, Pajarinen J **Characterization of macrophage polarizing ctokines in the aseptic loosening of total hip replacements**. *J Orthop Res* 2014;**32:**1241-1246.

68. Trindade R, Albrektsson T, Wennerberg A. **Current concepts for the biological basis of dental implants: foreign body equilibrium and osseointegration dynamics.** *Oral Maxillofac Surg Clin North Am* 2015;**27**:175-183.

69. Abusleme L, Dupuy A, Dutzan N, Silva N, Burleson J, Strausbaugh L, Gamonal J, Diaz P. **The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation**. *ISME J* 2013;**7**:1016-1025.