INVESTIGATIONS ON THE MICROBIAL COMMUNITY ASSOCIATED WITH PERI-IMPLANTITIS IN SMokers AND NON SMOKERS.

THESIS

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ABSTRACT

Background: Peri-implantitis is a biofilm-induced disease that leads to destruction of implant-supporting structures and may result in loss of the implant. It is known that smokers are at higher risk for implant failure than nonsmokers. We have previously demonstrated that even in states of clinical health, smokers have a pathogen-enriched periodontal biofilm that increases their susceptibility to disease. In addition, although early studies indicated that peri-implant microbiota may be similar to subgingival microbiota, evidence is emerging, that the microbial profiles of peri-implant health and peri-implantitis are different from periodontal health and periodontitis. The ultimate goal of the present study is to identify subject-specific differences between implants and teeth and answer the question whether altered subgingival microbial profiles in smokers are attributable to smoking per se or occur secondarily to peri-implant disease.

Methods: Subgingival and submucosal plaque samples were collected from forty current and forty never smokers with peri-implantitis, peri-implant mucositis and peri-implant health. Bacterial DNA was isolated, amplified and sequenced using 16S pyrotag sequencing. Chimera-depleted sequences were compared against a locally-hosted curated database for bacterial identification. UniFrac distances were compared using community ordination methods. ANOVA with Tukey HSD were used to compare the microbial profile across groups.

Results: No statistically significant differences were seen between smokers and nonsmokers in any of the demographic characteristics. Based on the health status of
the implants, the only significant differences were found in terms of history of implant loss and bruxism in peri-implantitis groups compared to peri-implant mucositis and healthy sites. Non-smokers were found to have higher gingival inflammation in the peri-implantitis cases and these differences were statistically significant. Overall 2.72 millions sequences were identified with Firmicutes accounting for 56.7% of all sequences. The evaluation of the shared species between teeth and implants revealed that 40 to 85% of the species were shared between the two groups and there was significantly more bacterial diversity on teeth compared to implants. Irrespective of the implant health status, the most predominant gram status in all cases was the gram positive anaerobes, followed by gram negative anaerobes but no statistical significant differences were found in health and disease. The most predominant species in the peri-implant communities in non-smokers belonged to the families *Unclassified Streptococcus, Unclassified Fusobacterium, Unclassified Clostridiaceae*. Smokers presented similar species apart from peri-implantitis cases that presented a completely different microbial profile including the genera *Acinetobacter, Kingella* and *Methylobacterium*.

**Conclusions:** Peri-implant disease in smokers is distinct from that of non-smokers, as well as healthy peri-implant biofilms in smokers demonstrate differences compared to that of non-smokers. In addition, the microbial profile of peri-implantitis includes several previously unsuspected species. Lastly, even though teeth and implants share species, they remain microbiologically different.
Dedicated to:

Konstantinos, Evita and George
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FIELDS OF STUDY

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CHAPTER 1

INTRODUCTION

Dental implants as viable treatment options for the replacement of missing teeth

In spite of a marked decline in the prevalence of both partial and total tooth loss in adults since the late 1950’s, significant disparities are still present, particularly among lower socioeconomic groups. By age 50, nearly 70% of the population has lost one tooth and less than 10% has more than 25 natural teeth present. This number increases in older individuals with a tenth of the population being completely edentulous.¹

Over the last few decades, among the various options available to replace missing or lost teeth, dental implants have become a commonly used treatment alternative to removable and conventional fixed partial dentures with better oral rehabilitation outcomes. Dental implants present advantages such as increased stability, prevention of alveolar bone loss and better esthetic results when compared to conventional removable or fixed dental restorations. Additionally, the long term survival rate of dental implant supported restorations is reported as similar as or even higher than traditional crowns, bridges and/or dentures.²⁻⁴ Recent evidence suggests that by 2005 more than 8 million implants were placed only in the United States and there are now over 400,000 implants inserted annually. This number is expected to increase at a sustained growth of 18% yearly for the next several years.⁵
Definitions of implant health and disease

The increase in popularity of dental implants in the last 25 years has been linked with an equivalent growth in the complications and failures seen around implants. Biological failure of implants can be defined as the inability of tissue to establish or maintain osseointegration. These failures are classified into early (failure to establish osseointegration) and late (failure to maintain osseointegration). One way to differentiate early and late failures is to define the early group as implants removed before prosthetic restoration, while those occurring after prosthetic rehabilitation are classified as late. The principal cause of early failure is the lack of osseointegration and it may be unrelated to infection. However, the most common causes of late failures are related to fractures, implant overload and peri-implant infection, specifically peri-implantitis.

Late complications (those that occur around an implant in function) may range from reversible changes in the mucosa to irreversible loss of the surrounding bone or the implant itself. Implant failure may be referred to as the status of implant performance that, when using some quantitative measurements, falls below an acceptable level. This definition encompasses clinical situations, ranging from mobile implants to implants showing more than 0.2mm of peri-implant bone loss after the first year of loading or bleeding in more than 5mm of probing depth. The distinction between failed, failing and ailing implant is clinically important. The lack of osseointegration is generally characterized by implant mobility and peri- fixtural radiolucency. In this situation, the implant is considered to be “failed”. On the other hand, the failure process might be slow and continuing. Therefore, an “ailing” implant has been defined as a clinically stable implant affected by bone loss with pocketing. The major difference between an “ailing” and a “failing” implant is the outcome of
the therapy. In fact, if an “ailing” implant is resistant to therapy it becomes “failing.”

In other words, the term “ailing” implies a somewhat more favorable prognosis than “failing.”\(^9\) Later on, other definitions of an ailing implant were also given. For instance, Krauser et al\(^{10}\), as well as Esposito et al,\(^9\) considered an implant to be “ailing” when affected by soft tissue aberrations without loss of supporting bone. Currently, this definition is widely accepted.

In general, the inflammatory lesions that develop in the tissues around implants in response to a bacterial biofilm are collectively recognized as peri-implant diseases. In accordance with the classification of periodontal diseases at teeth, peri-implant disease includes two entities: peri-implant mucositis that corresponds to gingivitis and peri-implantitis that corresponds to periodontitis. Peri-implant mucositis is defined as a reversible inflammatory reaction in the soft tissues surrounding a functioning implant and may be identified clinically by redness and swelling of the soft tissue, but bleeding on probing is currently recognized as the important feature. On the other hand, peri-implantitis is defined as inflammatory reactions associated with loss of supporting bone around an implant in function. Clinically, the mucosal lesion is often associated with suppuration and deepened pockets, but always accompanied by loss of supporting marginal bone. Finally peri-implant health is defined as an implant that demonstrates no mobility, less than 2 mm of radiographic bone loss from the prosthesis installation with no pain on function or history of exudate.\(^{11}\)

**Prevalence of peri-implant diseases**

The prevalence of peri-implant diseases is currently a controversial issue. Conflicting statements have been made with regards to the magnitude and the long-term consequences of this problem. Roos- Jansaker et al\(^{12}\) reported that peri-implant...
mucositis occurred in about 79% of the subjects and 50% of the implants. In a study by Fransson et al\textsuperscript{13} BOP was found in more than 90% of the implants without a history of bone loss.

The prevalence of peri-implantitis has been addressed in numerous publications with an average function time of 5-11 years.\textsuperscript{6,14,15} On subject level basis, prevalence ranging from 12.4 to 56% has been reported with a great heterogeneity being present in most of the studies. Based on the most recent evidence suggested by Mombelli et al\textsuperscript{15} one may state that the prevalence of peri-implantitis seems to be in the order of 10% implants and 20% patients during 5–10 years after implantation, but this statement needs to be taken with caution as the individual reported figures are rather variable, not easily comparable and not suitable for meta-analysis. Factors that have been shown to affect prevalence figures are the disease definition, the threshold for peri-implantitis and differences in the composition of study populations.\textsuperscript{15}

**Efficacy of current treatment modalities**

One of the key factors for the long-term success of oral implants is the maintenance of healthy tissues around them. Bacterial plaque accumulation induces inflammatory changes in the soft tissues surrounding oral implants and it may lead to their progressive destruction and ultimately to implant failure. In the case of peri-implant diseases, various interventions (often combined) have been suggested, including: a) mechanical debridement b) pharmaceutical therapy (subgingival chlorhexidine irrigation, local or systemic antibiotics) and c) surgical procedures including: open flap debridement aimed at 1) removing bacteria (also using soft lasers) 2) smoothing the implant surface (to decrease surface roughness) and removing unsupported implant threads that protect bacterial plaque, 3)
‘decontamination’ or ‘detoxification’ of the implant surface using various chemical agents or laser beam. After the primary goal of surgical intervention has been achieved, it may be necessary to correct the anatomical conditions for improving plaque control and for eliminating the favorable environment for anaerobic bacteria. In order for this to be achieved, either resective procedures or alternatively grafting procedures, including soft tissue grafting, guided bone regeneration, autologous or allogenic bone grafts, etc., have been suggested.

However, the predictability and efficacy of all the above-mentioned treatment modalities remains unclear until today. Based on the consensus report of the 6th European Workshop on Periodontology, it seems that in cases of peri-implant mucositis, non surgical mechanical debridement in conjunction with antimicrobials can have a positive effect but it still remains unpredictable. In cases of peri-implantitis there is limited evidence that debridement and decontamination can resolve the problem and there is no evidence that so called regenerative procedures have additional beneficial effects. In a similar study by Esposito et al it was concluded that there is no reliable evidence suggesting which could be the most effective interventions for treating peri-implantitis and that follow-up longer than 1 year suggested recurrence of peri-implantitis in up to 100% of the treated cases for some of the tested interventions. Thus, there is an increasing need to better understand the mechanism(s) involved in the pathogenesis of the disease, which will allow us to carefully design more effective treatment approaches.

**Colonization of the peri-implant crevice (pristine pocket)**

After exposure of an osseointegrated implant in the oral cavity through a transmucosal abutment, an acquired pellicle is formed on the implant surface through
selective adsorption of the environmental macromolecules such as a-amylase and serum albumin.\textsuperscript{18} This pellicle is derived from components in the saliva, as well as bacterial and host tissue products. It acts as a substrate for bacterial colonization, which occurs as early as 30 minutes after implant exposure in the oral cavity.\textsuperscript{19} In comparison to natural teeth, the acquired pellicle on dental implants has a lower albumin adsorption capability, which according to some authors contributed to the lower plaque formation around implants.\textsuperscript{20} Biofilm represents an organized structure in which microorganisms interact metabolically as a community. Biofilm formation around implants occurs in a similar way as teeth. After formation of the acquired pellicle, bacterial attachment with initial colonizers followed by cell-to-cell adhesion with secondary colonizers occurs on the implant surface.\textsuperscript{21} Biofilms are the preferred method of growth for most bacteria because they facilitate exchange of nutrients and protect the bacterial community from competing microorganisms.\textsuperscript{22} Moreover, biofilms also contribute to the spread of antibiotic resistance unless the biofilm is mechanically disrupted.\textsuperscript{21}

**Microflora in peri implant health and disease**

Similar to the healthy periodontium around natural teeth, many studies suggest that the microorganisms associated with healthy implants are predominantly Gram-positive cocci and rods.\textsuperscript{19} The dominant species are members of the yellow and purple complexes or are independent of the complexes such as *Actinomyces naeslundii* or *Actinomyces viscosus*.\textsuperscript{23} Gram-negative bacteria can be found in smaller proportions and include *Prevotella intermedia*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella nigrescens*, and *Campylobacter rectus*. This suggests that certain species are indigenous, host-compatible organisms. Moreover, the composition of the supragingival and submucosal microflora is similar around healthy implants.\textsuperscript{23}
An adequate permucosal seal of the soft tissue to the implant surface protects the base of the sulcus against the penetration of chemical and bacterial substances. With the loss of this initial seal, the peri-implant mucosa may progress from health to mucositis and possibly to peri-implantitis. As the peri-implant tissues undergo the process from a state of health to that of disease, differences in bacterial numbers and morphotypes can be found. A shift from a Gram-positive facultative dominated flora to a Gram-negative anaerobic biofilm occurs.\textsuperscript{24} The failing implant is characterized by a greater proportion of red and orange (\textit{P. intermedia} and \textit{Fusobacterium nucleatum}) complex, as well as \textit{Aggregatibacter actinomycetemcomitans} and \textit{Eikenella corrodens} with a lower proportion of the flora associated with health.\textsuperscript{25-27} Hence, the main differences between health and disease are in the proportions of \textit{Actinomyces}, orange and red complex species. Furthermore, increasing peri-implant probing depth has been significantly associated with higher total anaerobic cultivable microbiota and the frequency of detection of \textit{P. gingivalis}.\textsuperscript{28} Van Winkelhoff et al\textsuperscript{29} studied the early colonization of the peri-implant pockets by putative periodontal pathogens in 20 partially edentulous patients. The authors found that most periodontal pathogens (\textit{P. gingivalis}, \textit{F. nucleatum}, \textit{P. intermedia}, and \textit{T. forsythia}) were already identified as early as 6 months after loading. In particular, \textit{P. gingivalis} was significantly associated with the presence of fistulas and implant loss. The study also demonstrated that although 2 patients exhibited \textit{A. actinomycetemcomitans} during their baseline examination, \textit{A. actinomycetemcomitans} was not isolated from any implant pocket during the experimental period. Other microorganisms such as \textit{Staphylococcus aureus}, \textit{Candida albicans}, and enteric rods have also been associated with peri-implantitis.\textsuperscript{26} \textit{S. aureus} has high adhesion to titanium surfaces, and its presence has been associated with suppuration and bleeding on probing.\textsuperscript{25} \textit{C. albicans} is the most
common fungus found in the oral cavity, and its presence is strongly associated with oral candidiasis especially in patients wearing dentures. *C. albicans* has high adhesion to dental implants\textsuperscript{30} although an in vitro study showed lower levels of this microorganism on sandblasted titanium surfaces. Infection of dental implants with these opportunistic microorganisms should be recognized especially in the immunocompromised patients.\textsuperscript{31}

Regarding the bacterial profile differences between peri-implant mucositis and peri-implantitis studies using checkerboard DNA–DNA hybridization revealed that *P. gingivalis, T. forsythia, P. intermedia, Fusobacterium ssp.*, *S. sanguinis, S. gordonii, V. parvula* and *actinomycetes* were detected at elevated levels in periimplantitis. *C. ochracea, Neisseria mucosa, P. gingivalis, P. nigrescens, Fusobacterium ssp.* and *Actinomycetes* were detected at elevated levels in mucositis.\textsuperscript{15} Only three species were found at significantly different levels in samples from mucositis or peri-implantitis: *T. forsythia* (higher levels in the peri-implantitis group), *A. gerencseriae* and *C. ochracea* (lower counts in the peri-implantitis group).\textsuperscript{15}

The analysis with various methods has shown that the microbiota associated with peri-implant disease is (i) mixed, (ii) rather variable, and (iii) in most cases dominated by diverse Gram-negative anaerobic bacteria.

**Factors affecting peri-implant disease (implant type, surface characteristics)**

Current knowledge of the factors related to implant characteristics and failure are mostly gleaned from *in vitro* studies. Identified strong evidence in the literature suggests that poor oral hygiene, history of periodontitis and cigarette smoking are strong indicators for peri implant disease. On the other hand, even though surface characteristics may influence the amount and composition of biofilm formation,
(rough surface implants -titanium plasma sprayed (TPS)- are more likely to develop peri-implantitis than minimally rough implants once exposed to the oral environment) so far, there is not enough evidence to make definitive conclusions on the clinical implications.32

**Differences between periodontal and peri-implant histopathology**

While comprehensive information exists regarding histopathological characteristics of human periodontitis lesions, few studies have evaluated peri-implantitis lesions in human biopsy material. Experimental peri-implantitis lesions were evaluated in 10 studies and three of the studies included comparisons to experimental periodontitis. Based on human biopsy material, it has been found that the apical extension of the inflammatory cell infiltrate (ICT) is more pronounced in peri-implantitis than in periodontitis and in most cases it is located apical of the pocket epithelium.33 The predominant cells in both types of lesions are plasma cells and lymphocytes, whereas neutrophil granulocytes and macrophages occur in larger relative proportions in peri-implantitis lesions. In addition, it has been suggested that that neutrophil granulocytes apart from residing in pocket epithelium, they are also included in perivascular compartments in apical portions distant from the pocket area around implants.33 Furthermore, experimental studies have shown that placement of ligatures together with plaque formation result in loss of supporting tissues and large inflammatory cell infiltrate around implants and teeth. Following ligature removal, a “self limiting” process occurred in the tissues around teeth with a connective tissue capsule that separated the inflammatory cell infiltrate from bone, while in peri-implant tissues it extended to the bone crest. Finally, peri-implantitis lesions, in
contrast to periodontitis lesions, exhibited signs of acute inflammation and large amounts of osteoclasts that lined the surface of the crestal bone.\textsuperscript{33}

**Differences between periodontal and peri-implant microflora**

The transmucosal abutment of osseointegrated dental implants serves as a surface for bacterial colonization of microbial biofilms. Like the gingival crevice around the natural tooth, the peri-implant mucosa, which covers the alveolar bone, is closely adapted to the implant. In partially edentulous subjects, the developing microbiota around implants has been found to closely resemble the microflora of naturally remaining teeth.\textsuperscript{34, 35} A history of periodontitis and the presence of putative periodontal pathogens are factors that can influence the condition of peri-implant tissues in partially edentulous subjects. Quirynen and Listgarten\textsuperscript{36} used phase-contrast microscopy to evaluate the impact of periodontitis around remaining teeth and probing depth around the implants on the composition of the peri-implant subgingival flora in partially edentulous subjects. The investigators found that the subgingival microflora around implants harbored increased spirochetes and motile rods compared with teeth present in the same jaw. Samples from deep peri-implant pockets in the residual dentition of patients with chronic or refractory periodontitis showed significantly higher proportions of spirochetes and motile rods than samples from periodontally healthy patients with comparable probing pocket depths. Papaioannou et al\textsuperscript{37} also using phase-contrast microscopy and DNA probes, determined the prevalence of putative periodontal pathogens in partially edentulous and edentulous patients with a history of periodontal disease. The microbiologic profiles were similar
around teeth and dental implants of equal pocket depth, which may indicate that pockets around teeth can serve as a reservoir for putative periodontal pathogens. This finding was confirmed in several studies on partially edentulous patients. As early as 1 month after implantation, putative periodontal pathogens were detected around the implants of partially edentulous patients. Most of the evidence suggests that implant failures due to infection are characterized by a complex peri-implant microbiota resembling that of adult periodontitis. In edentulous subjects, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* are not as frequently associated with peri-implant infection as in dentate subjects. Danser et al reported that after total extraction in patients with severe periodontitis, *Porphyromonas gingivalis* could no longer be detected on the mucosal surface of edentulous patients. Furthermore, *A. actinomycetemcomitans* and *Porphyromonas gingivalis* could not be isolated at the peri-implant pockets in these patients after insertion of implants. In addition to the dark-pigmented, gram-negative anaerobic rods, other bacterial species are associated with peri-implant infections (eg, *Bacteroides forsythus*, *Fusobacterium nucleatum*, *Campylobacter*, *Peptostreptococcus micros* and *Prevotella intermedia*). Organisms that are less frequently associated with periodontitis, such as *Staphylococcus spp*, enterics, and *Candida spp*, have also been found in peri-implant infections. Longitudinal data on implants in partially edentulous persons with a history of periodontal disease, however, have shown no association between periodontal pathogens and loss of attachment at implants after 36 months of function. This finding corresponds to the situation observed in periodontitis: putative periodontal pathogens can also be detected in apparently healthy periodontal pockets and at sites with no periodontal progression. Thus, it has been suggested that
the pathogens in peri-implant infections propagate from the periodontopathic bacteria of natural teeth into the saliva and become transmitted to the vicinity of implants.

**Smoking and implant outcomes**

Smoking is a risk factor for general health and oral health. Smoking has a long term chronic effect on many aspects of the inflammatory and immune systems. The deleterious effects of smoking include impaired wound healing, reduced collagen production, impaired fibroblast function, reduced peripheral circulation and compromised function of neutrophils and macrophages. By altering the host microenvironment in many different ways smoking may thus contribute to preferential colonization by certain bacteria. For example, bacteria require iron for successful colonization and proliferation; which would normally be withheld by powerful innate immune responses within the host. By causing a situation of iron-load in the host; smoking increases the levels of bacteria with poor iron-acquisition abilities. Exposure to cigarette smoke increases bacterial adherence to epithelial cells, favoring increased levels of species with normally poor attachment abilities. Smoking has been shown to reduce oxygen tension in the subgingival microenvironment. Low-oxygen tension microbial communities demonstrate a significantly different profile than normal oxygen tension communities. Smoking also markedly reduces the levels of protective nasopharyngeal bacteria, which predisposes to colonization of this niche by potential pathogens.

The effect of smoking on the peri-implant tissues has been documented in a number of studies. Its role as a risk factor for periodontal disease progression has been confirmed, and current data suggest that smokers have at least a threefold increased risk of developing periodontitis. The possible relationship between smoking and
implant failures has been evaluated in several retrospective and prospective clinical studies.\textsuperscript{50} In a retrospective analysis of the outcome of 2194 implants placed in 540 subjects, Bain and Moy\textsuperscript{51} reported that a significantly greater percentage of implant failures occurred in smokers than in nonsmokers. Smokers had an overall failure rate of 11.3\%, whereas only 4.8\% of the implants placed in nonsmokers failed. Gorman et al\textsuperscript{52} found that implant failures were twice as common in smokers as in nonsmokers at second-stage surgery. In general, it can be concluded that smoking has a negative effect on implant survival, especially during the early healing period after implant installation.

The effect of smoking on marginal bone loss has also been evaluated. Cigarette smoking was associated with significantly greater marginal bone loss at implants used in the treatment of edentulous mandibles.\textsuperscript{53} The 10- and 15-year follow-up reports on this group of edentulous patients showed that bone loss, although limited, was related to several factors, among which smoking and oral hygiene were the most important. Haas et al\textsuperscript{54} compared the association between smoking and peri-implantitis in 107 smokers compared with 314 nonsmokers. Smokers had higher bleeding scores, more signs of clinical inflammation, deeper probing pocket depth, and more radiographic bone loss around implants than nonsmokers. The investigators further stated that the effect of smoking on the condition of peri-implant tissues was more pronounced in the maxilla. While cigarette smoking is not an absolute contraindication for implant placement, with the majority of studies reporting implant survival in the range of 80\% to 96\%, smokers should be informed that there is an increased risk of implant loss and peri-implantitis (reported odds ratios 3.6 to 4.60)
In light of all this evidence, it is clear that although both bacterial plaque and smoking are reported to play important roles in peri-implant disease, associations between smoking and individual species or bacterial consortia have not been well elucidated. Recent cultivation-independent explorations of the subgingival microbial community, have revealed that the majority of species are uncultivated and hence, unrecognized when using traditional, cultivation-based approaches. Therefore, it is important to examine the subgingival microbial profile of this high-risk population using molecular approaches that circumvent the need for cultivation.

The purpose of this study is

- To identify subject-specific differences between implants and teeth
- To answer the question whether altered subgingival microbial profiles in smokers are attributable to smoking per se or occur secondarily to peri-implant disease.

**Null Hypothesis:** The bacterial profile between healthy and diseased implants is similar in smokers and nonsmokers.
Specific Aims:

1. **To compare the peri-implant microbial community in current smokers with healthy and diseased peri-implant tissues.** We propose to compare the levels of species/phylotypes in smokers with peri-implant disease and peri-implant health to elucidate the association between peri-implant health status and selected bacteria or bacterial consortia in this high risk population.

2. **To compare the peri-implant microbial community in never smokers with healthy and diseased peri-implant tissues.** We will compare the levels of health and disease-associated species in never smokers to identify the bacteria that play a more important role in peri-implant infections and whose elimination could lead to improvement of the clinical condition.

3. **To compare the levels of subgingival bacteria in current and never smokers with healthy peri-implant tissues.** We propose to compare the levels of selected species/phylotypes in current and never smokers that maintain both healthy peri-implant tissues, so as to identify certain microflora changes associated with smoking per se.

4. **To compare the levels of subgingival bacteria in current and never smokers with peri mucositis and peri-implantitis.** We will compare the levels of health and disease-associated species between smokers and nonsmokers with peri-mucositis and peri-implantitis. This will elucidate whether altered microbial profiles in smokers are associated with smoking per se or are merely secondary to disease.

**Significance:** Identification of putative pathogens and health- associated bacteria in this high-risk population is an important step in studying their interactions with the human host. This could lead to the development of clinically useful disease markers and predictors, as well as biological interventions for prevention of disease.
CHAPTER 2

MATERIALS AND METHODS

Study population and study design:

The overall study design was observational case control study. Patients presenting with at least 1 dental implant in function for a minimum of one year were recruited from the patient pool of Graduate Periodontology Clinic, Implant Clinic, Advanced General Dental Clinic, Pre-Doctoral Dental Clinic and Dental Faculty Practice at The Ohio State University, between March 2011 and November 2012.

Inclusion criteria included: 1) Adult patients, age ≥ 18 years old; 2) patients who presented with peri implant health or peri-implant disease in at least one functioning implant for a minimum of one year, bounded by teeth on either side, 3) smokers and nonsmokers; 4) patients who are able and willing to provide consent for the study protocol.

Exclusion criteria included 1) diabetic patients 2) pregnant women 3) HIV infected patients 4) use of immunosuppressant medications, bisphosphonates or steroids 5) antibiotic therapy or oral prophylactic procedures within the last 3 months 6) need for antibiotic coverage before dental treatment and 7) less than 20 teeth present in the dentition. The study protocol and informed consent forms were approved by the Institutional Review Board of the Ohio State University (2011H0023). Written consent was obtained from all participants before entry into the study.

The study design consisted of a single visit. During this visit, participants’ medical status and dental history were reviewed. All participants were given oral and
written informed consents for the study protocol. The signed informed consents were collected.

Participants classified as smokers were individuals who are currently smoking and have smoked over 100 cigarettes in their lifetime, whereas nonsmokers were considered subjects that smoked none or less than 100 cigarettes in total and do not currently smoke. After a thorough clinical and radiographic examination, each one of the subjects was further divided in one of the following groups: peri-implant mucositis, peri-implantitis or peri-implant health.\textsuperscript{11} Peri-implant health was diagnosed when the implant demonstrated no bleeding on probing, suppuration or mobility and no radiographic evidence of more than 2 mm of marginal bone loss after being at least 1 year in function. Implants with only clinical signs of inflammation and absence of radiographic bone loss were classified as peri-implant mucositis, whereas implants with presence of both inflammation and radiographic evidence of more than 2 mm bone loss since the prosthesis installation were diagnosed as peri-implantitis cases.

\textit{Clinical Data collection}

Detailed demographic information, medical and dental history were collected from all patients recruited to the study (see Figure 1). Clinical information about the implant and adjacent teeth was also collected (see Figure 2).

\textit{Sample collection}

The selected sites were dried and isolated with the use of two cotton rolls that were placed on the floor of the mouth of each subject for 1 minute. Subgingival and submucosal plaque samples were collected by inserting a total of 10 sterile endodontic
paper points (Caulk- Dentsply) into the peri implant crevice for 10 sec. Similarly, 6 sterile endodontic paper points were placed into the sulci or periodontal pocket of each adjacent tooth. In case any of the adjacent teeth was not present the first available tooth mesially or distally was sampled. Samples were placed in 1.5 ml microcentrifuge tubes and frozen at -80°C until further analysis.

DNA isolation

DNA was isolated from each sample by placing the paper points in a 1.5mL sterilized collection tube with 200μL of PBS then agitated for 20-30 minutes. The paper points were then removed and placed into a 0.5mL collection tube with a hole punctured in the base and this tube was returned to the 1.5mL tube to be centrifuged for 3 minutes to allow the fluid from the paper points to return to the PBS solution and the paper points were saved in -80°C. 180μL of Buffer ATL and 40μL of Proteinase K were added to the PBS/Bacteria solution, and agitated for 15 seconds to mix the solutions. The collection tube was then incubated at 56°C in a hot water bath for at least 2 hours. Following incubation, 200μL of Buffer AL was added to the collection tube, agitated for 15 seconds, and incubated at 70°C in a dry bath for 10 minutes.

Once this was completed, 200μL of 100% Ethanol was added to the tube, agitated for 15 seconds, the solution was then transferred to a QIAamp Spin Column nested inside of a 2.0mL collection tube and centrifuged for 1 minute. The filtrate was then discarded and 500μL of Buffer AW1 was placed into the spin column and centrifuged for 1 minute, filtrate was discarded, 500μL of Buffer AW2 was added to the spin column and centrifuged for 3 minutes. Once completed the collection tube was discarded, and the spin column was placed in a new sterile 2.0mL collection tube.
50μL of Buffer EB/AE was added to the column, incubated at room temperature for 5 minutes and centrifuged for 1 minute, the sample was then moved to a sterile 0.2mL collection tube.

Pyrosequencing

Multiplexed bacterial tag-encoded FLX amplicon pyrosequencing was performed using the Titanium platform (Roche Applied Science, Indianapolis, IN, USA) as previously described in a commercial facility (Research and Testing Laboratories, Lubbock, TX, USA). Briefly, a single step PCR with broad-range universal primers and 22 cycles of amplification was used to amplify the 16S rRNA genes as well as to introduce adaptor sequences and sample-specific bar-code oligonucleotide tags into the DNA. Two regions of the 16S rRNA genes were sequenced: V1–V3 and V7–V9. The primers used for sequencing have been previously described. Adaptor sequences were trimmed from raw data with 98% or more of bases demonstrating a quality control of 30 and sequences binned into individual sample collections based on bar-code sequence tags, which were then trimmed. The resulting files were denoised with Pyronoise and depleted of chimeras using B2C2 (http://www.researchandtesting.com/B2C2.html). Sequences <300 bp were discarded and the rest were clustered into species-level operational taxonomic units (s-OTUs) at 97% sequence similarity and assigned a taxonomic identity by alignment to locally hosted version of the Greengenes database using the Blastn algorithm. Phylogenetic trees were generated and visualized using FastTree. Unifrac and community diversity metrics were computed as previously described. All analysis were conducted using the QIIME pipeline.
Statistical analysis:

Shannon diversity index was computed using s-OTU data. A variance stabilizing transformation was used to create normal distribution of the data. The proportion (p) of each s-OTU in the community of each subject was expressed as $X = \sin^{-1}(\sqrt{p})$ and ANOVA with Tukey HSD were used to compare the means of this transformed variable X across groups. Unweighted Unifrac distances were used to examine similarities between communities.
CHAPTER 3

RESULTS

A total of 80 subjects fulfilled the inclusion criteria and were recruited for the study. Table 1 shows the demographic details of the study population. The non-smoker patient population consisted of 16 female and 24 male subjects with a mean age of 55.2 years. The smoker patient population consisted of 21 female and 19 male subjects with an average age of 57.7 years. The differences between smokers and non-smokers based on age, sex, ethnicity, history of implant loss, history of bruxism, oral hygiene, number of implants present, implant type, implant location and years in function were not statistically significant. Based on the health status of the implants, the only statistical significant differences were found in terms of history of implant loss and bruxism. Specifically, history of implant loss was statistical significant more prevalent in peri-implantitis cases compared to peri-implant mucositis, and bruxism was statistical significant more common in diseased compared to healthy sites (p<0.05, Chi-square analysis).

Table 2 shows the clinical characteristics of the study population. The differences in the implant sites, between smokers and non-smokers, in terms of modified gingival index, modified plaque index, mobility, bleeding on probing, probing depth, keratinized gingiva, presence of suppuration, threads exposed, pain, redness and radiographic bone loss were not statistically significant. Non-smokers were found to have higher gingival inflammation (as evidenced by the presence of
swelling), in the peri-implantitis cases and these differences were statistically significant (p<0.05, Chi-square analysis).

Figure 3 shows the distribution of the sequences by taxonomic level. A total of 2.72 million sequences were used in the analysis. Overall, these sequences represented the phyla: Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Synergistetes and TM7; with Firmicutes accounting for 56.7% of all sequences. These sequences represented 116 genus level OTUs and 242 s-OTUs. 2% of sequences were not classifiable at the genus level and were placed in higher order classifications. Uncultivable phylotypes accounted for an average of 18% and 16.58% of the healthy biofilm, 8.22% and 12.9% of the peri-implant mucositis biofilm and 8.67% and 11.62% associated with peri-implantitis in smokers and nonsmokers respectively.

Figure 4 shows the distribution of the shared species between teeth and implants for all 80 subjects. In an attempt to relate the percentage of overlap between the groups with the abundance of the microbial communities different cut off values were used. Figure 4A shows the proportion of overlap at 0.0% cut off. Overall, it was observed that 40 to 85% of the species were shared between the two groups with half of the patients sharing less than 65% of the species. Figure 4B demonstrates the proportion of overlap in the bacterial species, evaluated at 0.01% cut-off (if a species was found in tooth and implant at an abundance of 0.01%, it was considered shared). The percentage of shared species in the two groups dropped to a range of 35-75% in the overall population. Figure 4C shows the proportion of overlap at 0.1% cut off. A greater decrease of shared communities was evident. More specifically, it was found that less than 45% of the bacterial flora was similar between teeth and implants with a minimum overlap value close to 12%. Figure 4D demonstrates the proportion of overlap in the bacterial species, evaluated at 1% cut-off. Maximizing the abundance
of shared species to 1% resulted in a decrease of the overlapping microbial population to less than 18% between the groups. The percentage of shared species between teeth and implants was also evaluated based on the health status of the examined sites, in four different groups: Healthy Tooth/ Healthy Implant (HT/HI), Healthy Tooth/ Diseased Implant (HT/DI), Diseased Tooth/ Healthy Implant (DT/HI), Diseased Tooth/ Diseased Implant (DT/DI). Figure 2E shows that no statistical significant differences were found in the percentage of similarity of species between the groups (p>0.05, ANOVA with Tukey HSD).

Figure 5A shows the overall Shannon Diversity Index differences in Shannon Diversity Index between tooth-implant pairs in each subject. Overall, there was statistically significant more bacterial diversity on teeth compared to implants (p<0.05, Wilcoxon signed- rank test). Figure 5B shows the relative contributions of tooth and implant health to the difference in diversity. In three of the groups (HT/HI, HT/DI, DT/DI) there is a greater diversity in the microbial community of teeth compared to implants with the Healthy Tooth/ Healthy Implant group showing a more pronounced diversity. In the Diseased Tooth/ Healthy Implant group the diversity of the bacterial species is less evident.

Figure 6 shows the principal coordinate analysis of weighted Unifrac distances of the peri-implant microbiomes in health and disease. A distinct partitioning of the bacterial communities associated with healthy and diseased implants is evident. A higher level of partitioning was observed between the microbial profiles of healthy and diseased implants. A greater degree of similarity was observed between peri-implant mucositis and peri-implantitis sites than between healthy and peri-implantitis cases. Overall the peri-implantitis microbial profile represented a more homogenous bacterial community compared to the other groups, both in smokers and nonsmokers.
Figure 7 shows the average abundance of bacterial species found in the implant sites based on their gram stain characteristics. Figure 7A shows the average abundance of the bacterial species found in nonsmokers in the different implant groups. Irrespective of their health status, the most predominant group in all cases was the gram positive anaerobes, followed by gram negative anaerobes as well as gram negative and positive aerobes in lower proportions. In the peri-implantitis group the discrepancy between gram positive and negative anaerobes was less evident. No statistically significant differences were found in the gram status and aerobes or anaerobes in health or disease in nonsmokers (p>0.05, ANOVA with Tukey HSD).

Figure 7B shows the average abundance of the bacterial species based on their gram status in the healthy and diseased implant groups in smokers. Similarly, the gram positive and negative anaerobes are the predominant groups in all cases with the gram positive aerobes being the least prevalent group. No statistically significant differences were found in the average abundance of bacteria with the same gram status between the implant groups (p>0.05, ANOVA with Tukey HSD).

Figure 8 shows the number of bacterial species identified in the different implant groups in smokers and nonsmokers. The number of species varied from 47 to 136 per sample with the minimum number found on a healthy nonsmoker subject and the maximum number was identified in a peri-mucositis smoker subject. There were no statistical significant differences between implant groups in either smokers or nonsmokers (p>0.05 ANOVA with Tukey HSD).

Figure 9 shows the overall and relative abundances of genera in the three groups of implant samples. Figure 9A represents the species found in the nonsmoker group. Most of the species identified were present in both healthy and diseased implant sites in a different abundance. Certain species such as *Enterococcus*
casseliflavus, Lactobacillus refteri, Lactobacillus vaginalis, Desulfovibrio D168 were present only in healthy implants, others like Sphingomonas leidya, Unclassified Desulfomicrobium and Bacillus humi were found only in peri-implant mucositis sites, while peri-implantitis cases were associated with unique species such as Bifidobacterium breve, Parascardovia denticolens and Unclassified Bilophila. Figure 9B shows the species in the smoker population. Similarly, there are certain species associated only with health and disease in this high risk population. Healthy implants were found to have species such as Lactobacillus helveticus, Bacillus badius, and Unclassified Dorea, peri-implant mucositis cases were related to Staphylococcus aureus, Unclassified Bacillus and Moryella indoligenes and peri-implantitis cases were found to have unique species such as Campylobacter ureolyticus, Aggregatibacter actinomycetemcomitans and Acinetobacter johnsonii. Overall, the number of unique species present on healthy implants in nonsmokers was 15 and there was a minimum increase up to 16 in peri-implant mucositis and peri-implantitis. The number of unique species in smokers was 15 for the healthy group, 19 for the peri-implant mucositis group and dropped to 9 in the peri-implantitis cases. The unique microbial communities remained distinct between the groups.

The evaluation of figures 9A and B reveals species that were not represented by any of the implant groups, but were identified only on teeth sites. In nonsmokers, teeth presented with unique genera such as Enhydrobacter, Staphylococcus and Xenococcaceae family, while in smokers the unique microbial profile was represented by genera such as Alicycliphilus, Variovorax and Methylobacteriaceae family.

Figure 10 represents the most abundant species seen in the different implant groups. Figure 10A shows the most abundant species in nonsmokers. Bacteria such as Unclassified Fusobacterium, Unclassified Clostridiaceae and Unclassified
Streptococcus, were the most predominant in all groups but in different levels. Figure 10B demonstrates the most abundant species in smokers. Similarly, species such as Unclassified Clostridiaceae and Unclassified Streptococcus were the most abundant in healthy implants and implants with peri-implant mucositis. However in the peri-implantitis group the bacterial profile is completely different. In these cases the most predominant bacteria were Acinetobacter rhizosphaerae, Acinetobacter lwoffii and Acinetobacter johnsonii.
### Tables and Figures:

#### Figure 1: Patient Demographic Information - Collection Form

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</tr>
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<td>3. Ethnicity</td>
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</tr>
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<td>4. Smoking status</td>
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<td>5. History of previous implant loss</td>
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<td>7. Number of Implants</td>
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<td>8. Type of Implant</td>
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<td>9. Implant Location</td>
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</tr>
<tr>
<td>10. Years in function</td>
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</tr>
<tr>
<td>11. Bruxism /mouth guard use</td>
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<td>12. Type of home care oral hygiene</td>
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*Figure 1: Patient Demographic Information- Collection form.*
**Figure 2:** Clinical and radiographic findings-Collection form A: Implant site. 

B: Tooth site.
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*B: Brushing  
*F: Flossing  
*M: Mouthrinse

**Table 1:** Demographic Data
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<td>4%</td>
<td>4%</td>
<td>2.75%</td>
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**Table 2:** Clinical Characteristics.
Figure 3: Overall diversity of each taxonomic level (N=80).
**Figure 4:** A: Shared microbial species between implants and teeth in health and disease with 0.0% cut off B: Shared microbial species with 0.01% cut off. C: Shared microbial species with 0.1% cut off. D: Shared microbial species with 1.0% cut off. E: Percentage of similarity based on health status of teeth and implants at 0.0% cut off value (p>0.05, ANOVA with Tukey HSD).
**Figure 5:** Shannon diversity of the microbial communities between teeth and implants based on health status. A: Differences in Shannon Diversity between Tooth-Implant Pairs (p<0.05, Wilcoxon signed-rank test). B: Shannon diversity based on health status.
Figure 6: Principal Coordinate Analysis of UniFrac distances. Community characteristics of the peri-implant microbiomes in health and disease in smokers and nonsmokers (N=80).
Figure 7: Average abundance of bacterial species found in the different implant groups based on their gram status. A: Average abundance found in nonsmokers B: Average abundance found in smokers (p>0.05, ANOVA).
Figure 8. Number of bacterial species identified in the different implant groups in smokers and nonsmokers.
Figure 9: Detection frequency and overall abundances of genera in the three groups of samples. The phylogenetic trees were created using the Interactive Tree of Life.

A: Species detected in nonsmokers.
Figure 9: Detection frequency and overall abundances of genera in the three groups of samples. The phylogenetic trees were created using the Interactive Tree of Life.

B: Species detected in smokers.
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<td>Unclassified_Corynebacterium</td>
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<td>Unclassified_Capnocytophaga</td>
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<td>Veillonella_parvula</td>
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<tr>
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<td>Unclassified_Leptotrichia</td>
<td>Unclassified_Streptococcaceae</td>
</tr>
</tbody>
</table>

**Figure 10:** Most abundant species present in different implant groups. A: Most abundant species in nonsmokers.
<table>
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<tr>
<th>Healthy</th>
<th>Mucositis</th>
<th>Peri-implantitis</th>
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</thead>
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<td>Veillonella_parvula</td>
<td>Acinetobacter_schindleri</td>
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<td>Unclassified_Kingella</td>
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<td>Acinetobacter_venetianus</td>
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<td>Asticcacaulis_biprosthecium</td>
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<td>Unclassified_Veillonellaceae</td>
<td>Haemophilus_influenzae</td>
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<td>Unclassified_Micrococcaceae</td>
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</table>

10B

**Figure 10:** Most abundant species present in different implant groups. B: Most abundant species in smokers.
CHAPTER 4

DISCUSSION

The present investigation examined the subject specific differences of the periodontal and peri-implant biofilms in health and disease in 40 smokers and 40 nonsmokers. The study design allowed for comparisons of clinical indices of peri-implant health, identification of subgingival bacteria levels and evaluation of species characteristics in the different groups. These results provided a comprehensive exploration of peri-implant microbial communities, in health and disease both in smokers and nonsmokers and revealed a completely new picture of the peri-implant microbiome.

The analysis of the demographic information of the subjects revealed no statistical significant differences between smokers and nonsmokers in any of the characteristics evaluated. History of implant loss and bruxism were the only characteristics that were statistically significantly more prevalent in the peri-implantitis and diseased cases respectively. This finding is in agreement with previous studies reporting that subjects with one implant failure would be likely to have others, as well as a 30% increase should be expected in the probability of removal of a second implant in patients with multiple implants presenting with one failure.\textsuperscript{61, 62} Bruxism on the other hand remains a controversial issue. Several studies have reported that para-functional activities (bruxism, clenching, etc.)\textsuperscript{63} are correlated with implant bone loss/failure and implant fractures.\textsuperscript{63, 64} However, currently there is no
evidence-based, implant-specific concept of occlusion and overload and no definite conclusions can be drawn regarding their effect on the bony support around implants.\textsuperscript{65, 66}

The present study revealed no statistically significant differences between smokers and nonsmokers in any of the clinical characteristics apart from higher level of inflammation in terms swelling, in the nonsmoker population. These results are in contrast with the ones reported by Haas\textsuperscript{54}, Ataoglu\textsuperscript{67} and Gruica\textsuperscript{68} where smokers represented the group with higher level of peri-implant mucosal inflammation, probing depth, as well as swelling and suppuration. This difference could potentially be explained by the fact that in all three studies mentioned there was a big discrepancy in the number of patients recruited as smokers and nonsmokers. In addition a limitation of the current investigation is the fact that the number of cigarettes smoked and the classification of our patients as light or heavy smokers was not evaluated, which could have affected the clinical presentation of the subjects.

The main findings of the present investigation focus on the composition differences between peri-implant and periodontal communities in health and disease as well as the shifts of the microbial ecosystem on implant sites in smokers and nonsmokers.

16 s pyrotag sequencing was used to identify the bacteria present. This method is an open ended, global approach for the examination of peri-implant and subgingival microbial communities that targets the 16s gene of the ribosomal RNA. This gene is considered housekeeping in all organisms; it’s a mosaic of highly conserved and variable regions, representing a unique fingerprint for each organism. 16s sequencing is different compared to other methodologies used for bacteria identification.
(anaerobic culturing, DNA-DNA checkerboard, micro assays, etc) since it does not use primers and probes targeted to specific organisms. Therefore, with this technique, all the bacterial signatures are compared to the Green Genes database and even previously unknown and unsuspected species can be detected.69

The present investigation showed that the distribution of the phyla in this study population was mainly dominated by the Firmicutes, which was accounting for 56.7% of all sequences. There were a large number of OTUs (116) that were identifiable at the genus level and several of the OTUs were uncultivable. This finding confirms the results previously reported by Kumar et al70 that the microbial profile of peri-implant communities includes several previously unsuspected and unknown species.

The biofilm composition around teeth and implants has been previously reported in multiple investigations, suggesting microbial similarity between subgingival and peri-implant communities both in health and disease. Mombelli71, Sanz72, Botero26 and Renvert25 have identified these similarities by comparing levels of known pathogens between teeth and implants. Recent reports have indicated that peri-implant infections may occasionally be linked to a microflora with a different profile, such as high numbers of Peptostreptococci or Staphylococci.73 Our data suggests that in our study population, teeth and implants shared 40 to 85% of their species, with half of the population sharing less that 65%. Tannerella forsythia was the only species that was found in 95% of the cases, in 0.01% abundance. For this reason a further evaluation was conducted regarding the shared species and the level of their abundance. Overall, it was found that the more we increase the abundance cut off value, the less the shared species between the communities. With the hypothesis that the health status of the teeth could have an additional effect on the colonization of
the implant surface the similarity between teeth and implants with different health status was evaluated. Our data suggests that the health status of the sites does not have any significant effect on the percentage of shared communities. This is in partial agreement with previous studies supporting that the indigenous oral bacteria on the remaining teeth serve as reservoirs for colonization of implants. Furthermore, the Shannon Diversity Index was used so as to provide a composite value of the microbial communities around teeth and implants, in the different groups, in terms of species richness (amount of a particular species present) and evenness (relative proportion of species present). It was shown that irrespective of the sites’ health status the diversity of the microbial profile around teeth remains significantly higher compared to that of the implants. This result confirms the information provided recently by Kumar. In conclusion, teeth and implants share bacterial species in health and disease but this does not make them same microbiologically. There are still species found only on implant surfaces such as Rs-045 family, Butyrivibrio, Hylemonella, Bacillaceae family and similarly species such as Alicycliphilus Variovorax and Methylobacteriaceae family found only on tooth surfaces.

Profile analysis of the peri-implant microbiome in health and disease; with the use of Principal coordinate analysis of Unifrac distances showed that in general peri-implantitis communities demonstrate a greater homogeneity compared to health or peri-implant mucositis. This can justify the term ‘simple infection’ for the characterization of the peri-implantitis groups. The data also suggests that peri-implantitis just like periodontitis, is also a predominantly Gram negative infection, a finding that collaborates previous evidence. However an interesting finding is the increased abundance of Gram negative anaerobes present in mucositis and especially healthy implant sites, both in smokers and nonsmokers. This is in contrast with
previous investigations,\textsuperscript{71,72} a fact that can be explained by the limitations of the microbial analysis used in these studies. Furthermore the idea that there is a shift from Gram-positive facultative dominated flora to a Gram negative- anaerobic biofilm in the transition from health to disease is slightly evident in our results, less obvious in the smoker group but overall not statistical significant different in any of groups.\textsuperscript{24}

In the analysis of the most predominant species present in the microbial community in peri-implant health of nonsmokers, it was found that species belonging to the families \textit{Unclassified Streptococcus}, \textit{Unclassified Fusobacterium}, \textit{Unclassified Clostridiaceae}, as well as \textit{Streptococcus sobrinus} and \textit{Veillonella dispar} were present. During the transition from health to peri-implant mucositis certain new species were added to the community such as \textit{Atopobium rima}, \textit{Dialister invisus} and \textit{Unclassified Leptotrichia}. The transition from peri-implant mucositis to peri-implantitis showed a similar effect with some new species added such as \textit{Unclassified Corynebacterium}, \textit{Unclassified Capnocytophaga} and \textit{Unclassified Lachnospiraceaerepresents} but differences were found in the abundance levels of the species in common. The shift from health to disease in nonsmokers represents a community substitution with some new species being added in the microbiome, but with different species being predominant in each group. Our findings are in a partial agreement with other investigations showing that the most predominant species on the diseased state of implants belong to the genera \textit{Fusobacterium} or \textit{Capnocytophaga}, but remain in contrast with most of the studies suggesting that \textit{P.intermedia}, \textit{P.nigrescens}, \textit{Bacteroidaceae}, \textit{P. gingivalis} and other periodopathogenic bacteria are the most common in the implants sites.\textsuperscript{77,78,27}
Evaluating the same transition from health to disease in smokers resulted in some interesting findings. Peri-implant health was related with species such as *Unclassified Fusobacterium*, *Unclassified Clostridiaceae* and *Streptococcus*, which also remained present in the peri-implant mucositis cases, though in different abundance levels. Few species such as *Unclassified TG5*, *Unclassified Porphyromonas*, and *Unclassified Micrococcaceae* were added in this group justifying a community substitution in this transition as well. Moreover, a completely different community was found in the peri-implantitis cases of smokers. Bacteria belonging to the genera *Acinetobacter*, (*Acinetobacter johnsonii*, *Acinetobacter lwoffii*, *Acinetobacter rhizosphaerae*), *Kingella* (*Kingella kingae*, *Kingella oralis*) and *Methylobacterium* (*Methylobacterium adhaesivum*, *Methylobacterium hispanicum*, *Methylobacterium komagatae*) were the most predominant in this group and no similarities were noticed in the microbiome with any of the other groups in smokers or nonsmokers. This transition corresponds to a replacement community and demonstrates a dynamic shift with completely different bacteria present in this high risk population.

*Acinetobacter* species are strictly aerobic nonfermentative Gram-negative bacilli, which are frequently isolated in nosocomial infections, and are especially prevalent in intensive care units, where both sporadic cases as well as epidemic and endemic occurrence is common. Certain species of these genera have been implicated in various other infections including skin and wound infections, bacteremia and meningitis. *Acinetobacter* can also cause life-threatening infections.

*Kingella* organisms colonize the human respiratory tract. They cause skeletal infections, endocarditis, and bacteremia and, rarely, pneumonia, epiglottitis, meningitis, abscesses, and ocular infections. *Kingella* are short, non-motile, gram-
negative coccobacilli that are slow-growing and fastidious. Among *Kingella* species, *Kingella kingae* is the most frequent human pathogen; these organisms frequently colonize the respiratory mucous membranes. Children aged 6 months to 4 years have the highest rates of colonization and invasive disease from this pathogen.

*Methylobacterium* is non-motile rod-shaped, obligate aerobic that can be found mostly in soils, on leaves, and in other parts of plants and, even though its mechanisms aren’t fully understood, is highly studied, important organism. Certain species have been found living inside the human mouth. *Methylobacterium* forms a strong cohesive mat at fuel/water interfaces, such as those that might occur in storage tanks for middle distillate fuel-oils for heating or diesel engines. These chemofilms promote biofilm formation.

To the best of our knowledge, this is the first study evaluating the effect of smoking in the peri-implant biofilm during the transition from health to disease. The significant composition of the peri-implantitis biofilm in smokers is the beginning of a new research journey regarding the approaches and treatment modalities we can use in order to prevent the occurrence of peri-implant disease.
CHAPTER 5

SUMMARY & CONCLUSIONS

The results of the present study show that implants and teeth share few bacterial species in their microbial communities both in smokers and nonsmokers and the percentage of shared communities is not affected by the health status of the sites. Teeth represent a more diverse microbial profile compared to implants in all cases and there are certain species found only on teeth and only on implant surfaces in all groups. Even though teeth and implants share species, they remain different microbiologically.

The implant related microbial ecosystem shows a shift during transition from health to disease both in smokers and nonsmokers. This transition represents a community substitution in the nonsmoker group where few species are added and differences are seen mainly in the abundance levels between health and diseased sites. The transition from health to disease in smokers represents mainly a replacement community where a completely different microbial profile is evident. Peri-implantitis microbiome represents a more homogenous community compared to any other health status.

Overall, peri-implant disease in smokers is distinct from that of nonsmokers, as well as healthy peri-implant biofilms in smokers demonstrate differences compared to that of nonsmokers. In addition, the microbial profile of peri-implantitis includes several previously unsuspected species.
REFERENCES


