Stony Brook University

An *In Vivo* Assessment of the Effects of Using Different Implant Abutment Occluding Materials on Implant Microleakage and the Peri-Implant Microbiome

A Thesis Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biomedical Sciences

(Concentration - Oral Biology and Pathology)

Stony Brook University

August 2015

Stony Brook University

The Graduate School

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Abstract of the Thesis

An *In Vivo* Assessment of the Effects of Using Different Implant Abutment Occluding Materials on Implant Microleakage and the Peri-Implant Microbiome

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2015

Microleakage may be a factor in the progression of peri-implant pathology. Microleakage in implant dentistry refers to the passage of bacteria, fluids, molecules or ions between the abutment-implant interface to and from the surrounding periodontal tissues. This creates a zone of inflammation and reservoir of bacteria at the implant-abutment interface. Bone loss typically occurs within the first year of abutment connection and then stabilizes. It has not yet been definitively proven that the occurrence of microleakage cannot contribute to future bone loss or impede the treatment of peri-implant disease. Therefore, strategies to reduce or eliminate microleakage are sought out. Recent evidence demonstrates that the type of implant abutment channel occluding material can affect the amount of microleakage in an *in vitro* study environment. Thus, we hypothesize that different abutment screw channel occluding materials will affect the amount of observed microleakage, *vis-à-vis* the correlation between the microflora found on the abutment screw channel occluding material those found in the peri-implant sulcus.

Additional objectives include confirming the presence of microleakage *in vivo* and assessing any impact that different abutment screw channel occluding materials may have on the peri-implant microbiome. Finally, the present study provides an opportunity to further characterize the peri-implant microbiome.

Eight fully edentulous patients restored with at dental implants supporting screw-retained fixed hybrid prostheses were included in the study. At the initial appointment (T_1), the prostheses were removed and the implants and prostheses were cleaned. The prostheses were then inserted with polytetrafluoroethylene tape (PTFE, Teflon®), cotton, polyvinyl siloxane (PVS), or synthetic foam as the implant abutment channel occluding material and sealed over with composite resin. About six months later (T_2), the prostheses were removed and the materials collected. Paper points were used to sample the peri-implant sulcus bacteria. All samples were then submitted to DNA purification, polymerase chain reaction (PCR), and sequencing protocols to assess relative numbers of bacterial species. Periodontal parameters were collected at both time points.

Overall, our findings support several conclusions. Different implant abutment channel occluding materials appear to have no effect on the amount of observed microleakage and the peri-implant microbiome. Evidence for microleakage was found in the present study, corroborating existing in vivo evidence. Finally, we gained several insights regarding the peri implant microbiome. Of note, the peri-implant microbiome is well described by the classical periodontal microbial complexes, but a large portion consists of bacteria not previously classified into the microbial complexes.

TABLE OF CONTENTS

List of Figures

List of Tables

Acknowledgements

I would like to acknowledge several individuals who have been instrumental in the development and execution of this thesis. We had a team from various disciplinary backgrounds at Stony Brook University. The completion of this work would not be possible without the help Dr. Stephen Finch, and his graduate students, Sunny Shang and Jesse Colton (Department of Mathematics and Statistics). These individuals were responsible for aiding us in our study design, ensuring randomization and adequate power, and for the statistical analysis of our data. From the School of Marine and Atmospheric Sciences, Dr. Jackie Collier was responsible for helping to select our DNA analysis protocols and the interpretation of the raw data. From the Department of Oral Biology and Pathology, Dr. Stephen Walker was consulted for his expertise in microbiology and connected us to Dr. Collier. Dr. Walker also confirmed our control samples. There is no lack of gratitude for my research mentor, Dr. Julio Carrion, for believing in this work and encouraging its completion. I would like to especially acknowledge Dr. Vincent Iacono and Dr. Marcia Simon for their administrative and consulting roles for this project. Without their efforts, the joint program in Advanced Education in Periodontics and in the Oral Biology and Pathology of the Graduate School would not be possible. Finally, I would be amiss to leave out thanks to my research partner, Dr. Brandon Katz. Dr. Katz provided his support for all phases of this project, especially in its design and clinical and laboratory procedures.

Introduction

I. Overview

Dental implants are medical devices inserted into alveolar bone whose main purpose is to support a dental prosthesis. In 2010, the global dental implant market was worth about $3.2 billion and is expected to reach $4.2 billion in 2015. There is an estimated compound annual growth rate of 6.0% from 2010 to 2015. North America is expected to account for 31% of the market revenue, surpassed only by Europe with 42% of the market revenue [1]. Dental implants have revolutionized dentistry owing to their high predictability for incorporation, about 97-98%, and survival rates, approximately 89% after 10 years of function [2, 3].

The clinical and economic success of dental implants relies on the development of the concept of osseointegration, first introduced by P. I. Brånemark [4]. Osseointegration has been defined in several ways. The most formal is "a direct functional and structural connection between living bone and the surface of a load carrying implant," which is apparent at the light microscope level [5]. A clinical definition is "a process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading" [6]. Another term for osseointegration is "functional ankylosis," which describes the rigid fixation of the dental implant to bone [7]. Indeed, the most definitive success criterion for dental implants is the complete absence of clinical mobility.

Dental implants are commonly made of titanium or its alloys and can vary in size, shape, and surface properties. Most implants that are used today are root form implants, meaning they mimic the shape of dental roots. Implants may be either one-piece or two-piece. A one-piece implant has a prosthetic connection, called an abutment, formed directly to the main part of the implant, known as the implant body. This is by far the less common of the two types. A two-

piece implant requires that the abutment be screwed into the implant body using a prosthetic screw tightened into a channel inside the abutment. The prosthetic screw is typically protected by placing a dental material inside the abutment channel. Common materials include cotton, foam, polyvinyl siloxane (PVS), or polytetrafluoroethylene (PTFE, Teflon®) tape. Several configurations of prosthetic restorations can then be joined to the dental implant. These include removable prostheses, designed to replace several teeth or a full arch. These prostheses can be removed by the patient. Fixed prostheses which can support as little as a single tooth ("unit") or as much as full arch of prosthetic teeth are also available. These cannot be removed by the patient under normal circumstances, but, if necessary, they can be removed by a dentist. The prostheses themselves can be either cement-retained or screw-retained. A cement-retained prosthetic is one that is secured onto the abutment by various adhesive materials. A screw-retained prosthetic is one that the prosthetic crown is screwed into the implant body. There are two assembly types. In one, the prosthetic assembly is screwed directly to the implant platform (Figure 1). In the other, an intermediary part, called a multiunit abutment, is screwed down first and then the prosthetic crown screwed to this abutment (Figure 2). In the case of a screw-retained prosthesis, one of the aforementioned dental materials is typically placed into the prosthetic channel and then sealed over by a composite resin material. The presence of a multiunit abutment places the dental material further from the implant-abutment interface. Two-piece systems inherently have an implant-abutment interface, the importance of which will be discussed in great detail [8-11].

Despite great scientific advancement and research efforts, dental implants are not impervious to disease or failure. Peri-implant disease is classified as peri-implant mucositis or peri-implantitis. Peri-implant mucositis is defined as "a disease in which the presence of

inflammation is confined to the soft tissues surrounding a dental implant with no signs of loss of supporting bone following initial bone remodeling during healing." Peri-implantitis is defined as "an inflammatory process around an implant, which includes both soft tissue inflammation and progressive loss of supporting bone beyond biological bone remodeling." Bleeding on probing and/or suppuration, with probing depths of greater than or equal to 4 mm could be found in both types of peri-implant disease, however peri-implant mucositis appears to be reversible and limited to the soft tissues, while peri-implantitis is irreversible extends to the loss of peri-implant bone [12]. A classification system for the severity of peri-implantitis has been proposed by Froum and Rosen in 2012. This classification was created to aid in describing disease severity only, as it still needs evidence based studies for validations of its use. The authors utilize bleeding on probing (BOP), probing depth (PD), and radiographic bone loss as compared to the time of prosthetic loading to differentiate between early, moderate, and advanced peri-implantitis. The complete definitions are found in Table 1. Failure can be defined as a removed, lost, mobile, or fractured implant [13].

The precise prevalence of peri-implant diseases is disputed. In a systematic review by Zitzmann and Burglundh in 2008, it was found that with a follow up of at least 5 years peri-implant mucositis affects 80% of patients and 50% of implants while peri-implantitis affects 26% and ≥56% of patients and in 12% and 43% of implants [14]. The most recent systematic review was conducted by Atieh in 2012. It states that with a mean follow up of 5-13 years, peri-implant mucositis is estimated to affect 63% of implant patients and peri-implantitis is estimated to affect 19% of implant patients. This translates to an estimated 31% of implants with peri-implant mucositis and 9.5% of implants with peri-implantitis [15]. Since then, a cross-sectional study was published by Daubert *et al* in 2014. Ninety-six patients with 225 implants placed

between 1998 and 2003 were included in the study. The mean follow up time was 10.9 years and the implant survival rate was 91.6%. Peri-implant mucositis was found in 48% of patients and 33% of implants. Peri-implantitis was found in 26% of patients and 19% of implants [16]. The differences in these studies can be owed in part to differences in disease criteria, especially thresholds used to note radiographic bone loss [17]. Despite these inconsistencies, it cannot be disputed that peri-implant diseases affect a significant portion of patients who receive dental implants.

There are several risk factors that may lead to the initiation and progression of peri-implant diseases. These include previous periodontal disease, poor plaque control, residual cement, smoking, genetic factors, diabetes, and occlusal overload. The most commonly accepted etiology of peri-implant diseases is the dysbiosis of the oral bacterial biofilm and the host immune response. This is similar to the etiology of gingivitis and periodontitis [12]. The role of the biofilm in gingivitis and periodontitis as compared to peri-implant diseases is discussed in the following section.

II. Peri-Implant Microbiology

Several researchers have attempted to identify the typical microbiological characteristics of dental implants in health and in disease. Some of the challenges in this undertaking include possible differences in implants placed in fully edentulous patients versus partially edentulous patients, whether there is a history of periodontal disease, and the health status of the implant. While new data are constantly pouring in over the topic of the microbiome of implants, a popular notion is that the microbiological composition of the peri-implant sulcus of a healthy implant is similar to the subgingival composition in periodontal health. Specifically, both are highly dominated by Gram-positive facultative anaerobic bacteria. Equally, the microbiological

composition in disease about teeth and implants are thought to be similar, being dominated by Gram-negative anaerobic bacteria and putative periodontal pathogens [18]. The following will discuss the microbiome around natural teeth, the microbiome around healthy implants, the apparent association between periodontal disease history and failing implants, the microbiome of implants with peri-implant diseases, and briefly the current treatment modalities for peri-implant diseases.

A. Microbiome around Natural Teeth

Classic experiments have determined the role of bacterial accumulation on teeth in inducing gingival and periodontal diseases. One such classic study was carried out by Löe *et al* in 1965. Twelve subjects were monitored for clinical and microbiological changes for 21 days of ceasing oral hygiene. All subjects began the experiment with healthy gingival condition and good oral hygiene. All subjects developed soft debris in large quantities and gingivitis, albeit at different rates. After reintroducing oral hygiene, gingival inflammation resolved in about a week. Bacteria collected were examined by light microscopy. At the start of the experiment, cocci and short rods dominated the flora. As the experiment progressed, coccal flora increased dramatically, followed by a preponderance of filamentous bacteria and slender rods. After reintroducing oral hygiene, the bacterial samples were again dominated by cocci and short rods [19].

Since this experiment, the following trends have been established. In clinically healthy sites, the microbiome is complex, and contains especially Gram-positive facultative species that are commonly associated with those found in the nose and throat [20]. For example, *Streptococci* and facultative species of *Actinomyces* can account up to 85% of the cultivable oral flora [21]. In gingivitis, the composition of the oral samples becomes more complex and diverse

between subjects. In general, Gram-negative and spiral bacteria are found in higher counts in gingivitis than in health [20, 22].

Several studies have examined the microbiota in periodontitis versus health using different methods of analysis. For example, one study using culture methods found that *A. actinomycetemcomitans, P. gingivalis, E. nodatum, F. nucleatum, P. intermedia, and P. nigre*scens appeared to be associated with periodontal diseases and *Actinomyces naeslundii, C. gingivalis, N. mucosa, S. oralis, Streptococcus salivarius, S. sanguinis* and *V. parvula* appeared to be associated with periodontal health or stability. These conclusions are based on a significant correlation between the percentage of total isolates either increasing or decreasing with increased disease severity [23].

A seminal study by Socransky and Haffajee *et al* in 1998 analyzed subgingival plaque using a high throughput DNA method, checkerboard DNA-DNA hybridization. Whole genomic DNA probes for 40 subgingival taxa were incubated on membranes with fixed DNA to determine their presence and level in 13,261 plaque samples. The researchers then attempted to cluster the species by their frequency of being present in a specific site. These data were also correlated with clinical examinations. Five major complexes of bacteria were consistently observed and named: red, orange, yellow, green, and purple (Figure 3). The red complex, consisting of *P. forsythia, P. gingivalis,* and *T. denticola,* was found to be most highly related to clinical measures of periodontal disease, namely increasing probing depth and bleeding on probing. The members of the red complex were also rarely found in the absence of those in the orange complex, which includes *Fusobacterium* species and *P. intermedia* [24].

Socransky and Haffajee followed this study with one that analyzed supragingival plaque with the same DNA-DNA hybridization techniques. The complexes found in supragingival

plaque were similar to those found in subgingival plaque. This provided insight into the ecological succession to a mature biofilm. In contrast to the subgingival plaque, the most closely related complexes to each other were those in the yellow complex, such as *S. mitis* and *S. oralis*, and *Actinomyces* species [25]. This added to the body of support for the notion that colonization of the supragingival environment is initiated by members of the yellow complex with a slower accumulation of *Actinomyces* species. Species of the orange complex and late colonizers of the red complex require more time to establish their communities within supragingival biofilm. Furthermore, the development and composition of the supragingival microbiota is influenced by the presence of inflammation and deep pockets in adjacent sites. Other factors such as the nature of the surface (i.e. enamel, cementum, dentin, or acrylic), tooth position, and plaque mass can also influence the microbial composition of supragingival biofilm. This could ultimately affect the composition of subgingival biofilm [26].

It is important to note that the above conclusions were drawn from analytical methods that require cultivable bacterial species. For example, even DNA methods require the cultivation of bacterial species because DNA probes are typically selected from a panel of previously known bacteria. Several uncultivable species have been identified using culture-independent methods such as pyrosequencing or PCR of the highly conserved 16S ribosomal RNA (rRNA). The first study to investigate the oral microbiota via next-generation sequencing (pyrosequencing) estimated that 19,000 phylotypes may be present in the human oral microbiota [27], a major increase since earlier estimates of 700 taxa [28] as determined by culture and non-culture methods, including PCR of 16S rRNA. A recent review by Curtis discusses the role of new technologies in specifically analyzing the periodontal microbiome, which are expanding the number of putative periodontal pathogens [29]. Uncultivable species, may especially play a role

in periodontal disease, however their role has not yet been elucidated. For example, in 2012 Griffen *et al* [30] used pyrosequencing to compare subgingival biofilms from healthy patients to periodontitis patients. The group reported that uncultivated taxa were more abundant in samples from diseased sites but 81% of the sequences were mapped to cultivated species. One could argue that the 19% of uncultivated taxa could play a major role in periodontal disease, of which we were not previously aware. On the other hand, using the same data, one could argue that because the majority came from cultivated species that our knowledge based on cultivated species is still valid when assessing the oral microbiome as it relates to periodontal disease. Other culture-independent studies have shown that there was relatively infrequent detection of established periodontal pathogens, such as *P. gingivalis* and *T. forsythia* [31-33]. As this is an emerging technology, more studies should be carried out before dismissing the overwhelming body of work establishing the role of putative periodontal pathogens, such as the red and orange complex bacteria. Furthermore, it is important to note that these DNA/RNA dependent methods only detect the presence of bacteria and do not give any insight as to whether they are viable. There should be a continued search for relevant uncultivated or unrecognized species using emerging technology, which could potentially complement the findings of cultivatable species. Certainly a challenge, future researchers must tease out taxa which might be transient, rare, or results of artifacts when employing new technologies.

B. Microbiome of Healthy Implants

The microbes on successfully integrated implants in fully edentulous patients were first assessed using dark-field microscopy in the late 1980's by the Mombelli group. The main findings were coccoid morphotypes with low proportions of spirochetes, fusiforms, and motile and curved rods [34]. The same group also did a microbial analysis of healthy implants used as

abutments for overdentures in function for two years. They found a high prevalence of Gram-positive facultative cocci and *Actinomyces* and *Veillonella* species. There were low total anaerobic counts, low levels of Gram-negative anaerobic rods, a low frequency of *Fusobacterium* species and "blackpigmented *Bacteriodes* (now known to include *P. intermedia*)," and no detection of *P. gingivalis*. The group studied these implants further, for up to five years, and did not find significant microbial changes within the same patient over time [35]. A three-year longitudinal prospective study consisting of sixteen fully edentulous patients with ninety-five implants under fixed-detachable ("hybrid") restorations in function analyzed subgingival microbiological samples. The implants were all deemed healthy by the examiners over the course of the study period, with no mobility, probing depths of 3mm or less and not above 5mm, and only mild soft tissue reactions as determined by histology. The authors found that coccoid cells and non-motile rods comprised 93% of the thirty-two samples taken [36].

There are several studies showing that this profile for healthy implant microbiota is also seen in partially edentulous patients. Supragingival and subgingival biofilm appears to be of similar composition at healthy implant sites as determined by checkerboard DNA-DNA hybridization in patients with at least one implant restored and functioning for at least 2 years. Specifically, the supragingival biofilm on healthy implants was dominated by *Actimomyces naeslundii*, *Streptococcus intermedius*, *Streptococcus mitis*, and *Fusobacterium periodontinticum*, while the subgingival biofilm was dominated by *Veillonella parvula*, *Streptococcus gordonii*, *Streptococcus intermedius*, and *Fusobacterium periodontinticum* [37]. In 2012, Kumar *et al* took subgingival and submucosal plaque samples from forty subjects with periodontitis, peri-implantitis, periodontal and peri-implant health and analyzed them with 16S pyrosequencing. Healthy implants showed the highest levels of Gram-negative anaerobes when

compared to peri-implantitis sites and natural diseased or healthy teeth. Healthy implants had higher levels of *Prevotella*, *Treponema*, *Letotrichia*, *S. mutans*, *Butyrivibrio*, *Catonella*, *Priopionbacter*, and *Lactoccocus*, and lower levels of *Arthrobacter*, *Synergistes*, *Corynebacterium*, *Neisseria*, *Veillonella*, *Dialister*, *Granulicatella*, *Actinomyces*, *Fusobacterium*, and non-*mutans Streptococcus* when compared to healthy teeth. The high level of Gram-negative anaerobes around healthy implants found by 16S pyrosequencing is contradictory to the findings previously discussed. The authors explain that this can be owed to a large fraction of the microbiome of healthy implants being uncultivated and that further confirmation studies will be needed [38]. On the whole, the body of data shows that healthy implant sites are dominated by facultative Gram-positive cocci and rods and have low levels of putative periodontal pathogens, making the microbiomes of clinically healthy implant fixtures similar to those that colonize the teeth in healthy periodontal sites.

One hypothesis for the infrequent occurrence of putative periodontal pathogens in fully edentulous patients is that they are eradicated due to the elimination of their main reservoir – the subgingival environment. For example, in 2002 Sumida et al demonstrated that the teeth were the reservoir for bacteria colonizing implant surfaces. In this study, pulsed field gel electrophoresis patterns demonstrated that DNA samples from *P. gingivalis* and *P. intermedia* from implants were the same as from teeth within the same patient [39].

Several authors have refuted the notion that teeth were required to harbor periodontal pathogens. *A. actinomycetemcomitans* and *P. gingivalis* were not detected by culture methods in twenty patients with a history of periodontitis who had become edentulous and subsequently restored with dental implants [40]. However, other putative periodontal pathogens, such as *P. intermedia* and *Fusobacterium* species, were found around implants in the same study. A recent

study using culture techniques and quantitative real-time PCR showed that three months after full mouth extractions resulted in reduction of *A. actinomycetemcomitans* and *P. gingivalis*. While the majority of previously positive patients had reductions below detection level, the study also showed that *A. actinomycetemcomitans* and *P. gingivalis* could persist in the edentulous oral cavity [41]. Socransky and Haffajee examined the microbes from edentulous patients without implants using checkerboard DNA-DNA hybridization. They determined that the soft tissues of edentulous patients are the most likely source for periodontal pathogens that colonize dental implants after placement in fully edentulous patients. In this study, the dorsum of the tongue harbored the most species and *A. actinomycetemcomitans* and *P. gingivalis* were found on the keratinized oral mucosa and other oral soft tissue sites, such as the tongue and labial vestibules [42]. In 2005, checkerboard DNA-DNA hybridization methods revealed the presence of small amounts of these and other key periodontal pathogens in implants placed in fully edentulous patients, and in amounts less than those found in dentate individuals [43]. Overall, it is accepted that periodontal pathogens can persist in those who are fully edentulous and can colonize the subgingival environment around dental implants.

C. Influence of Past Periodontal Disease on the Microbiome of Dental Implants

It can be hypothesized that more periodontal pathogens can be found on implant surfaces in patients with a history of periodontal disease than those with a history of health. The Mombelli group investigated the bacterial colonization of dental implants in partially edentulous patients who were previously treated for periodontal disease. They took subgingival plaque samples from the deepest residual probing depth around natural teeth in each quadrant before placement of single stage implants or before second stage surgery was performed. The implants and teeth were then sampled at 3 and 6 months after the implants were exposed to the oral

environment. These samples were then analyzed using dark-field microscopy. The results were that at 3 months, samples from the dental implants had a distribution of bacterial morophotypes similar to those found in the residual pockets. These morphotypes and findings were stable at 6 months [44].

A set of prospective studies by the Mengel group took samples from implants placed in patients with generalized aggressive periodontitis, generalized chronic periodontitis, and periodontally healthy patients. The microbiological samples were analyzed by dark-field microscopy and DNA probes for *A. actinomycetemcomitans*, *P. gingivalis,* and *P. intermedia*. The patients with disease were treated and all patients were followed for up to 5 years. The results were that at three years, the microbiological composition in the periodontitis patients (both aggressive and chronic) was similar to that in the healthy patients in that it was dominated by coccoid species. Patients with generalized aggressive periodontitis had an increase in spirochetes, motile rods, filaments, and fusiforms from year 4 to 5. *P. gingivalis* and *P. intermedia* also increased in these patients after the third year. Most profoundly, those with generalized aggressive periodontitis had a lower implant success rate than the other subjects in the study [45, 46]. This work by Mengel *et al* demonstrates a link between periodontal disease history, microbiology, and implant success.

D. Microbiome of Dental Implants in Peri-Implant Disease

There have been many studies establishing stark differences between healthy implants and diseased implants. One study of experimental peri-implant mucositis by Pontoriero *et al* in 1994 established a link between plaque and the disease. Twenty partially edentulous patients were treated for moderate to advanced periodontal disease before having dental implants placed. Baseline oral health parameters and subgingival microbiological samples were taken two months

after the implants were exposed to the oral cavity and had prosthetic abutments placed. The patients were asked to refrain from oral hygiene for three weeks and then clinical data and subgingival microbiological samples were obtained. After the three week period, oral hygiene was reinstituted. During the three week period of no oral hygiene, all clinical parameters indicated the presence of inflammation around natural teeth and implants and were similar between tooth and implant sites. As determined by phase-contrast microscopy, the percentages of coccoid cells decreased while the percentage of motile rods and spirochetes increased in peri-implant sites after the three week no hygiene period. Again, this was not significantly different than the microbiological shift about teeth after the same period of no oral hygiene. This is in agreement with the experimental findings by Löe *et al* in 1965. The authors concluded that there is a similar cause and effect relationship between the accumulation of plaque and the development of peri-implant mucositis as there is between the accumulation of plaque and the development of peri-implant mucositis [47].

Several papers were published in the 1980's correlating increasing probing depth with changes in the peri-implant microbiome. In the same three-year longitudinal prospective study of peri-implant tissue under fixed-detachable restorations mentioned above, the authors noted that gingival inflammation was correlated with filiforms and small spirochetes and that deeper probing depths around implants were significantly correlated with increased presence of spirochetes [36]. The Mombelli group also contributed much knowledge on this subject. They examined plaque samples from healthy and failing implants with dark-field microscopy and culture methods. Failing implants had pocket formation of at least 6mm, suppuration, and radiographic bone loss when compared to implant sites with no signs of infection in the same individuals. They found that stable implants were colonized mostly by coccoid cells, fusiforms

and motile rods were present at very low levels, and there were no spirochetes found. Spirochetes and fusiforms were found in low proportions from healthy implants in patients who had peri-implantitis affected implants in other sites. There were no differences between the microbiology of samples from healthy implants in patients with or without peri-implantitis. The significant difference occurred about implants with peri-implantitis. These sites contained higher levels of motile rods, spirochetes, and fusiforms and a relatively lower level of coccoid cells. Failing sites had significantly higher numbers of *P. intermedia* and *Fusobacterium* species while the proportions of *Streptococci* and *Actinomyces* species were reduced. Interestingly, *P. gingivalis* was not found in any of the samples in this study, even when assessed both culturally and by indirect immunofluorescence [48].

Further studies were carried out to assess the relationships between clinical conditions and the microbiology of the peri-implant environment. A study in 1991 assessed thirty two failing implants by direct phase-contrast microscopy and culture. In comparison to implants showing only mobility and peri-implant radiolucency, high proportions of spirochetes and motile rods and *P. gingivalis*, *P. intermedia*, *Campylobacter rectus*, and *Fusobacterium* species were found when at least one of the following clinical signs was present around implants: bleeding, suppuration, pain, high plaque and gingival indices, and granulomatous tissue when implants were removed. *A. actinomycetemcomitans* and *Parvimonas micra* (*Peptostreptococcus micros*) were only detected in samples from this group [49]. In a retrospective study, forty-five partially edentulous patients with a total of sixty-four implants, with varying times of last dental visit and maintenance therapy ranging from three to twenty-four months were included. All implants were in function between six and eleven years. Nine implants experienced one episode and six implants experienced two episodes of peri-implantitis, as defined by bleeding on probing and/or

suppuration, pocket formation greater than 4mm, and bone loss. Four of these implants had *P. gingivalis* and two were positive for *A. actinomycetemcomitans*, as detected by culture methods. There appeared to be a significant relationship between peri-implant probing depth and the total anaerobic microbiota cultivated and the frequency of detecting *P. gingivalis* [50]. The frequency of detecting periodontal pathogens seems to increase with probing depth even in implants in fully edentulous patients. When probing depth was at least 5mm, *P. intermedia* could be found, while when probing depth was 4mm or less, *P. intermedia* could not be found [40].

Checkerboard DNA-DNA hybridization has been employed to examine the microbiology of the peri-implantitis lesion. Salcetti *et al* found that out of 44 known subgingival taxa, only 4 species were found to be associated with peri-implantitis. These were *Prevotella nigrescens, Peptostreptococcus micros, F. nucleatum ss vincentii,* and *F. nucleatum ss nucleatum*. There was also a higher prevalence of *P. gingivalis, T. forsynthia,* and *T. denticola,* although this was not statistically significant [51]. Another group using checkerboard DNA-DNA hybridization noted that *P. gingivalis, T. forsythia, T. denticola,* and *P. nigrescens* were present at significantly higher levels in both supra- and subgingival microbiological samples taken from implants with peri-implantitis than those taken from healthy implants. Higher mean counts of *F. nucleatum, P. intermedia, P. nigrescens, T. denticola, Selenomonas noxia,* and *T. forsynthia* were found in the subgingival biofilm of implants with peri-implantitis as compared to the supragingival biofilm from the same sites. They concluded that there is a marked presence of red complex species and lower mean proportions of host-compatible microbial complexes (purple, yellow, green, actinomyces species) in the implants with peri-implantitis [37].

As mentioned above, Kumar *et al* 2012 also took subgingival and submucosal plaque samples from peri-implantitis sites and analyzed them with 16S pyrosequencing. Peri-implantitis was associated with lower levels of *Prevotella* and *Leptotrichia* and higher levels of *Actinomyces*, *Peptococcus*, *Campylobacter*, non-mutans *Streptococcus*, *Butyrivibrio* and *Streptococcus mutans* than healthy implants. They concluded that, in general, peri-implantitis is a microbiologically heterogeneous infection with predominantly Gram-negative species, and is less complex than periodontitis [38]. This article represents one of few studies that do not support a strong similarity between the microbes found in periodontal disease and in peri-implant diseases, and the authors suggest a possible different pathophysiologic process than that of periodontal disease. It does, however, support the importance of a microbiologic etiology, as is in periodontal disease.

Other microorganisms have been associated with peri-implantitis and include *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas* species. It has been shown that *S. aureus* and *C. albicans* have a high adhesion to titanium. In addition, *S. aureus* has been associated with suppuration and bleeding on probing around dental implants [52-54]. A study with microbial samples collected from 166 patients with peri-implantitis and 47 with healthy implants and analyzed by DNA-DNA checkerboard hybridization showed that *P. aeruginosa* and *S. aureus* were found more frequently in peri-implantitis sites, along with periodontal pathogens, such as *T. forsythia* and *A. actinomycetemcomitans* [55]. In 2014, a study using culture and PCR targeting 16S rDNA methods analyzed periodontal pathogens and opportunistic pathogens, such as *S. aureus*, enteric bacteria, *Pseudomonas*, and yeasts, around dental implants with peri-implantitis in partially edentulous patients. *Staphylococcus*, *P. aeruginosa*, and *C. albicans* were found in five out of thirty-three patients. *P. aeruginosa* was found in four of these patients, and

S. aureus and *C. albicans* were found in one patient each. The authors conclude that along with putative periodontal pathogens, inclusive of *P. gingivalis, T. forsythia, P. intermedia,* and *T. denticola*, but exclusive of *A. actinomycetemcomitans*, these opportunistic microbes may play a role in peri-implantitis [56].

E. Treatment of Peri-Implant Diseases

Current treatment methods for peri-implant diseases are based on the opinion that there is microbial similarity between implants and teeth in health and disease. This idea has been overwhelmingly supported by the above summary. The notion then translates to: "If the microbiology is similar to periodontal diseases and plays a sizeable role in the etiology in peri-implant diseases, then we can treat peri-implant diseases with traditional periodontal therapy."

Applying this concept to peri-mucositis appears to be successful. For example, Salvi *et al* in 2011 conducted a prospective study in which patients that had at least one molar or premolar in the mandible refrained from oral hygiene practices for three weeks, and then followed them for another three weeks after hygiene was reinstated and a prophylaxis was performed. The results were that gingival and plaque scores increased about the implants and natural teeth after three weeks of no home care and that the condition of the gingival tissues about implants improved after treatment, however not as complete as about natural teeth. This occurred on a clinical, microbiological, and inflammatory marker level (GCF and plaque samples were collected in additional to clinical data) [57]. Therefore, elimination of the biofilm from implant surfaces should be the primary objective when treating peri-implant mucositis.

In contrast, there appears to be only a modest success in treatment of peri-implantitis. Non-surgical methods alone have not been shown to be effective in treatment of this type of lesion; however plaque control remains paramount as a component of treatment [58-60].

Surgical treatment could include respective or regenerative procedures [61-65]. In terms of regeneration, many different modalities have been attempted: from the application of tetracycline and enamel matrix derivative to a complex combination approach utilizing surface decontamination, enamel matrix derivative, a combination of platelet-derived growth factor with anorganic bovine bone or mineralized freeze-dried bone, and coverage with a collagen membrane or a subepithelial connective tissue graft [66, 67]. Treatment complications include possible implant surface damage and exposure of implant threads causing esthetic compromise. There is also a question of "implant surface detoxification." That is, there is controversy as to what extent of detoxification is necessary to achieve resolution of the lesion and how to best accomplish this. From curettes made from various materials, to tetracycline, to lasers, to titanium brushes, it appears that there is no consensus to the answers for the above questions [68]. In addition, emerging information on the role of opportunistic organisms in peri-implantitis lesions could prove a treatment challenge, depending on their precise involvement. For example, it has been shown that common systemic antibiotic regiments for the treatment of periodontal disease and peri-implantitis, such as amoxicillin/clavulanic acid and metronizole, are not effective against *P. aeruginosa* and *C. albicans* [69].

The greatest obstacle in applying traditional periodontal treatments to the management peri-implant disease is arguably the lack of knowledge for the exact mechanism of these disease states. Although there appears to be similarity in the microbiological etiology, the treatment goals for periodontitis and peri-implantitis are inherently dissimilar. That is, in periodontitis the ultimate goal is for regeneration about a natural tooth, while in peri-implantitis, the ultimate goal is re-osseointegration of the implant. Long-term controlled studies are of paramount necessity to validate optimal treatments for different clinical presentations of peri-implant diseases. It is

arguably more effective to attempt to prevent peri-implant diseases before necessitating surgical interventions with unpredictable long term success rates. The following section will discuss the concept of microleakage and argue that choice of implant abutment channel occluding material may affect its occurrence and overall implant health.

III. Microleakage

A. Overview

Microleakage in implant dentistry refers to the passage of bacteria, fluids, molecules or ions between the implant-abutment interface to and from the surrounding periodontal tissues. From an engineering perspective, gaps between implant and prosthetic components are unavoidable when different manufactured parts are fitted together. Several studies have assessed the presence of this "microgap" and associated microleakage at the implant-abutment interface.

The earliest mention of these concepts in the literature was published in an abstract by Traversy and Birek in 1992. They did an *in vitro* study that showed bidirectional leakage of fluid and *Streptococcus sanguis* between the implant-abutment interface of the original Brånemark implant design [70]. This was followed shortly by an *in vivo* study by Quirynen and van Steenberghe assessing the presence of microorganisms in the inner aspect of the Brånemark implant. Nine patients who had implants placed within at least two years of the study and no history of peri-implant bone loss or clinical signs of implant or periodontal disease were included. Three patients were fully edentulous and six were partially edentulous. After a chlorhexidine disinfection protocol of the implant and surrounding soft tissue, sterilized abutment screws and abutments were installed. Three months later, clinical assessment and microbial sampling were performed. The authors found a significant quantity of microorganisms on the apical part of the abutment screw, with the majority (mean 86%) being coccoid cells and

no significant differences between edentulous and partially edentulous patients. The authors rule out baseline contamination of the implant body at the time of insertion and removal of the test prosthetic components due to their controlled insertion and removal techniques. In addition, if the screw access channels were hermetically sealed, then theoretically there would be no access to nutrients for any bacteria that could have contaminated the implant body at baseline to thrive over years of function. Because implants in this study were all clinically healthy and other studies at the time supported the excellent long-term prognosis of implants, these particular authors state that the presence of microbial leakage could have limited clinical relevance [71]. A literature review in 2013 has reiterated this notion, citing lack of studies related to progressive bone loss for its limited clinical relevance [72]. However, both papers state that it could be responsible for the approximate 1 mm bone loss that was typical within the first year of this and other implant systems and that it could be a reservoir of microorganisms, possibly interfering with treatment of peri-implant disease [71, 72].

Persson *et al* further identified bacteria found in the implant body in twenty-eight Brånemark implants in ten patients which were in service for one to eight years. The samples were collected from the abutment screw and inside the abutment using a sterilized plastic stick and cotton pellet. They found a heterogeneous and primarily anaerobic microbiota, generally consisting of facultative and anaerobic streptococci, Gram-positive rods (including *Actinomyces* species), and Gram-negative anaerobic rods (including *Fusobacterium*, *Prevotella*, and *Porphyromonas* species). Individual samples showed great variation, but in some cases similarities could be seen between samples taken from implants within the same patient. No association between type and length of abutment, abutment stability, bone loss, and microbial data could be found. The authors suggest that the presence of bacteria could be from

contamination at implant installation or the time of abutment installation or from transmission during function of the dental prosthesis. They state that the heterogeneity of the microbes found could suggest that the selection and establishment of bacteria within the implant body may occur in a random fashion [73].

Thus far in the literature, the only implant studied was the original Brånemark implant. As implants grew in popularity as a treatment option, other implant designs emerged. These differences range from differences in implant body type (tapered vs parallel walled), implant surface properties, to type of implant-abutment connection and more. Designs have emerged to specifically achieve a tight implant-abutment connection with the goal of preventing microbial penetration into the implant body, but have limited success rates in achieving this goal. In an in vitro study by Jansen *et al* in 1997, thirteen different implant-abutment combinations derived from nine different implant systems were tested. Some implants in the study had conical connections, where the implant abutment extends into the implant body, while others had flat connections, where the abutment sits to a butt joint to the surface of the implant head. Ten implants from each system had the inner implant body inoculated with *Escherichia coli*, which measures 1.1 to 1.5 microns in diameter and 2 to 6 microns in length. The tip of the abutment screw was also inoculated prior to connecting the abutment to the implant body. The implants were then suspended in growth media. When the media was cloudy, it was plated to confirm bacterial growth. Scanning electron microscopy was also used to assess the width of the gap between the implant the abutment. All implant types showed microbial leakage and the gap was less than 10 microns in all systems, regardless of connection type [74].

Another *in vitro* study was carried out by Gross *et al* in 1999. Five implant systems were assessed by spectrophotometry using colored probes pushed through the implant abutment by a

closed two atmosphere pressure system. An attempt to seal the implant-abutment interface was made by using plumber's isolation tape, made of PTFE, and the occlusal portion was attempted to be sealed with wax. All systems showed evidence of microleakage. A system with a conical connection showed more evidence of microleakage than those with flat connections. In addition, these authors showed that with the appropriate recommended closing torque of abutments, microleakage could be minimized. Multiple subsequent *in vitro* studies have shown that there is no connection type that completely blocks fluid and/or bacterial flow to and from the internal aspect of an implant [75].

A dynamic *in vitro* model was developed to assess bacterial microleakage in 2005. Dynamic loading could potentially decrease the stability of the implant-abutment connection and further cause leakage at the implant-abutment interface. In this model, the internal aspect of five different implant systems were inoculated with *E. coli* and eight standard abutment combinations for single molar crowns were connected with the manufacturer's recommended closing torque. The assemblies were then placed in a nutrient broth and loaded with 1.2 million cycles at 120 N force (a force within physiologic range) in a chewing simulator. The nutrient broth was then plated to assess for presence of *E. coli*. The conclusions were that outward leakage was detected for each system and that cycle number at which microleakage was detected was different with each system. This amount could depend on the precise fit, the degree of micromovement, and closing torque forces of the implant and abutments [76].

Microleakage has been described in terms of monoculture up to this point. One *in vitro* study evaluated three different implant connections using saliva under loaded and unloaded conditions. Sixty implants, with external hexagon, internal hexagon, or conical connections, were evaluated. The abutments were attached and submerged in human saliva. Half were

loaded with 500,000 cycles at 120N force and the other half left static. Microorganisms were found in the internal aspects of all implants, with the conical connection implants having the fewest. Loading increased the microbial counts with external or internal hexagon connections [77].

There is a single *in vivo* study which attempts to relate the microbes found in the peri-implant sulcus and those contaminating the prosthetic components. Of particular interest, the screw channel material is harvested to carry out their investigation. Fifty-eight implants with no clinical signs of peri-implantitis supporting screw-retained full-arch bridges for an average of 9.6 years were included. All implants were of the original Brånemark design. Two of the patients were fully edentulous and the remaining six had periodontally healthy natural dentition in the mandible. Subgingival plaque was collected from the peri-implant sulcus with a sterile paper point. After removing the coronal seal of the screw access channel, the cotton pellet from in the channel was collected. The cotton pellet sat upon a prosthetic screw, which connected the prosthetic bridge to abutments connected directly to the implants. This prosthetic screw was not analyzed. Finally, the prosthesis was removed and the abutment screw was collected. All samples were analyzed using checkerboard DNA-DNA hybridization using probes for forty bacterial species. About 75% of the cotton pellets sampled showed *Streptococcus intermedius*, *C. showae*, *F. nucleatum*, *F. periodonticum*, *Leptotrichia buccalis*, and *Prevotella melaninogenica*. More than half of the screws showed *C. showae*, *F. nucleatum*, and *F. periodonticum*. There was a significant correlation between the peri-implant sulcus and cotton pellets for A. *actinomycetemcomitans* Y4, *F. nucleatum sp. polymorphum*, and A. *actinomycetemcomitans* a29523. The cotton showed similar detection frequencies and levels to the peri-implant sulcus overall. A significant correlation between the peri-implant sulcus and

screw was found for sixteen out of the forty species tested. However, the majority of species were found less frequently and in lower numbers at the screw than in the peri-implant sulcus. There was only a significant correlation between *F. nucleatum sp. polymorphum* between the cotton pellets and screw. The authors note that bacterial contamination was higher in the cotton pellets than in the screw. The composition in the cotton pellets was highly varied showing *Helicobacter pylori* and *Staphylococcus aureus* in at least one third of the samples and anaerobic species found frequently. With correlation of only three species out of forty between the peri-implant sulcus and cotton pellets, the authors hypothesize that the leakage occurred primarily through the margin of composite placed at the most coronal portion of the screw access channel. The authors however point to the implant-abutment interface and abutment-prosthesis interface as the main points of contamination of the abutment screw [78].

The ability of viable bacteria to flow in a bidirectional manner to and from the internal aspect of the implant body has been discussed at length. It has been suggested that other bacterial byproducts or nutrients required for bacterial growth could also be transported along the implant-abutment microgap [75]. A static *in vitro* study (Harder 2009) investigated the ability for endotoxins to flow through two conical internal connection implant systems. There was only one implant out of sixteen tested that showed no indication of endotoxin contamination. This determined that other bacterial products could flow to the internal aspect of the implant, even when mechanically tight connections with minimal micromovement were tested [79, 80]. In summary of this section, there is overwhelming evidence for the existence of microleakage, as determined by both *in vitro* and *in vivo* experiments.

B. Microleakage and Peri-implant Bone Loss

Authors have argued that the microgap and associated microleakage creates a zone of inflammation, contributing to the incidence of peri-implant bone loss. Ericsson *et al* examined this histologically in a dog model in 1995 [81]. Twelve months after abutment connection, the study animals were sacrificed and block sections including bone and soft tissue surrounding the installed Brånemark implants were prepared. The researchers found that there was an inflammatory cell infiltrate at the area of the connective tissue facing the implant-abutment interface, irrespective of whether they were kept devoid of plaque or were allowed to accumulate plaque. This inflammatory cell infiltrate in the connective tissue, or "abutment ICT," was found to be about 1.5mm high and 0.5 mm wide, with its apical border about 1mm from the alveolar crest. The abutment ICT was also found to be generally centered at the implant abutment-interface, with about 0.75mm coronal and 0.75mm apical to the interface (Figure 4). The authors attribute this zone to likely presence of microorganisms within the inner part of the implant, citing the article by Quirynen and van Steenberghe in 1993 [81, 70].

Available data suggest that once the abutment ICT becomes established, further bone loss does not occur. This is supported by several studies which showed the presence of microbiota within the implant body and the implants studied being clinically healthy [69, 70, 72]. In fact, Ericsson *et al* showed that very few osteoclasts were found at the alveolar crest in his study sample of implants in function for one to eight years [81]. In these studies, the implants were placed at the level of the alveolar crest as per the Brånemark protocol.

The hypothesis that the location of the abutment ICT could influence bone loss was tested by placing implants either 1 mm coronal to, at, or 1 mm apical to the alveolar bone crest in a foxhound dog model. Abutments were connected three months after implant placement. All

implants used had a conical connection. At 4, 8, and 10 weeks, the abutments were loosened then tightened to simulate prosthetic procedures. Six months after initial placement, histologic samples were obtained. The study showed that the more apical the abutment ICT was, the greater the magnitude of peri-implant inflammation. The greatest amount of inflammation also happened to at or immediately coronal to implant-abutment interfaces, regardless of the level of placement of the implant in relationship with the alveolar bone crests. Bone loss was significantly correlated to inflammatory cell infiltration below the original alveolar bone crest and not with infiltration above the original alveolar bone crest. The authors conclude that the implant-abutment interface position determines the amount of inflammatory cell infiltration and that this infiltrate contributes to the extent of alveolar bone loss around dental implants, at least within the first six months [82].

A prospective clinical trial by Hartman and Cochran supports the above information. In this study, it was found that bone loss did not develop until the abutment was connected to the implant. Furthermore, the closer the implant-abutment interface was to the alveolar crest, the greater the bone loss. This was in comparison to implants placed more coronally [83].

Despite the scientific efforts on microleakage, microgap, abutment ICT, and alveolar bone levels, these factors still remain controversial. A review in 2013 cites lack of studies related to progressive bone loss and failure to demonstrate a direct relationship between marginal bone loss and implant-abutment interface size as a reason for the continued controversy [71]. The demonstrated long term stability of implants has been often used to argue against microleakage as being a factor for developing peri-implant disease. One study was found to support the hypothesis that microleakage may be a factor in implant loss. Covani *et al* assessed the distribution of bacteria into the internal and external surfaces of fifteen failed implants using

histology. The implant-abutment interface harbored heavy accumulations of bacteria and the internal surfaces of the implants also harbored bacteria, although heterogenous in nature. The authors suggest that the microgap could facilitate colonization and proliferation for bacteria found in peri-implant disease, as bacteria in the internal surface of implants are protected from the host response [84]. There are also no studies directly exploring the hypothesis set forth by Quirynen *et al* in 1993, stating that microleakage could interfere with the treatment of peri-implant disease [71]. More studies are needed to evaluate the role of microleakage in peri-implant diseases. As of now, it is critical to search for ways to avoid microleakage until it is completely clear that it does not relate to the initiation or progression of peri-implant disease and subsequent implant loss.

Changing the design or placement of dental implants has been tested to this end. The role of connection type on influencing microleakage has been discussed above. While a conical connection did not prevent microleakage, its incidence is reduced the most and implants with conical connections appear to have less marginal bone loss than nonconical systems in most cases [85, 86]. Broggini *et al* in 2003 tested the degree of inflammatory cell infiltration in peri-implant tissues in an implant system that did not have an implant-abutment interface, such as that in a one-piece system. They noted limited inflammatory cell infiltrate in peri-implant tissues in these types of systems [82]. Other studies, one in an animal model and one in humans, showed that when the implant-abutment interface was placed approximately 3mm above the alveolar crest that minimal bone loss was found over time [87, 88]. These design and placement elements are not without problems. A one-piece system limits prosthetic options. For example, the abutment allows the clinician to change the angle of the implant prosthetic screw channel if the implant had to be placed in a suboptimal restorative position. Having a supracrestal margin

could cause esthetic concern if soft tissue recession ever occurs because the abutment, typically made out of metal, would then show. Therefore, the majority of common implant systems today prescribes placing the implant head at the level of the alveolar bone crest and is comprised of two-piece systems. The use of a conical connection appears promising, but does not preclude peri-implant bone loss. Other strategies to eliminate or reduce the effects of the abutment ICT must be investigated.

C. Microleakage and Implant Abutment Screw Sealing Material

One strategy to reduce microleakage is to assess materials frequently used to seal the abutment screw channel. Currently, the choice of material is highly dependent on the clinician's preference rather than scientific evidence. For example a survey of US dental schools found that the use of cotton pellets with composite resin was the most common choice [10]. This could be due to their low cost and ability to be sterilized. However, the use of cotton pellets often is associated with malodor if they need to be removed [11]. This is also empirically found to be true of synthetic foam by the investigators of this current study. PVS can be easily manipulated but it cannot be sterilized. PFTE tape as an abutment screw sealing material has come into use owing to its ease of manipulation and removal. It has also been used previously in dental applications, especially as a mechanical barrier [11]. The composite resin placed at the most occlusal portion of the screw access channel serves to create esthetics as well as attempt to further seal the channel [89].

There is one paper to date testing levels of microleakage in implants whose access holes have been sealed with different materials. This was an *in vitro* study testing cotton pellet, silicone sealing material, PVS, and gutta-percha as occluding materials. The materials were placed intimate with the abutment screw to a height of 6.5mm in implants with an internal-

hexagonal abutment-implant connection. Composite resin was placed at the occlusal portion, totaling 3mm of thickness, and the implants were restored with a temporary acrylic resin crown. The restored implants were then stabilized and submerged in 0.5% basic fuchsin solution. Basic fuchsin has a molecular weight similar to small molecules in the range of disaccharides and short peptides. Cyclic loading with 21 N at 1 Hz was applied 16,000 times to the specimens along the long axis of the tooth. This is roughly equivalent to seven days of mastication on molars. The different materials were removed and their absorbance was then measured by a spectrophotometer at 540 nm to determine the degree of microleakage. The authors found that the greatest microleakage occurred when the cotton pellet was used, then the silicone sealing material, then vinyl polysiloxane, and least with gutta-percha. There was no significant difference in the amount of microleakage between vinyl polysiloxane and gutta-percha. This paper showed that the type of access channel occluding material can affect the amount of microleakage in an *in vitro* study environment. It is important to note, that the leakage occurred only through the access hole and that the dye did not infiltrate at the implant-abutment interface. This has been hypothesized in another context, using findings from a previous study testing microleakage using cotton pellets *in vivo* [78]. The major criticism of the *in vitro* study related to this finding is that the load force was not standardized to the higher forces used in other microleakage studies which demonstrated passage through the implant-abutment interface or that perhaps there were too few cycles, as implant restorations are typically in function for years as opposed to a week. Nevertheless, this study shows that the material chosen can influence the amount of microleakage into the implant abutment screw channel.

Aims of the Research

1. **To assess any association between the microflora found in the inner aspects of a restored implant *vis-à-vis* the abutment screw channel occluding material and that found in the peri-implant sulcus, thus testing the concept of microleakage.** We hypothesize that there will be a correlation between the microflora in the inner aspects of a restored implant and that found in the peri-implant sulcus.
2. **To assess any variations in the observed microleakage as different abutment screw channel occluding materials are used.** We hypothesize that different materials will have an effect on the amount of observed correlation between the dental material microbiota and peri-implant sulcus microbiota.
3. **To assess any impact on peri-implant microbiota that different abutment screw channel occluding materials may have.** We hypothesize that different materials will affect peri-implant microbiota differently.

Research Design and Methods

I. Study population and design

The Committee on Research Involving Human Subjects (CORIHS) at Stony Brook University approved all protocols involving human subjects (IRB Net ID 409069). Informed consent was obtained prior to the beginning of the study. Eight fully edentulous subjects with implants supporting a full arch prosthesis were included in the study. All patients had both arches restored by dental implants. No natural dentition was present in any of the patients. Ages of the subjects selected ranged from 18-85 years old, with no preference for male or female. Exclusion criteria included: poor oral hygiene and motivation, uncontrolled diabetes, pregnant or lactating, substance abusers, current smokers, psychiatric problems or unrealistic expectations, acute infection in the area intended for implant sealing, positive to HIV and hepatitis B and C, affected by autoimmune diseases, under chronic treatment with steroids or non-steroidal anti-inflammatory drugs.

This was a randomized controlled trial that examined the correlation between the bacterial DNA found on four dental materials used to seal prosthetic implant abutment channels (cotton, PTFE tape, synthetic foam, PVS) and that found in the peri-implant sulcus. Cotton, being the most commonly used material, served as the control. Secondary outcomes included the relationship between probing depth, gingival index, and plaque index, and the dental material used.

II. Screening

Patients were identified from our existing dental records in electronic databases, strictly based on our inclusion criteria. Patients were then be contacted by phone to determine their interest in participation in the study. Regardless of their participation in research, patients were

recommended to have a recall examination and cleaning every six months (standard of care procedures). Pregnant women were excluded from this study due to the length of the appointment (~2 hours). A pregnant woman might not tolerate being on a dental chair for an extended period of time, other than for emergency dental care. Pregnancy was assessed by asking the potential subject if they are pregnant or not. Oral hygiene was assessed prior to prosthesis removal.

III. Clinical Protocol

The study consisted of approximately three subject interactions over a period about 1 year from recruitment. Visit one consisted of being screened and consented. Subjects were invited for a screening appointment to review their medical and dental history and to ensure they understand the study and the importance of compliance with the protocol. Visit two consisted of removal of the prosthesis, debridement of the prosthesis and implants, replacement of the prosthesis, placement of the prosthetic channel occluding material, and sealing over the material with composite resin. Table 2 provides a summary of the dental materials used. Oral hygiene was also reviewed at this appointment. The materials were placed into the prosthetic channels according to a randomization scheme. Clinical data (probing depth, plaque index, gingival index) were collected at this visit while the prosthesis was removed. All clinical data was obtained using a UNC-15 periodontal probe. Probing depth (PD) was defined as the distance from the free gingival margin to the base of the peri-implant sulcus. Plaque index (PI) is a scale used to assess plaque accumulation. It also ranges from 0 to 3. A score of 0 is defined by no detection of plaque. A score of 1 is defined by being detected only by running a probe across the marginal surface of the implant. A score of 2 is defined by visible plaque. A score of 3 is defined by an abundance of soft matter. Gingival index (GI) is a scale used to assess the

bleeding tendency. It ranges from 0 to 3. A score of 0 is defined by no bleeding when a periodontal probe is passed along the gingival margin adjacent to the implant. A score of 1 is defined by isolated bleeding spots. A score of 2 is defined by blood forming a confluent red line on the margin. A score of 3 is defined by heavy or profuse bleeding [48]. Visit three consisted of material retrieval, at about six months. The composite resin sealing material was removed using a high-speed handpiece without irrigation in order to not disturb any biofilm present in the prostheses and periodontal tissues. The samples were retrieved with a sterile endodontic K-file or spoon excavator. The entire sample was placed into a sterilized Eppendorf tube and placed onto ice. Immediately after the removal of the prostheses, the implants were isolated using cotton rolls and the tissues dried. Sterile paper points were then placed into the peri-implant sulci for 10 seconds and then placed into sterile Eppendorf tubes. The tubes were then placed onto ice. The sulci were chosen based on the deepest probing depth on each implant as noted by the last clinical visit (T_1), so as not to disturb the biofilm. If multiple sites on an implant had the same probing depth, then the most mesial site was chosen. The paper points were pooled for each peri-implant site that supported the same dental material. The same clinical data collected during the second subject interactions and radiographs were obtained. The prostheses and implants were debrided, and the prostheses were re-inserted with PTFE tape and composite resin as the sealing materials. All patients were seen to the completion of the study and then placed on an individualized maintenance schedules or referred for appropriate treatment.

IV. Sample Storage

The samples were stored in a -80 Celsius freezer until they were processed for DNA extraction.

VI. Sample DNA extraction

The PowerSoil® DNA Isolation Kit was used in order to isolate genomic DNA from environmental samples with a high level of purity. The kit was selected to isolate the DNA from our dental material samples because it is intended for use with environmental samples, including difficult soil types. As this is a novel study, the research group was unsure what, if any, DNA could be isolated from the different materials. The kit has reported amplification of DNA and PCR analysis of various organisms, including bacteria, algae, and actinomycetes. The manufacturer's protocol was followed with the following alterations. The dental materials and paper points were first transferred to the PowerBead Tubes. The Eppendorf tubes in which they were contained were washed with the first solution in the protocol and then transferred to the appropriate PowerBead Tube. This was an attempt to obtain any DNA which may have been contained in fluid transferred with the samples that did not get transferred with the bulk dental material to the PowerBead Tube. Control samples, which were sterilized samples of each dental material and paper points were also placed into PowerBead Tubes. Care was taken to place similar volumes of these control materials to the volumes of the test materials into the protocol tubes. The transfer of materials was facilitated with sterile college pliers or pipette tips. The DNA was stored in a -20 Celsius freezer until they were sent out for PCR, sequencing, and analysis.

VI. PCR, Sequencing, and Analysis:

A Diversity Amplicon Study was carried out. The 16S rRNA primers selected were based on a study by Klindworth *et al* (Forward:S-D-Arch-0519-a-S-15 (A519F) CAGCMGCCGCGGTAA; Reverse: S-D-Bact-0785-b-A-18 (802R) TACNVGGGTATCTAATCC) [90]. A 30 cycle PCR using the HotStarTaq Plus Master Mix

Kit (Qiagen, USA) was run under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products were checked in a 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare a DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, sequences were joined, depleted of barcodes, then sequences <150bp removed, sequences with ambiguous base calls removed. Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity) – followed by removal of singletons and chimeras [92-97]. Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes [91], RDPII and NCBI (http://rdp.cme.msu.edu, www.ncbi.nlm.nih.gov).

Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes [91], RDPII and NCBI (http://rdp.cme.msu.edu, www.ncbi.nlm.nih.gov) and compiled into each taxonomic level into both "counts" and "percentage" files. Counts files contain the actual number of sequences while the percent files contain the relative (proportion) percentage of sequences within each sample that map to the

designated taxonomic classification. E.g. if there are 1000 sequences and 100 of the sequences are classified as Staphylococcus then we represented this as Staphylococcus being 10%.

VIII. Confirmation of Controls

A microbial test was devised to evaluate whether there were live bacteria on any of the dental materials used (PVS, PTFE, Foam, Cotton, Paper Point) without contact with patients. The materials were placed aseptically into sterile collection tubes and then nutrient broth was added. The tubes were allowed to incubate at room temperature for 4 days, which showed no turbidity or cells by microscopy. The tubes were then incubated at 37^{o}C for another 4 days. No turbidity or cells were found. Following this period, 1 mL of medium was removed from each tube and was concentrated via centrifugation. No bacteria-like objects were found in any sample except for the paper points when examined under light microscopy. After 20 days of incubation, none of the samples demonstrated bacterial growth. Therefore, the bacteria-like objects found on the paper points were nonviable.

IX. Statistics:

Prior to the start of the project, a power analysis was completed and it was determined that eight patients should be recruited to gain adequate statistical power. The materials were randomized according to a Latin Square design, with two samples of each material placed into two different implant sites for each patient, with eight implants in each patient. A total of sixty-four material samples, sixteen of each material, were obtained. Paper points were used per implant and pooled into the same Eppendorf tube representing implants that had the same dental materials placed. A total of 128 paper points were obtained. Thirty-two paper point samples, one of each correlating to a dental material type within the same patient, were analyzed.

To facilitate statistical analysis and interpretation, the bacteria identified were grouped into the Socransky and Haffajee bacterial complexes and then noted as "other" when not falling into any of the complex categories. T-tests were done to determine whether there was a difference between the clinical parameters (PD, PI, GI) taken at time one and time two and the proportion of complex bacteria on the occluding material and the peri-implant sulcus. The correlation coefficient (R) was calculated to investigate the relationship between the proportion of each bacterial complex on the occluding material and the peri-implant sulcus. Pearson coefficients were calculated to test the correlation between Red Complex bacteria and the probing depth at time two, and the correlation between each microbial complex found in the peri-implant sulcus. ANOVA was performed to determine the effect of several factors on the microbial complexes. The first was the effects of the material, position, and patient for each microbial complex found in the peri-implant sulcus. The second was the effects of the material, position, and patient for each microbial complex found in the material as compared to the microbial complex found in the peri-implant sulcus. The third and final was the effects of the material, position, and patient for each of the "other" bacteria found in the peri-implant sulcus.

X. Funding:

This study was funded by the Department of Periodontology of Stony Brook University.

Results

I. Patient Demographics

Six of the eight subjects fulfilled the criteria of eight implants under a maxillary prosthesis. One patient had only seven implants in the maxillary arch (Patient 6) and one had eight implants distributed under both maxillary and mandibular arches in an "All-on-Four" design for each arch (Patient 8). A summary of patient demographics can be found in Table 3. Of note, our study population was predominately white males with an average age of 60 years. The study prostheses were in function an average of 4.43 years, with the least function being 1.36 years (Patient 8) and most function being 7.94 years (Patient 6). The study time between the first clinical session and second clinical session in our study was on average 6.8 months, ranging from 175 days (Patient 7) to 265 days (Patient 5).

II. Summary of Clinical Data

Selected clinical data are summarized in Tables 4 and 5. The average probing depth at time one (PD at T_1) per site (as defined by material) ranged from 2.33mm (Patient 5, teflon) to 9.33mm (Patient 7, foam). The average PD at T_1 among patients was 3.87 mm (SD 1.58). 68.75% of all of the sites across all patients had probing depths below 4mm, 18.75% was at or greater than 4mm but less than 6mm, 9.375% was at or greater than 6mm but less than 8mm, and 3.125% was at 8mm or greater. The average probing depth at time two (PD at T_2) per site (as defined by material) ranged from 2.0mm (Patient 5, teflon) to 9.17mm (Patient 7, foam). The average PD at T_2 among patients was 3.96 mm (SD 1.69). 71.875% of all of the sites across all patients had probing depths below 4mm, 15.625% was at or greater than 4mm but less than 6mm, 6.25% was at or greater than 6mm but less than 8mm, and 6.25% was at 8mm or greater.

There was no statistical difference between the PD at T_1 and T_2, as determined by T-Test (P = 0.3306).

The Pearson Correlation Coefficient was calculated for PD at T_2 and Red complex. PD at T_2 is correlated with proportion of Red complex (Pearson Coefficient = 0.71258, P<0.0001, N=32). This is in agreement with the findings of Socransky and Haffajee in 1998 [24].

The Plaque Index at time one (PI at T_1) per site (as defined by material) ranged from 1 to 3. When comparing all patients, Patient 5 had the lowest average PI at T_1 across all sites (1), while Patient 2 had the greatest average PI at T_1 (2.5). The average PI at T_1 among all patients was 1.89 mm (SD 0.56). 21.875% of all of the sites across all patients had PI at T_1 of 1, 65.625% had an index of 2, and 12.5% had an index greater than 2. The Plaque Index at time two (PI at T_2) per site (as defined by material) ranged from 1 to 3. When comparing all patients, Patient 5 had the lowest average PI at T_2 across all sites (1), while Patient 1 had the greatest average PI at T_2 (2.38). The average PI at T_2 among all patients was 1.77 (SD 0.58). 31.25% of all of the sites across all patients had PI at T_2 of 1, 59.38% had an index of 2, and 3.38% had an index greater than 2. There was no statistical difference between the PI at T_1 and T_2, as determined by T-Test (P = 0.211).

The Gingival Index at time one (GI at T_1) per site (as defined by material) ranged from 1 to 3. When comparing all patients, Patient 5 had the lowest average GI at T_1 across all sites (1), while Patients 1, 4, and 8 had the greatest average GI at T_1 (2.25). The average GI at T_1 among patients was 1.97 (SD 0.46). 12.5% of all of the sites across all patients had GI at T_1 of 1, 75% had an index of 2, and 12.5% had an index greater than 2. The Gingival Index at time two (GI at T_2) per site (as defined by material) ranged from 1 to 3. When comparing all patients, Patient 5 had the lowest average GI at T_2 across all sites (1), while Patients 1, 4, and 8 had the greatest

average GI at T_2 (2.25). The average GI at T_2 among patients was 1.97 (SD 0.46). 12.5% of all of the sites across all patients had GI at T_2 of 1, 75% had an index of 2, and 12.5% had an index greater than 2. The measurements of GI at T_1 and T_2 for all sites and patients were identical, so no P-value was calculated.

Radiographic bone loss was difficult to assess because the radiographic record was either incomplete or not standardized based on when the prostheses were either placed or when the study started. Therefore, we defined percent radiographic bone loss as measured from the implant-abutment interface (IAI) to the level along the implant at T_2. In this definition, we assume that the implants were originally placed at the crest and that they had no bone loss prior to prosthesis insertion. Taking this definition, there were varying amounts of bone loss, ranging from none to about 50%. No implants were mobile.

All implant-abutment connections studied were flat, butt-joint connections. All but two patients had Nobel Replace implants, which have an internal triangular connection. Patient 1 had 3i Osseotite Certain implants, which have an internal hexagon connection. Patient 8 had Brånemark system implants, which have an external hexagon connection. 18 sampled sites had the prosthetic connection directly to the implant platform, 10 sampled sites had multiunit abutments screwed to the implant platform, and 4 sampled sites had mixed locations of the prosthetic connection (i.e. both types of assemblies). The multiunit abutment places the dental material farther from the IAI, which may play a role in our interpretation of the data.

In summary, the majority of the implants in our study were stable over the study period in terms of probing depth, plaque index, and gingival index. Despite being stable, there were moderate amounts of plaque accumulation and gingival inflammation, indicative of at least peri-

implant mucositis. This can place the implants at risk for further breakdown (ie. development of peri-implantitis) and possible loss of the implants.

III. Percent Microbial Complex Bacteria in Material versus Sulcus Samples Per Patient

Bar graphs depicting the relative proportions of the microbial complexes [24] in each patient per each sample and site can be found in figures 5A through 5H. Overall, there appeared to be a preponderance of early colonizing bacteria (Yellow, Blue, Green, Purple) within the dental materials, while the peri-implant sulcus appeared to be overwhelmingly composed of Orange complex bacteria. Paired T-tests were performed with a P value ≤ 0.05 denoting significant difference between occluding material and peri-implant sulci complex bacteria per site. The proportion of Red and Orange complexes found on the occluding materials was significantly less than in the peri-implant sulci. The proportion of Green, Yellow, and Blue complexes found on the occluding materials was significantly greater than in the peri-implant sulci. There was no significant difference for the Purple complex (Table 6).

The correlation between proportion of each bacterial complex on the occluding material and the peri-implant sulcus can be found in Table 7. The correlation between the proportion of bacteria between the occluding materials and peri-implant sulcus is significant for Red, Green, and Yellow complexes ($P \leq 0.05$). Therefore, the proportion of Red complex on the occluding materials appeared to be related to the proportion of Red complex in the peri-implant sulcus. This is also true of the Green and Yellow complexes. The correlation is not significant for Orange, Purple, and Blue complexes ($P \geq 0.05$). Therefore, there appears to be no relationship between the proportion of Orange complex on the occluding materials and the proportion of Orange complex in the peri-implant sulcus. This is also true of the Purple and Blue complexes.

None of the microbial complexes [24] found in the peri-implant sulci were significantly affected by the materials used as determined by ANOVA with a $P \geq 0.05$ when material is the independent variable (Tables 8A through 8F). The data was further studied by ANOVA to determine whether any of the dental materials had specifically produced a significant reduction in the proportion of complex bacteria found on the occluding material versus the peri-implant sulci. The material used did not have any specific effect (reduction or increase) on any of the complex bacterial proportions in the peri-implant sulcus, with a $P \geq 0.05$ (data not shown), as compared to other sulci. Patient differences had significant effects on the proportion of complex bacteria in the occluding material versus the peri-implant sulcus with a $P \leq 0.05$ for Red, Purple, and Green complexes (Table 9A-C). Patient 7 had a significantly higher proportion of Red complex bacteria in the peri-implant sulcus than in the occluding material than all other patients except for Patient 5 (HSD, $\alpha=0.5$, data not shown). Patient 8 had a higher proportion of Purple complex bacteria in the peri-implant sulcus than in the occluding material than all other patients, but this was not significant (HSD, $\alpha=0.5$, data not shown). Patient 3 had a significantly higher proportion of Green complex bacteria in the peri-implant sulcus than in the occluding material than only Patient 8 (HSD, $\alpha=0.5$, data not shown). *F. nucleatum* was most frequently detected among all samples. *C. rectus*, *E. nodatum*, *S. mitis*, *A. naeslundii*, *S. noxia*, and *E. corrodens* were not detected in any samples (data not shown).

IV. Non-Complex Peri-Implant Bacteria May Be Affected by Material

Five bacteria found in the peri-implant sulci not classified by Socransky and Haffajee may be significantly affected by the materials used as determined by ANOVA ($P \leq 0.05$, Tables 10A through 10E). These include *Aquabacterium spp*, *Comamonas testosteroni*, *Hydrogenophaga spp*, *Lewinella spp*, and *Wandonia haliotis*. For *Aquabacterium spp*, cotton

produced the lowest proportions but this was not significant (HSD, α=0.5, data not shown). For *Comamonas testosteroni, Hydrogenophaga spp,* and *Lewinella spp,* PVS produced the lowest proportions, but this was not significant (HSD, α=0.5, data not shown). For *Wandonia haliotis,* PVS produced the lowest proportions, but only significantly lower than cotton (HSD, α=0.5, data not shown). Cotton may produce the highest proportions of *Wandonia haliotis* and *Lewinella spp,* however the proportions of *Wandonia haliotis* was only significantly more than PVS (data not shown). These bacterial DNA sequences were not found in all patients at all sites and varied for their relative proportions per site. Overall, no specific sulcus bacterium appears to be affected by type of occluding material in the present study.

V. Peri-Implant Sulcus Bacteria

ANOVA tests comparing patient and different proportions of complex bacteria found in the peri-implant sulci were performed. The proportion of Red, Purple, Yellow, and Blue complexes were significantly different between patients ($P \leq 0.05$, Tables 8A, 8C, 8E, 8F). The proportion of Orange and Green complexes was not statistically different between patients ($P \geq 0.05$, tables 8B and 8D). Patient 7 had a statistically higher proportion of Red complex bacteria than all other patients (HSD, α=0.5, data not shown). Patient 8 had the highest proportion of Purple complex bacteria, but this was not significantly greater (HSD, α=0.5, data not shown). Patient 8 had the highest proportion of Yellow complex bacteria, and it was significantly greater than patients 1, 4, 5, 6, 7 (HSD, α=0.5, data not shown). Patient 8 had a statistically higher proportion of Blue complex bacteria than all other patients (HSD, α=0.5, data not shown). As determined by Pearson Correlation, Purple and Green complexes are significantly correlated, and Blue and Yellow complexes are significantly correlated in the peri-implant sulci of the study population (Table 11). This is in accordance with Socransky and

Haffajee in 2005, based on relative location of the Complex bacteria between the tooth surface and epithelium in a periodontal pocket (Fig 6) [99]. Red and Orange complexes were not significantly correlated, which is different from the findings of Socransky and Haffajee in their publications [24, 99].

An average of 43.12% (SD=10.72) of the DNA sequences representing the bacterial communities in the peri-implant sulcus are from bacteria not classified by Socransky and Haffajee. There appears to be no one novel bacteria in appreciable abundance for all patients at all sites. However, *F. nucleatum* was consistently found in the highest proportion for all patients at all peri-implant sulcus sites. This might reflect its importance as a "bridging species" in the ecological succession and maturation of oral subgingival biofilms [99].

Discussion

I. Evidence for Microleakage

Overall, the results support the inoculation of the implant abutment screw channel dental materials with bacteria. As part of the study design, it was ensured that viable bacterial components were not present on any of the materials used. Therefore, when bacterial DNA was identified on the control samples, we attribute the DNA to nonviable bacteria and low-level contamination arising from the DNA extraction and sequencing steps. Furthermore, the proportions of bacterial DNA found on the controls appear to be random (data not shown) versus the samples collected from the patients. Meticulous protocols were followed to decontaminate the implants and prostheses, and to place and collect the samples from the patients in an aseptic manner. All of these factors considered, the notion that the bacterial DNA identified on the study samples were from within the patient's oral cavity is favorable. This indicates the presence of microleakage at the level of the implant-abutment interface and/or at the coronal seal of the prosthesis.

The data suggests that the bacterial community found on the dental materials tested in this study is different from the bacterial community found in the peri-implant sulcus. Therefore, it is reasonable to assume that either there is hindrance of microleakage by physical blockage provided by the sealing materials or other mechanisms or there is environmental selection for certain bacterial types in each location. The latter can be modified by inflammation, plaque abundance, time, pH, oxygen levels, and other nutrients or factors. There appeared to be a correlation between some microbial complex bacteria [24] on both the dental materials and the peri-implant sulcus. Based on this information, it is reasonable to assume that at least a portion of the microleakage of bacterial DNA is related to the flow of DNA from the peri-implant sulcus

to the dental sealing material. In our study, the majority of prosthetic assemblies were screwed directly to the implant platform and all had butt-joint implant-abutment interfaces. Despite minor differences in the exact interfaces for two out of the eight patients, this was not statistically significant on the level analyzed. Pending statistical analysis investigating the influence of the assembly type, there is evidence that microleakage from the peri-implant sulcus to the implant abutment screw channel sealing materials occurs at the implant-abutment interface.

II. Implant Abutment Screw Channel Sealing Materials Appear to Have No Effect on the Peri-Implant Microbiome

The data has been analyzed for differences in the proportions of bacterial DNA between material type and peri-implant sulcus. There appeared to be no effect of different materials on the peri-implant sulcus microbiome. This is not in accordance with previously published data suggesting that material type could have an effect on microleakage and therefore influence the microbiota in the peri-implant sulcus [90]. The major difference between the present study and that already published is that this in an *in-vivo* model utilizing each patient's unique microbiomes found in oral fluids as the passage fluid versus an *in-vitro* model utilizing fuchsin dye as the passage fluid. As previously discussed, the selection of bacterial DNA into the abutment screw channel can be affected by several factors, whereas the fuchsin dye must only have a gap in which to flow.

III. Characteristics of the Peri-implant Microbiome

The factor which appeared to explain the differences in composition of peri-implant microbiome DNA was overwhelmingly the individual patients. Our data need to be analyzed further to question what patient factor or factors (such as periodontal clinical parameters, race, age, etc.) may influence the differences in composition further. This analysis, however, may be a

moot point. There is emerging data being published that liken oral bacterial communities to fingerprints, as they thought to be unique between individuals. Research endeavors, such as the Human Microbiome Project, are therefore underway [100-103]. Despite the variations among individuals, *F. nucleatum* stood out as the most common bacterial type. Other studies show a high frequency of *F. nucleatum*, but it does not appear as the most common species in those studies [103, 104].

In general, the classical oral bacterial complexes [24] were useful in describing the present data and their application to peri-implant microbiomes has been supported in this study. The bacterial DNA from species not included in the microbial complexes [24] appear to be a substantial component of the subgingival environment around implants. These species appear diverse, with no one species being consistently prevalent among patients. However, one of these "other" bacteria, *Atopobium* species, was the second most commonly detected bacteria after *F. nucleatum*. *Atopobium* species have been described in the peri-implant literature before [104]. *Atopobium* species are Gram-positive facultative anaerobic bacteria, which are either rod shaped or elliptical coccobacilli. They have also been detected in other pathologic conditions, such as bacterial vaginosis, dental caries, and endodontic lesions, and can be difficult to cultivate [104-110]. These species will have to be closely studied in the future to elucidate their role in disease processes and the microbial communities in which they are found.

The role of bacterial DNA in relation to the host response in periodontitis has been previously documented. For example, it has been demonstrated that DNA isolated from *P. gingivalis*, *A. actinomycetemcomitans*, and *Peptostreptococcus micros* stimulates macrophages and gingival fibroblasts to produce TNF-α and IL-6 in a dose-dependent manner. TNF-α and IL-6 are pro-inflammatory cytokines which contribute to the upregulation of matrix

metalloproteinases (MMPs). MMPs breakdown extracellular collagen matrixes. This facilitates the mobility of other cells and compromises the structure of connective tissues. IL-6 upregulates the receptor agonist for nuclear factor kappa beta ligand (RANKL), which binds RANK on pre-osteoclasts and leads to osteoclast differentiation. This leads to bone breakdown in the periodontium and subsequent attachment loss around teeth, the hallmark sign of periodontal disease. *Atopobium species* and other emerging relevant periodontal bacteria have to be studied to determine their specific effect on the host immune response and the pathway by which they have this effect [110].

Future Directions

We have added to the body of knowledge regarding the presence of microleakage into the implant screw channel. To enhance our results, the data need to be analyzed for any effect of probing depth, plaque index, gingival index, bone loss, and assembly type on the correlation between the bacteria in the peri-implant sulcus and on the implant abutment screw channel material. Of particular interest is the assembly type. If there is the same correlation between bacteria in the peri-implant sulcus despite assembly type, then the leakage is not exclusively at the abutment-implant interface. If there is a stronger correlation of the peri-implant sulcus bacteria and the dental sealing material bacteria in assemblies that screw directly into the implant, then there is support that more leakage occurs at the abutment-implant interface. It will also be interesting to analyze the data for specific bacteria that appear to be more correlated between the dental materials and peri-implant sulcus, as these would presumably be more readily able to leak in and out of the prosthetic assembly and/or be selected for the internal environment. It will be important to study these bacteria further, especially if they can be implicated in disease processes.

We have also demonstrated that in about a six-month period, there appears to be no effect of different implant abutment screw sealing materials on the microbial community within the peri-implant sulcus. However, weak associations for the five bacteria not classified by Socransky and Haffajee demonstrate a potential for material to have some effect [24]. The study by Park, *et al* [90] should perhaps be repeated in an *in vitro* model using human saliva to explore this potential further before repeating it in an *in vivo* experiment. The fact that the materials appear to have a significant greater proportion of early colonizers supports repeating the study with a longer study period. The ideal would be to design a prospective randomized trial in which

the study population is followed from the time of initial restoration and material placement, and in which half the study population has materials removed after 6 months and half of the study population has materials removed after 12 months. This can be difficult in terms of patient recruitment in terms of criteria and numbers needed. Another difficulty is that there is currently no specific guideline regarding when to perform maintenance on patients restored with full-arch fixed restorations, such as those encountered in this study, and therefore we would have to inform patients that we do not currently know the risks if their prostheses are not serviced in a prescribed interval. The data collected in the present study should be analyzed to help determine whether the time in which the patients were prescribed to remain in their prostheses without professional maintenance had any detriment to periodontal health as determined by the periodontal parameters collected.

Finally, we have attempted to describe the peri-implant microbiome in fully edentulous patients restored with dental implants supporting fixed, full-arch restorations. It is clear that the role of both highly studied periodontal bacteria, such as the Socransky and Haffajee complex bacteria, and emerging relevant periodontal bacteria must be studied further to characterize the peri-implant microbiome in edentulous patients in health and disease. The data need to be studied further for emerging relevant periodontal bacteria, especially those that seem to be correlated with parameters that are congruent with disease, such as increased probing depth.

It is important to note that this is a pilot study with no previous studies of similar design present in the literature. Several improvements can be made. One includes an even stricter isolation protocol used when placing and collecting patient samples, such as the use of rubber dam. Controls were not collected from the same sterilization packets or PVS dispensers that were used during patient interactions. More data can also be collected. For example, microbial

samples at time one can be taken in order to compare differences in microbiome in the peri-implant sulcus over the study period. The number of prosthetic disconnections-reconnections can also be determined from the patient's chart. An increased number of prosthetic disconnections-reconnections has been suggested to increase peri-implant bone loss, but this notion is still somewhat controversial [108, 109]. The massive amount of data collected in the present study and future studies derived from it will have implications for studying implant health on several fronts.

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Figures and Tables

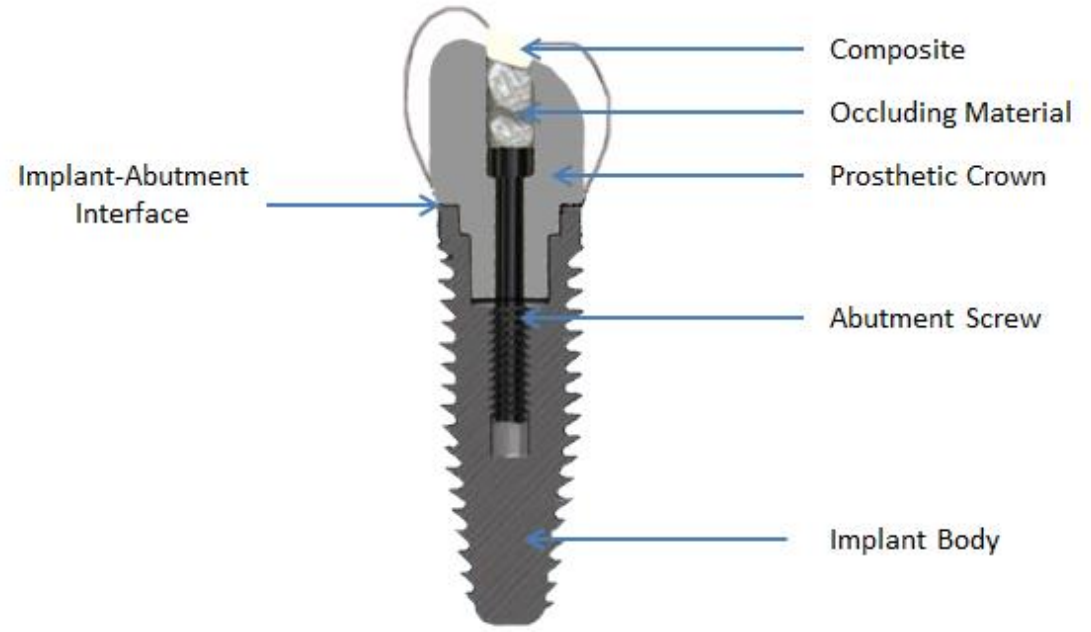

Figure 1: Example of Screw-Retained Prosthesis in which the prosthesis is screwed directly to the implant platform.

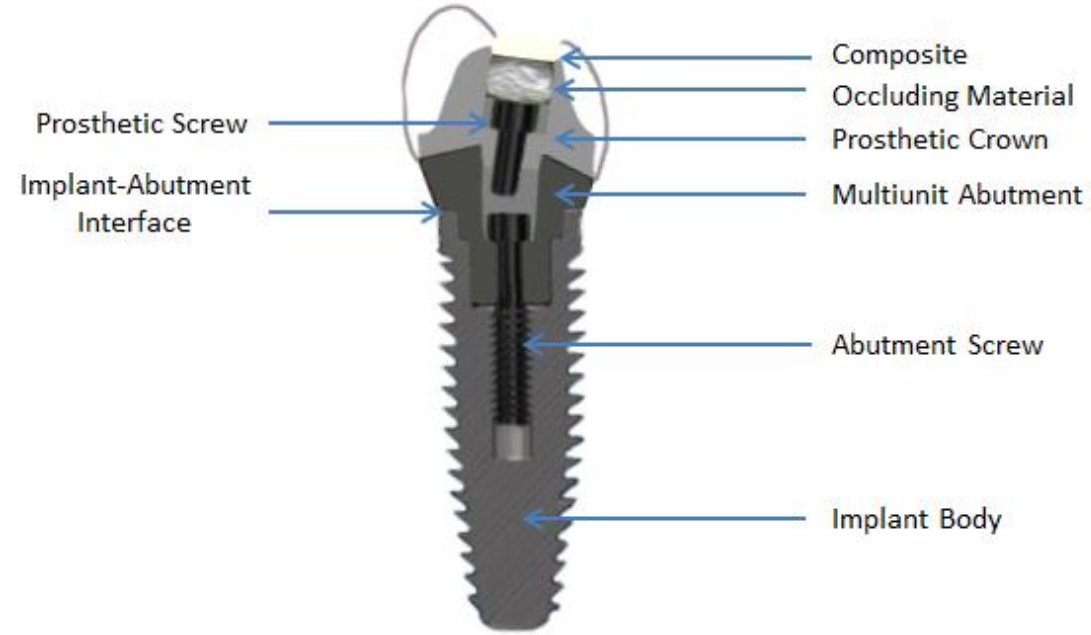

Figure 2: Example of Screw-Retained Prosthesis in which the prosthesis is screwed to an intermediary, called a multiunit abutment, instead of directly to the implant platform.

Table 1	Classification of peri-implantitis
Early	PD ≥ 4 mm (bleeding and/or suppuration on probing*) Bone loss < 25% of the implant length†
Moderate	PD ≥ 6 mm (bleeding and/or suppuration on probing*) Bone loss 25% to 50% of the implant length†
Advanced	PD ≥ 8 mm (bleeding and/or suppuration on probing*) Bone loss > 50% of the implant length†

*Noted on two or more aspects of the implant.
†Measured on radiographs from time of definitive prosthesis loading to current radiograph. If not available, the earliest available radiograph following loading should be used.

Table 1: A proposed system for the classification of peri-implantitis (Froum and Rosen 2012)

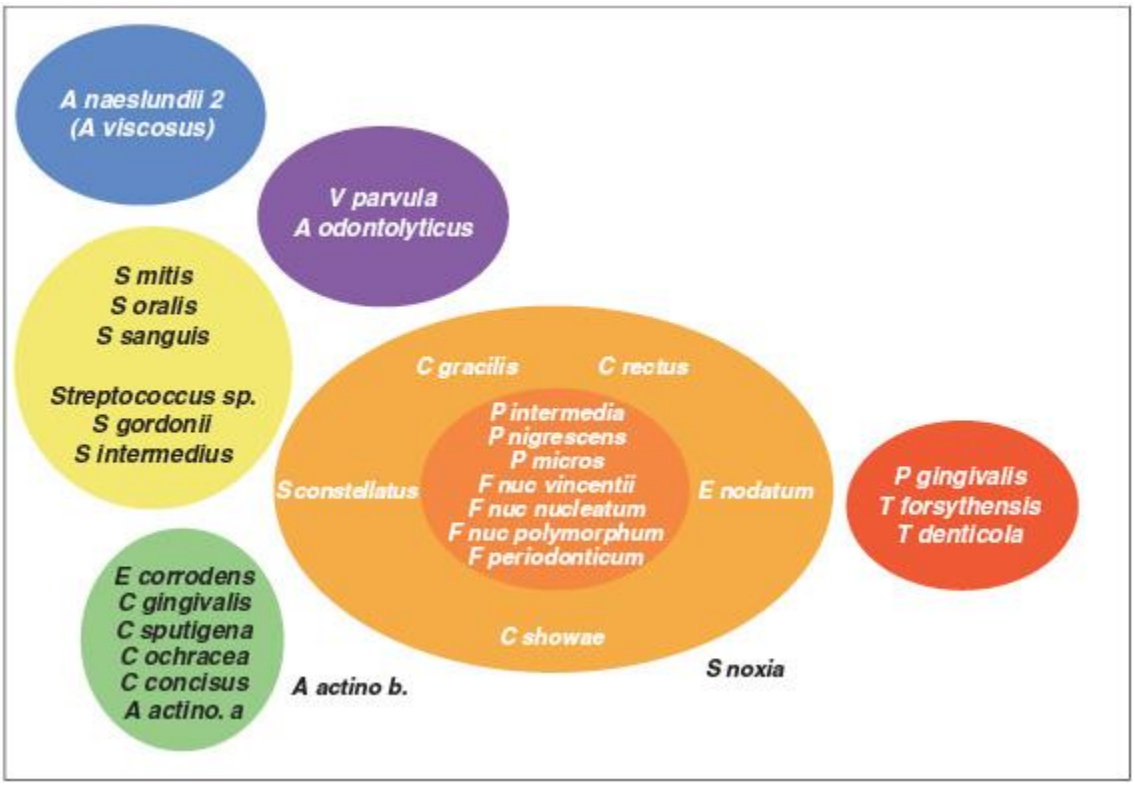

Figure 3: Bacterial Complexes (Socransky and Haffajee1998)

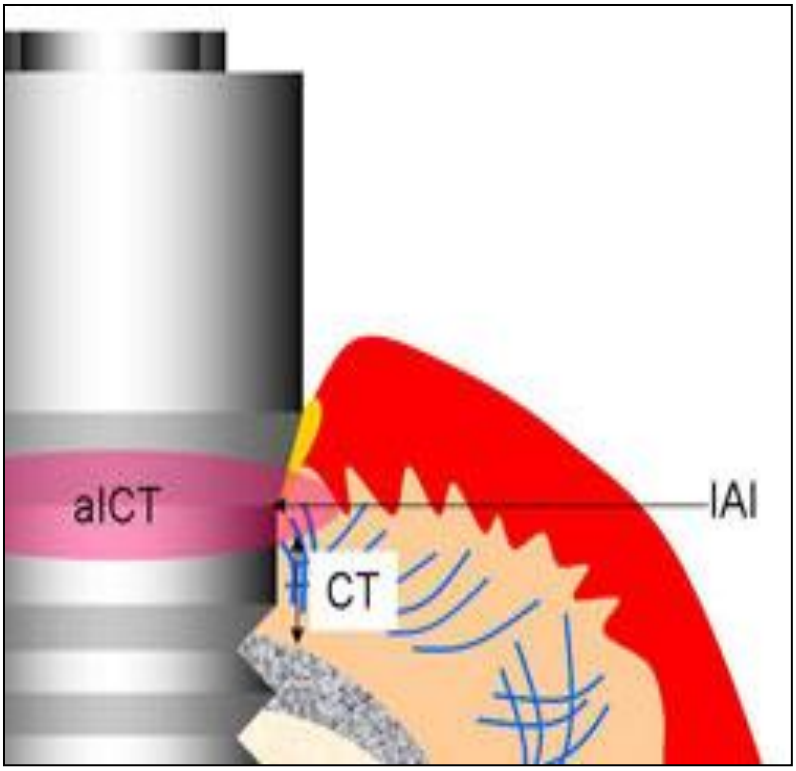

Figure 4: Inflammation at the Implant-Abutment Interface. IAI = Implant Abutment Interface, aICT = Abutment Inflammatory Cell Infiltrate (1.5mm = 0.75mm above IAI + 0.75mm below the IAI), CT = Zone of healthy connective tissue between the base of the aICT and crestal bone (1mm thick) (Ericsson I., *et al* 1995).

Material	Company	Product
Teflon® (PTFE) Tape	Merco Co.	Merco M55 Thread Seal Tape
Cotton Pellets	Richmond Dental	Bleached Cotton
Synthetic Foam	Jordco	Endoring Foam Inserts
Polyvinylsiloxane (PVS)	Dentsply	Aquasil
Composite	3M ESPE	Filtek Supreme Ultra Universal Restorative
Pink Composite	GC America Inc.	Gradia Gum

Table 2: Summary of Dental Materials

Patient Demographics		
Age (Years)	Range	53-73
	Mean	60
	Standard Deviation	6.52
Sex	Male	6
	Female	2
Race	White	5
	Black	2
	Hispanic	1
Time Study Prosthesis in Function (Years)	Range	1.36-7.94
	Mean	4.43
	Standard Deviation	1.97
Time Between T_1 and T_2 (Days)	Range	175-265
	Mean	206.86 (about 6.8 months)
	Standard Deviation	29.87

Table 3: Summary of Patient Demographics

Patient #	Location Defined By Material	Probing Depth at T_1 (avg mm)	Plaque Index at T_1 (avg)	Gingival Index at T_1 (avg)	Assembly Type
1	Foam	3.5	2	2	To platform
	Teflon	3.17	2.5	2.5	Mixed
	Cotton	2.92	2	2.5	Multiunit abutment
	PVS	3.08	2	2	To platform
2	Foam	3.08	3	2	To platform
	Teflon	3.25	2	2	To platform
	Cotton	4.42	2	2	To platform
	PVS	3.58	3	2	To platform
3	Foam	3.58	2	2	Multiunit abutment
	Teflon	4.0	2	2	To platform
	Cotton	3.25	2	2	To platform
	PVS	3.75	2	2	Multiunit abutment
4	Foam	4.83	2	3	Multiunit abutment
	Teflon	2.55	2	2	Mixed
	Cotton	3.0	2	2	To platform
	PVS	3.25	2	2	Multiunit abutment

Table 4A: Clinical Data at the first clinical appointment (T_1), Patients #1-4

Patient #	Location Defined By Material	Probing Depth at T_1 (avg mm)	Plaque Index at T_1 (avg)	Gingival Index at T_1 (avg)	Asssembly Type
5	Foam	3.08	1	1	To platform
	Teflon	2.33	1	1	Multiunit abutment
	Cotton	2.58	1	1	Mixed
	PVS	4.17	1	1	To Platform
6	Foam	4.58	2	2	To platform
	Teflon	3.5	2	2	To platform
	Cotton	4.5	2	2	To platform
	PVS	3.67	2	2	Mixed
7	Foam	9.33	2	2	To platform
	Teflon	6.08	2	2	To platform
	Cotton	6.42	2	2	To platform
	PVS	7.92	2	2	To platform
8	Foam	2.75	1	2	Multiunit abutment
	Teflon	2.58	1	2	Multiunit abutment
	Cotton	2.55	3	3	Multiunit abutment
	PVS	2.67	1	2	Multiunit abutment

Table 4B: Clinical Data at the first clinical appointment (T_1), Patients #5-8

Patient #	Location Defined By Material	Probing Depth at T_2 (avg mm)	Plaque Index at T_2 (avg)	Gingival Index at T_2 (avg)	Radiographic Bone Loss*
1	Foam	3.5	3	2	>50%
	Teflon	2.92	2.5	2.5	25-50%
	Cotton	3.08	2	2.5	25%
	PVS	3.08	2	2	25%
2	Foam	3.58	1	2	0%
	Teflon	3.58	2	2	<25%
	Cotton	3.67	1	2	<25%
	PVS	3.58	1	2	0%
3	Foam	2.92	2	2	25-50%
	Teflon	4.17	2	2	<50%
	Cotton	3.58	2	2	25%
	PVS	3.17	2	2	25-50%
4	Foam	4.67	2	3	25-50%
	Teflon	3.17	2	2	25-50%
	Cotton	4.25	2	2	<25%
	PVS	3.33	2	2	25%
*As measured from implant platform to level along implant at T_2					

Table 5A: Clinical Data at the time of dental material collection and paper point sampling (T_2), Patients #1-4

Patient #	Location Defined By Material	Probing Depth at T_2 (avg mm)	Plaque Index at T_2 (avg)	Gingival Index at T_2 (avg)	Radiographic Bone Loss*
5	Foam	2.42	1	1	<25%
	Teflon	2.0	1	1	0%
	Cotton	2.5	1	1	0%
	PVS	3.42	1	1	25-50%
6	Foam	4.58	2	2	25%
	Teflon	3.83	2	2	25%
	Cotton	5.67	2	2	25-50%
	PVS	3.33	2	2	25-50%
7	Foam	9.17	2	2	25-50%
	Teflon	6.42	2	2	25%
	Cotton	7.17	2	2	25%
	PVS	8.83	2	2	50%
8	Foam	2.83	1	2	<25%
	Teflon	2.75	1	2	<25%
	Cotton	2.67	3	3	<25%
	PVS	2.92	1	2	<25%
*As measured from implant platform to level along implant at T_2					

Table 5B: Clinical Data at the time of dental material collection and paper point sampling (T_2), Patients #5-8

Figures 5A-H: Percent Complex Bacteria in Material and Sulcus Samples Per Patient

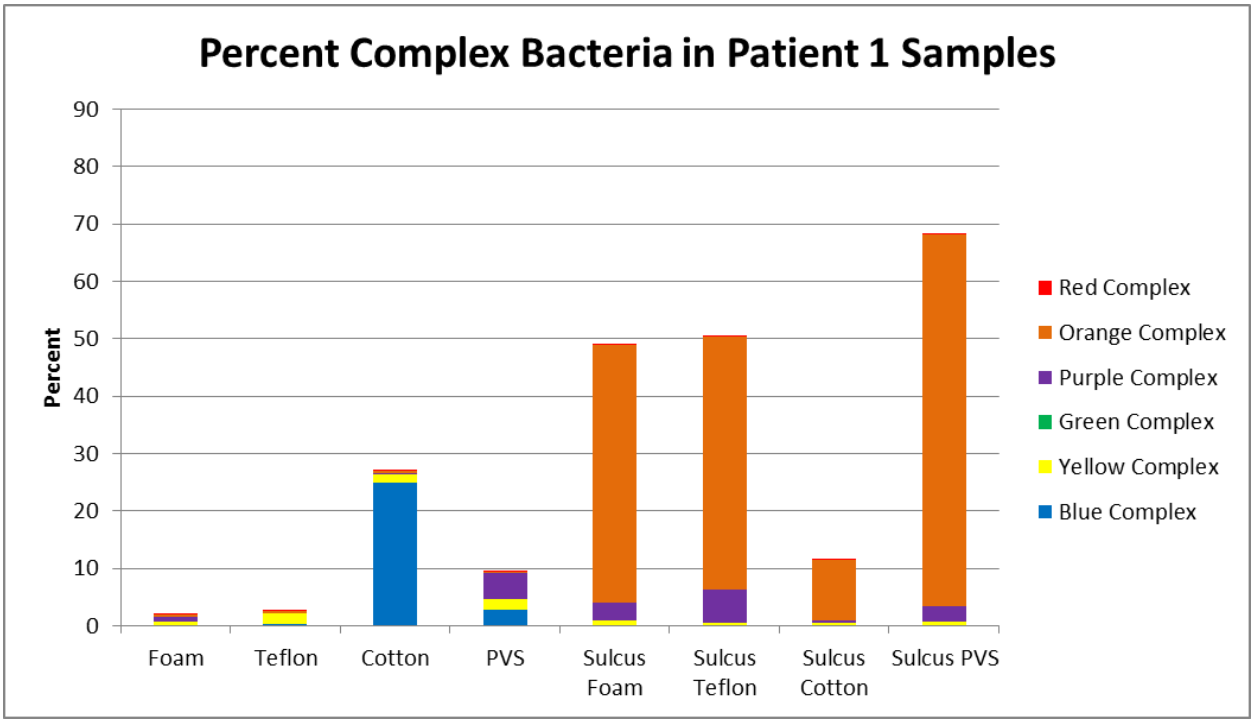

Figure 5A: Percent Complex Bacteria in Material and Sulcus Samples in Patient 1

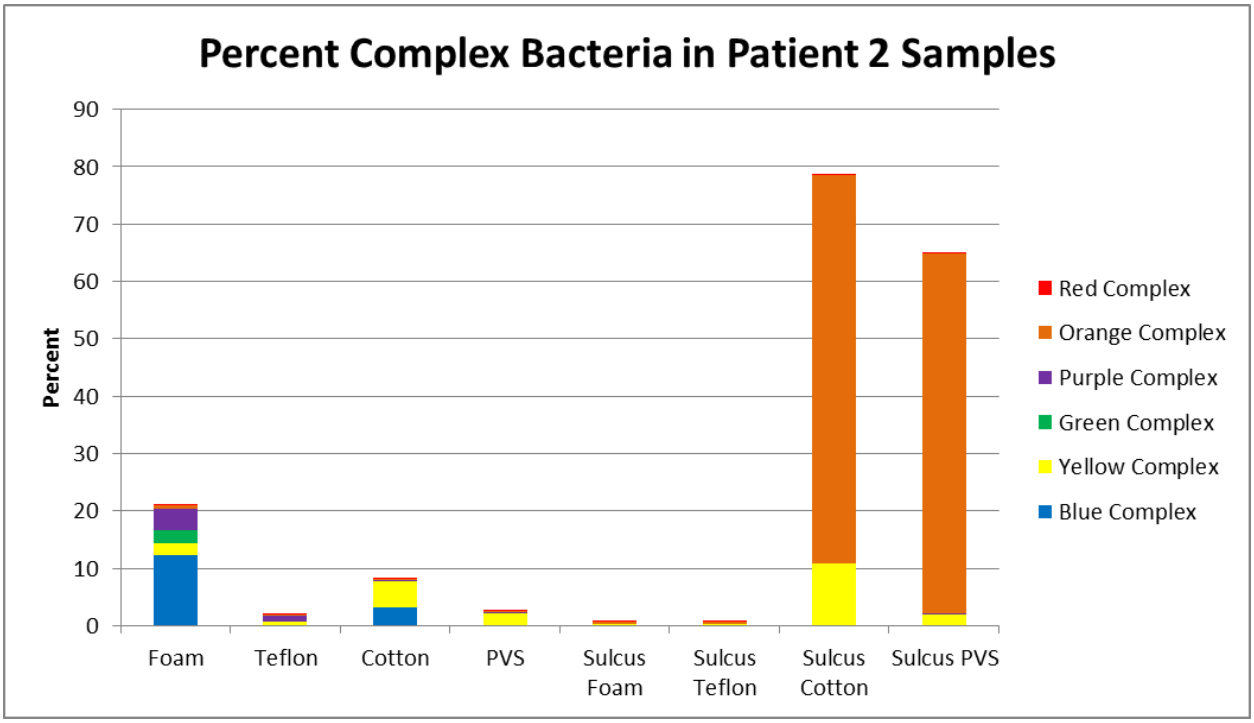

Figure 5B: Percent Complex Bacteria in Material and Sulcus Samples in Patient 2

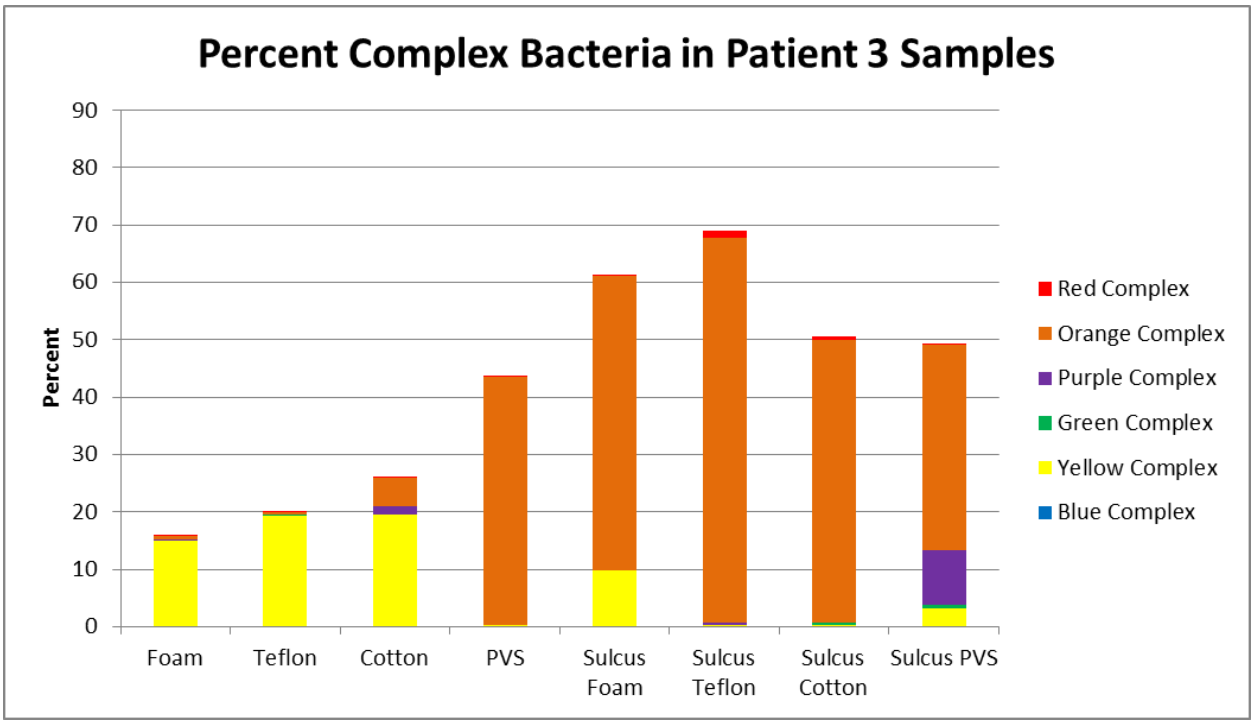

Figure 5C: Percent Complex Bacteria in Material and Sulcus Samples in Patient 3

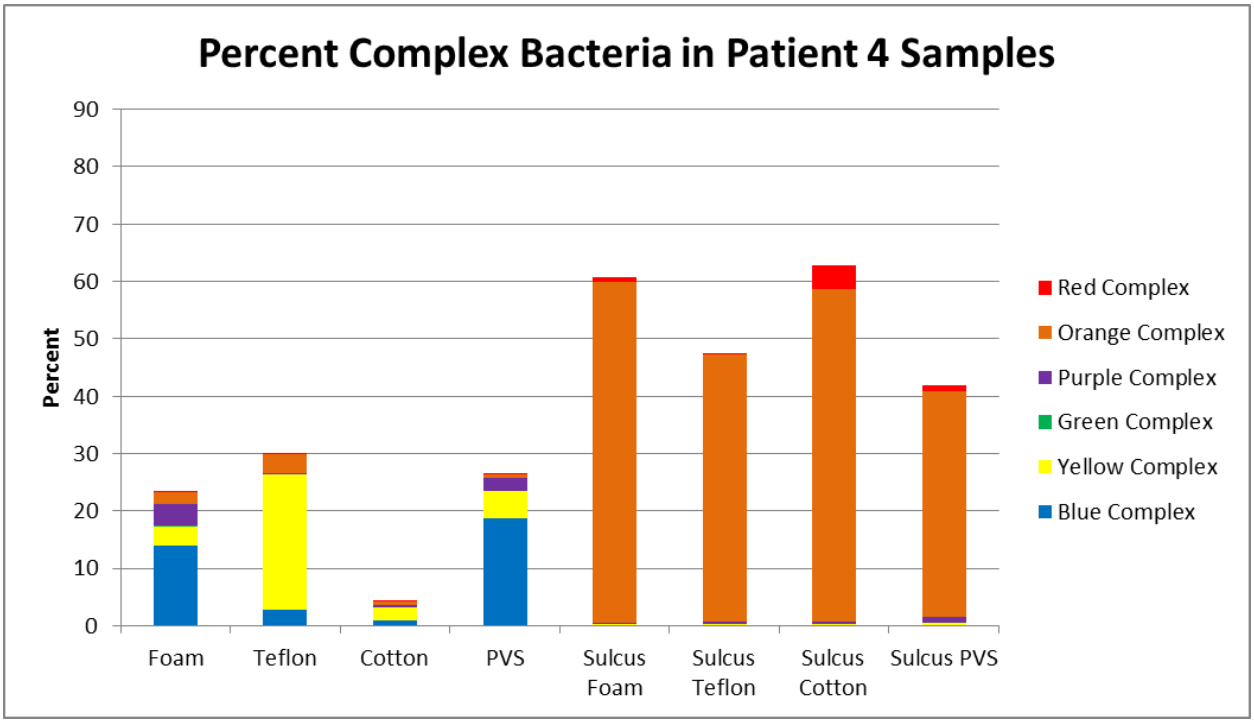

Figure 5D: Percent Complex Bacteria in Material and Sulcus Samples in Patient 4

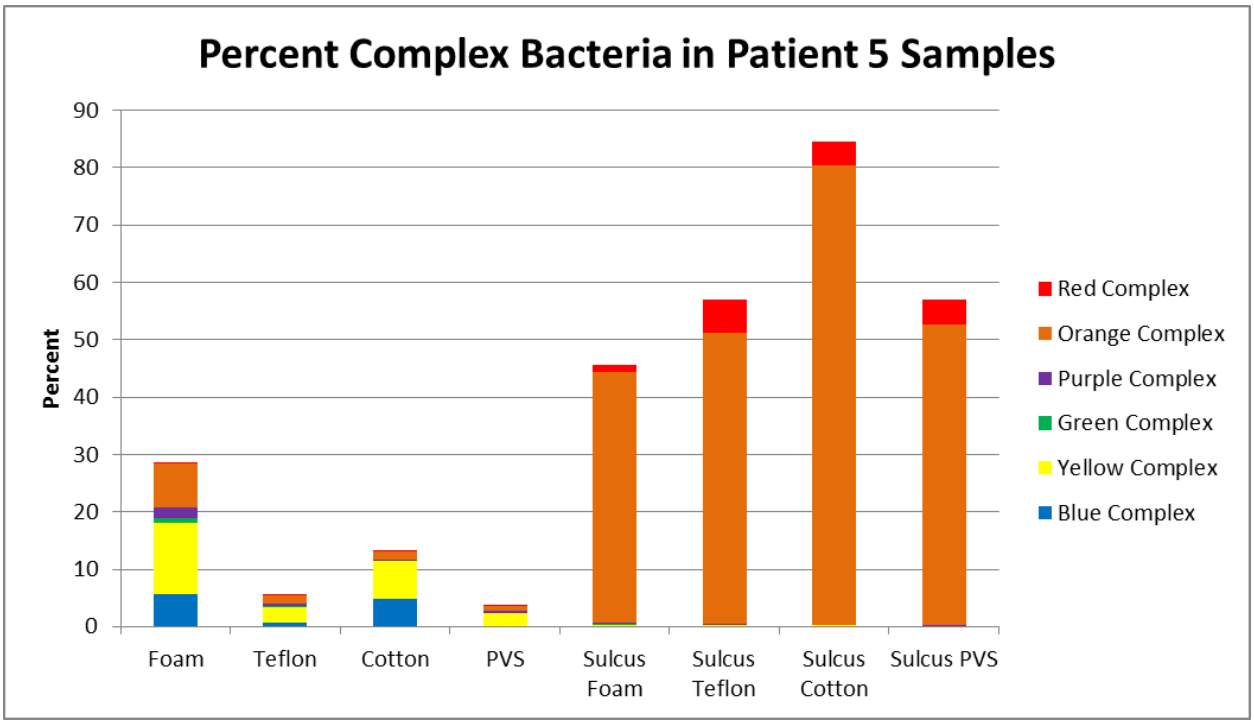

Figure 5E: Percent Complex Bacteria in Material and Sulcus Samples in Patient 5

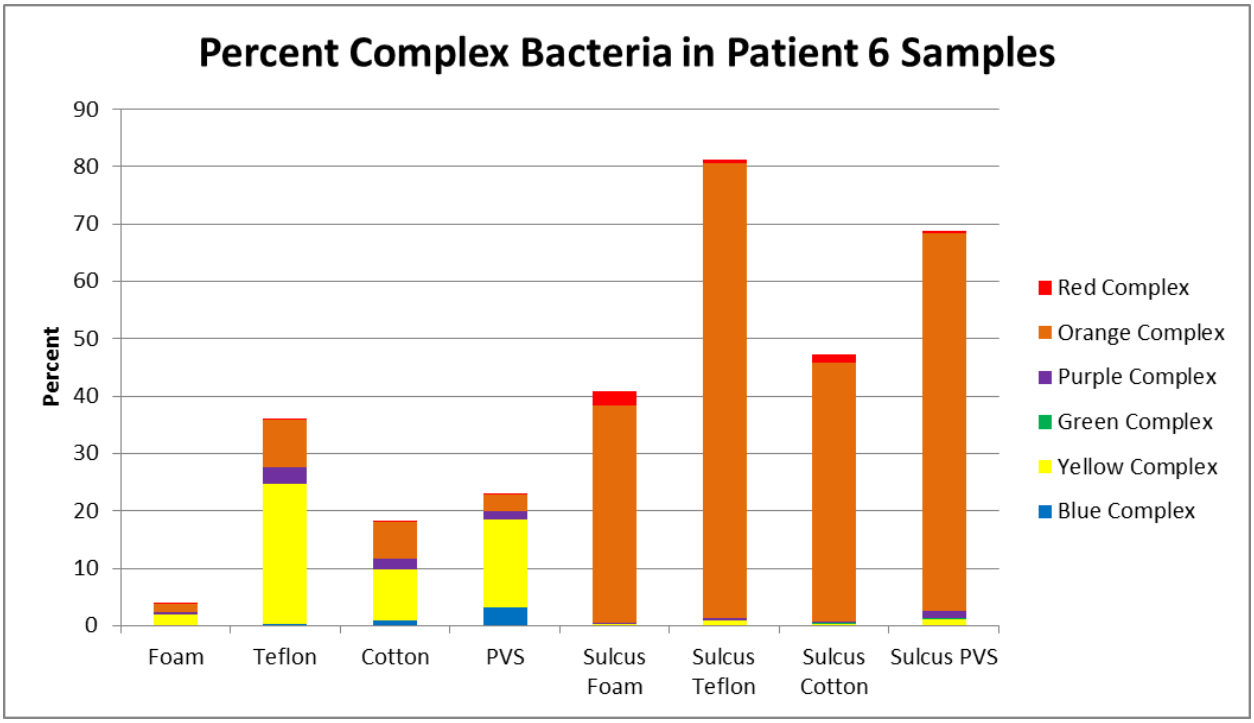

Figure 5F: Percent Complex Bacteria in Material and Sulcus Samples in Patient 6

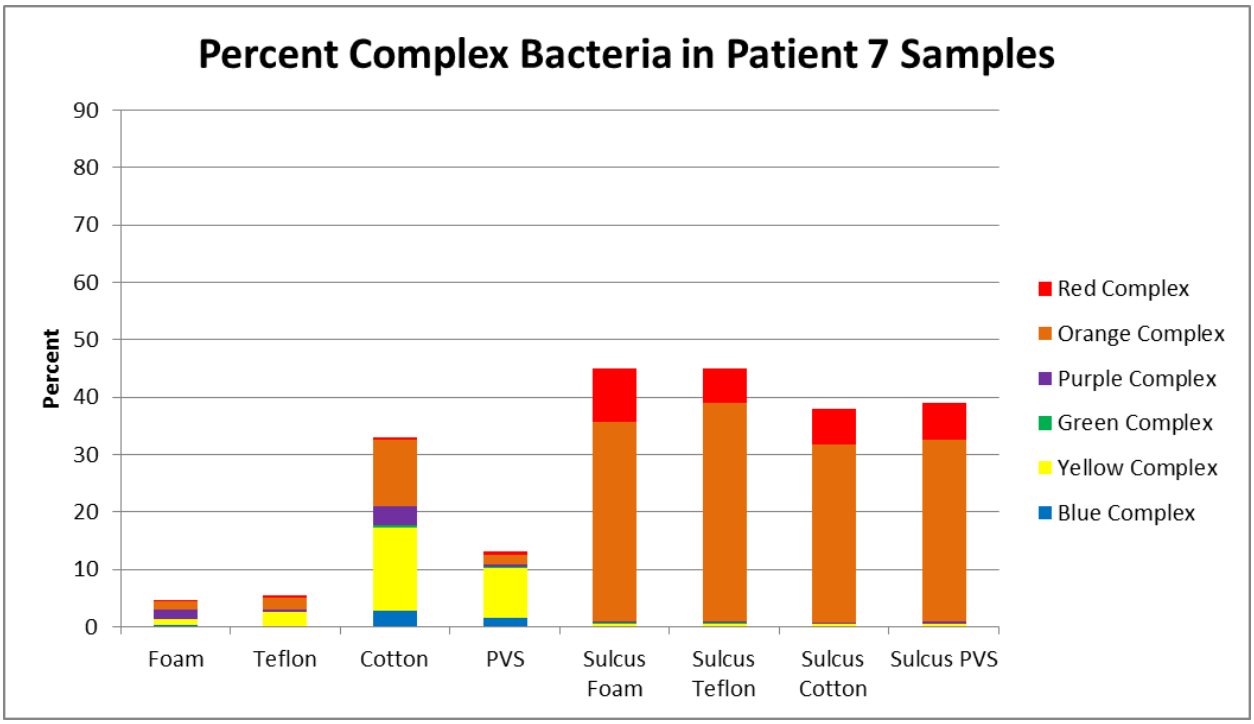

Figure 5G: Percent Complex Bacteria in Material and Sulcus Samples in Patient 7

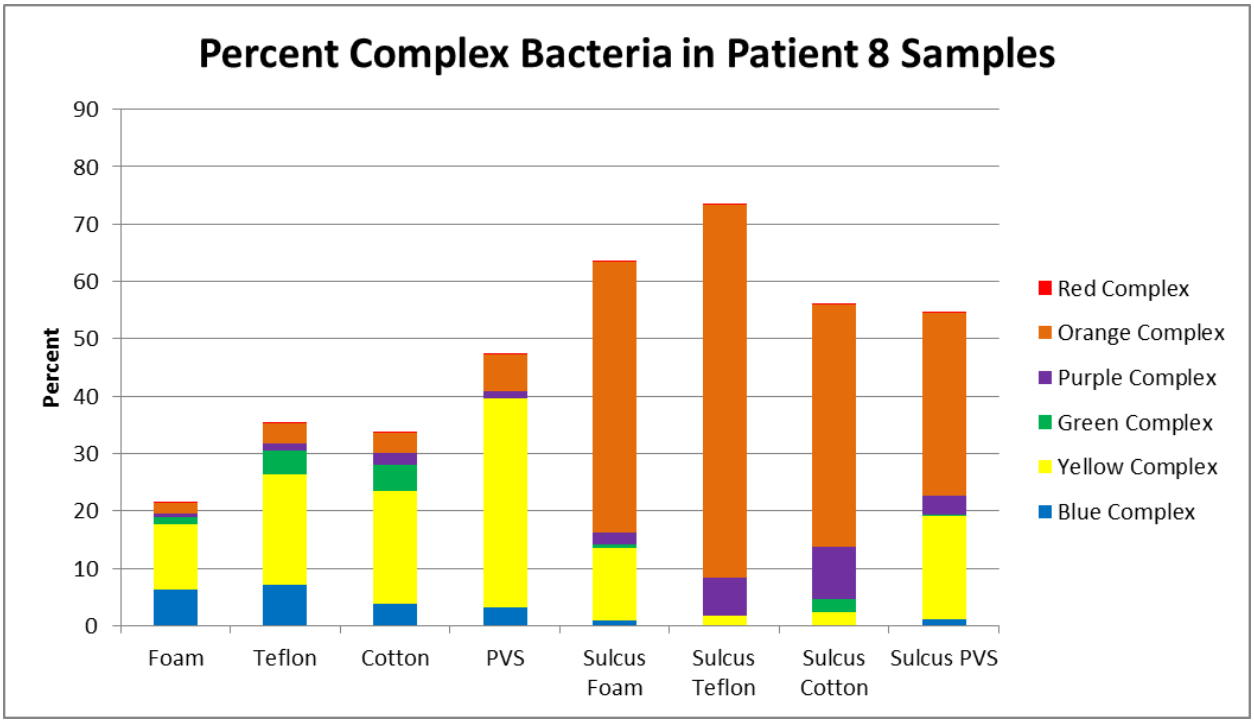

Figure 5H: Percent Complex Bacteria in Material and Sulcus Samples in Patient 8

Difference Between Proportion of Complex Bacteria on Occluding Material and Peri-Implant Sulcus				
Complex	Mean Difference of Proportion of Bacteria on Occluding Material vs Peri-Implant Sulcus	Standard Deviation	T Value	P Value
Red	-0.0168	0.0245	-3.86	0.0003
Orange	-0.4247	0.2128	-11.29	<.0001
Purple	-0.00294	0.0295	-0.56	0.2886
Green	0.00290	0.00913	1.80	0.0410
Yellow	0.0692	0.0829	4.72	<.0001
Blue	0.0366	0.0589	3.52	0.0007
*Negative T Value Indicates Greater Proportion in Peri-Implant Sulcus *Positive T Value Indicates Greater Proportion on Occluding Material				

Table 6: Difference between the proportion of complex bacteria on occluding material and peri-implant sulcus. Paired T-tests were performed to determine whether the differences in the mean proportions of each complex bacteria on the occluding material vs the peri-implant sulcus were significant. Significance was set at $P \leq 0.05$.

Bacterial Complex	Correlation R (Material, Sulcus)	P-value	Significant?
Red	0.6185839	0.0001609	Yes
Orange	-0.07675453	0.6763	No
Purple	-0.04339793	0.8136	No
Green	0.652335	0.00005217	Yes
Yellow	0.4296539	0.01412	Yes
Blue	0.03892543	0.8325	No

Table 7: Correlation between each proportion of each bacterial complex on the occluding material and the peri-implant sulcus as determined by Correlation Coefficient (R). Significance was set at $P \leq 0.05$

Table 8A-F: Complex bacteria are not affected by material. ANOVA was performed with significance was set at P≤0.05.

ANOVA for Red Complex Bacteria Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	0.00009443	0.00003148	0.20	0.8948
Position	3	0.00023973	0.00007991	0.51	0.6813
Patient	6	0.01185642	0.00197607	12.58	<.0001

Table 8A

ANOVA for Orange Complex Bacteria Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	0.06219080	0.02073027	0.50	0.6889
Position	3	0.10331297	0.03443766	0.83	0.4967
Patient	6	0.23473308	0.03912218	0.94	0.4922

Table 8B

ANOVA for Purple Complex Bacteria Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	0.00079666	0.00026555	0.51	0.6819
Position	3	0.00057626	0.00019209	0.37	0.7775
Patient	6	0.01023207	0.00170534	3.26	0.0238

Table 8C

ANOVA for Green Complex Bacteria Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	0.00003656	0.00001219	0.90	0.4610
Position	3	0.00003753	0.00001251	0.92	0.4499
Patient	6	0.00013773	0.00002295	1.69	0.1801

Table 8D

ANOVA for Yellow Complex Bacteria Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	0.00385515	0.00128505	1.20	0.3397
Position	3	0.01011661	0.00337220	3.14	0.0510
Patient	6	0.02351713	0.00391952	3.65	0.0152

Table 8E

ANOVA for Blue Complex Bacteria Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	0.00001268	0.00000423	1.07	0.3874
Position	3	0.00001226	0.00000409	1.03	0.4018
Patient	6	0.00009508	0.00001585	4.01	0.0101

Table 8F

Table 9A-C: There is a significant difference between the proportions of some microbial complexes found in the material samples in comparison to the peri-implant sulcus in different patients. ANOVA was performed with significance was set at P≤0.05.

ANOVA for Red Complex Bacteria Comparing Material Sample to Sulcus Sample					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	0.00008035	0.00002678	0.16	0.9233
Position	3	0.00029392	0.00009797	0.58	0.6374
Patient	6	0.01077424	0.00179571	10.58	<.0001

Table 9A

ANOVA for Purple Complex Bacteria Comparing Material Sample to Sulcus Sample					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	0.00172604	0.00057535	0.84	0.4913
Position	3	0.00114532	0.00038177	0.56	0.6513
Patient	6	0.01164187	0.00194031	2.82	0.0409

Table 9B

ANOVA for Green Complex Bacteria Comparing Material Sample to Sulcus Sample					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	0.00020835	0.00006945	1.19	0.3430
Position	3	0.00018839	0.00006280	1.07	0.3856
Patient	6	0.00103814	0.00017302	2.95	0.0346

Table 9C

Table 10A-E: Non-complex bacteria may be affected by material. ANOVA was performed with significance was set at P≤0.05.

ANOVA for *Aquabacterium spp* Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	1.2608897E-8	4.2029655E-9	3.88	0.0265
Position	3	2.4856581E-9	8.285527E-10	0.77	0.5280
Patient	6	1.5349773E-8	2.5582955E-9	2.36	0.0733

Table 10A

ANOVA for *Comamonas testosteroni* Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	1.5253631E-6	5.0845438E-7	3.43	0.0394
Position	3	8.7859575E-7	2.9286525E-7	1.97	0.1541
Patient	6	1.733667E-6	2.889445E-7	1.95	0.1277

Table 10B

ANOVA for *Hydrogenophaga spp* Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	1.4278079E-6	4.7593598E-7	3.66	0.0323
Position	3	7.4478455E-7	2.4826152E-7	1.91	0.1647
Patient	6	1.4949513E-6	2.4915855E-7	1.91	0.1336

Table 10C

ANOVA for *Lewinella spp* Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	2.1580727E-8	7.1935756E-9	3.65	0.0324
Position	3	2.4927092E-8	8.3090306E-9	4.22	0.0200
Patient	6	1.9841994E-8	3.3069991E-9	1.68	0.1836

Table 10D

ANOVA for *Wandonia haliotis* Found in Sulcus by Variable					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Material	3	1.3243526E-8	4.4145086E-9	3.91	0.0260
Position	3	1.7712318E-9	5.904106E-10	0.52	0.6721
Patient	6	6.6682167E-8	1.1113694E-8	9.84	<.0001

Table 10E

Pearson Correlation Coefficients for Sulcus Samples by Bacterial Complexes, N = 32 Prob > |r| under H0: Rho=0						
	Red Complex	Orange Complex	Purple Complex	Green Complex	Yellow Complex	Blue Complex
Red Complex	1.00000	-0.04931 0.7887	-0.34818 0.0508	-0.14283 0.4355	-0.29830 0.0973	-0.19004 0.2975
Orange Complex	-0.04931 0.7887	1.00000	-0.00457 0.9802	-0.05917 0.7477	0.02924 0.8738	-0.12428 0.4980
Purple Complex	-0.34818 0.0508	-0.00457 0.9802	1.00000	0.66137 <.0001	0.15376 0.4008	0.19070 0.2958
Green Complex	-0.14283 0.4355	-0.05917 0.7477	0.66137 <.0001	1.00000	0.09641 0.5996	0.16948 0.3538
Yellow Complex	-0.29830 0.0973	0.02924 0.8738	0.15376 0.4008	0.09641 0.5996	1.00000	0.80875 <.0001
Blue Complex	-0.19004 0.2975	-0.12428 0.4980	0.19070 0.2958	0.16948 0.3538	0.80875 <.0001	1.00000

Table 11: Correlation coefficients for sulcus samples by bacterial complexes. Significance was set at $P \leq 0.05$

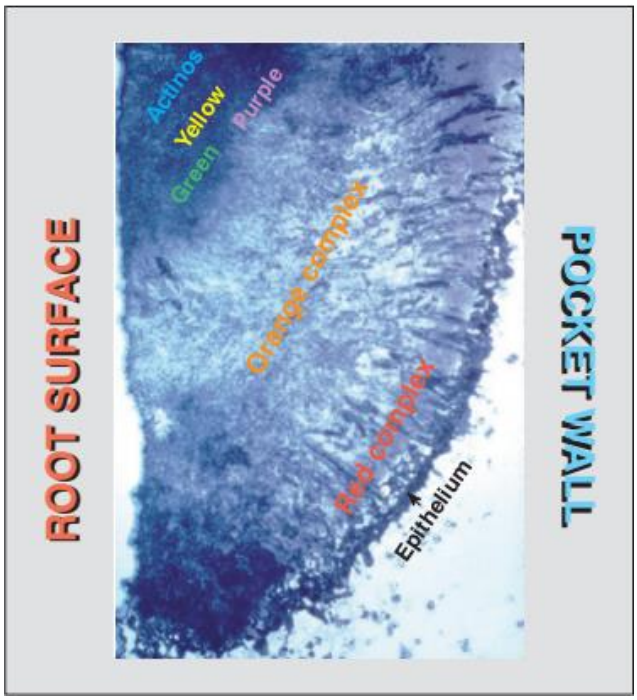

Figure 6: Relative location of the Complex bacteria between the tooth surface and epithelium in a periodontal pocket (Socransky and Haffajee 2005).