Bacterial Characterization in Health and Periodontal Diseases During Induced Gingival Inflammation

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ABSTRACT

ANDRE PAES BATISTA DA SILVA: Bacterial Characterization in Health and Periodontal Diseases During Induced Gingival Inflammation in Man

(Under the direction of Steven Offenbacher)

Objective: to characterize the plaque bacteria in subjects enrolled in five biofilm-gingival interface (BGI) groups, and to determine whether re-institution of oral hygiene regimen would lead to a change in the biofilm composition. **Methods:** participants from each of the five BGI groups abstained from brushing and flossing teeth in two posterior sextants during a three week period. Participants reinstated normal oral hygiene (Day 21), and were followed for four weeks. Clinical parameters were recorded, and subgingival plaque samples were analyzed by the Human Oral Microbe Identification Microarray (HOMIM). **Results:** at baseline *Synergystetes* were more abundant in BGI-P2 and BGI-P3 than BGI- P1 and -G ($p \leq 0.05$). Overall, at the peak of induction there was an increase in levels of Firmicutes ($p \leq 0.001$), Fusobacteria ($p=0.003$), Proteobacteria ($p \leq 0.001$), Synergistetes ($p=0.04$), and Bacteroidetes ($p \leq 0.001$). At the resolution phase, the Synergistetes did not rebound. When analyzing each BGI group, plaque induced inflammation prompted distinguished changes in subgingival biofilm composition in most of the BGI groups except BGI-P3. Removal of plaque significantly reduced to baseline levels most bacteria in all BGI groups except a few bacterial phylum, such as *Sinergistetes* in BGI-P1, which were also detected in increased levels in the high pocket depth responders. **Conclusions:** it is indicated that the Synergistetes phylum may be highly involved in the progression of periodontal disease.

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BGI=biofilm gingival interface

BMI=body mass index

BOP=bleeding on probing

GCF=gingival crevicular fluid

HOMIM=human oral microbe identification microarray

PCR=polymerase chain reaction

PD=probing depth

PMN’s=polymorphonuclear leukocytes

SD=standard deviation

Chapter 1. Introduction

Periodontal diseases are a heterogeneous group of conditions that reflects a cellular inflammatory response of the gingiva and surrounding connective tissue to the bacterial accumulations on teeth (Armitage et al, 1999; Loesche et al, 2007; Kornman et al, 2008). The classification of periodontal diseases remains based solely on history and clinical signs of disease. The current classification system (Armitage, 2002) does not characterize biologic systems. This leads to variation in prognosis and treatment results. Offenbacher (2007) characterized the biology of the biofilm–gingival interface (BGI). The authors identified new clinical categories that represented distinct biologic phenotypes based upon DNA checkerboard analyses, serum immunoglobulin G titers to plaque bacteria, and the gingival crevicular fluid (GCF) levels of inflammatory mediators. Five BGI clinical conditions were defined using probing depths (PDs) and bleeding on probing (BOP) scores.

Although many organisms are present in the subgingival biofilm, interestingly, the putative pathogens associated with gingivitis and periodontitis may comprise very small fractions of the total biomass (Benakanakere and Kinane, 2011). Previous studies indicated that there are several key bacterial species that play a role in the disease process, and which have been grouped into microbial complexes based on clinical parameters, the disease stage, and the site in the oral cavity (Socransky et al, 1998). Investigation of subgingival biofilm based of 16S rDNA cloning and sequencing showed 40% of bacterial species present to be novel species or phylotypes (Kumar et al, 2003).

Advances in molecular techniques have given rise to a much greater understanding of the diversity and complexity of human microbiota communities. Paster and Dewhirst have developed a molecular technique to detect the oral biofilm using a 16S rRNA-based microarray technology known as the human oral microbe identification microarray (HOMIM). HOMIM provides information on the nine most common bacterial phyla found in the oral cavity, including: *Bacteriodetes, Firmicutes, Proteobacteria, Synergistetes, Fusobacteria, Spirochaetes, Actinobacteria, SR-1*, and *TM-7* (Colombo et al, 2009). Ahn et al (2011), when comparing the oral microbiome community profiles assessed by the broad 16S rRNA pyrosequencing and custom 16S rRNA hybridization (HOMIM), showed that they were highly correlated at the phylum level and genus level

In this study we propose to use an induced gingivitis model as consequence of biofilm overgrowth (Löe et al, 1965) in healthy individuals, and induced inflammation in gingivitis and periodontitis patients. This inflammatory response is reversed during a resolution phase. In a recent study in our laboratory (Offenbacher et al, 2009), we have investigated changes in the patterns of whole-transcriptome gene expression that occur during the induction and resolution of experimental gingivitis in humans, and we showed that a small subset of the immune response genes analyzed was transiently activated in response to biofilm overgrowth, suggesting a degree of specificity in the transcriptome-expression response. Therefore, the specific bacteria of all five phenotypes (Offenbacher et al, 2007) should be indentified in order to further understand the mechanism by which biofilm is influencing gene expression.

Chapter 2. Background

Periodontal diseases – definition and etiology

Periodontal diseases are a heterogeneous group of conditions that reflects a cellular inflammatory response of the gingiva and surrounding connective tissue to the bacterial accumulations on teeth (Armitage et al, 1999; Loesche et al, 2007; Kornman et al, 2008). These diseases are broadly grouped into two major categories: gingivitis and periodontitis. Gingivitis is reversible and limited to superficial, gingival tissues, whereas periodontitis is non-reversible and features tissue destruction extending to also supporting tissues such as periodontal ligament and alveolar bone. Data derived from animal models indicate that gingivitis may work as a precursor to periodontitis (Heijl et al, 1976); however, human population studies clearly demonstrate that not all individuals with gingivitis linearly proceed to periodontitis (Socransky et al, 1984; Löe et al, 1986). Offenbacher (2007) characterized the biology of the biofilm–gingival interface (BGI). The authors identified new clinical categories that represented distinct biologic phenotypes based upon DNA checkerboard analyses, serum immunoglobulin G titers to plaque bacteria, and the gingival crevicular fluid (GCF) levels of inflammatory mediators. Five BGI clinical conditions were defined using probing depths (PDs) and bleeding on probing (BOP) scores.

Several studies have concluded that the etiology of periodontitis is complex (Socransky *et al.*, 1998; Paster *et al.*, 2001, 2006; Socransky and Haffajee, 2005; Ledder *et al.*, 2007). Although many organisms are present in the subgingival biofilm, interestingly, the putative pathogens associated with gingivitis and periodontitis may comprise very small fractions of the

total biomass (Benakanakere and Kinane, 2011). Previous studies indicated that there are several key bacterial species that play a role in the disease process, and which have been grouped into microbial complexes based on clinical parameters, the disease stage, and the site in the oral cavity (Socransky et al, 1998).

Methods of identification of bacteria

Biofilm was originally identified by dark field microscopy (Listgarten, 1978), which consisted of light entering a field obliquely, microorganisms are illuminated and glow against background. Dark field microscopy is easy to use, a low cost method and it can detect bacterial shape, size and motility. However, it is limited to about 10 morphotypes of bacteria and species cannot be distinguished. The culture technique (Tanner, 1979) is another method of bacterial identification and it consists of collecting dental plaque and cultivated in selective or non-selective medium. It is the only current method capable of determining in vitro antimicrobial susceptibility of periodontal pathogens. It identifies only live bacteria and it has been extensively used to detect *P.gingivalis* and *A. actinomicetecomitans* However, it is an expensive method, requires experience, the various methods to disperse plaque can favor growth of 1 specie, and there is no culture method that can recognize all bacteria. Immunodiagnostic methods, such as indirect immunofluorescence, flowcytometry, ELISA, use antibodies that recognizes specific bacterial antigen to detect target microorganisms. These methods do not require viable bacteria; less susceptible to variation in sample processing; less time consuming and easier to perform than culture. However, accuracy depends greatly on the quality of reagents used, and poorer detection limit when comparing to nucleic acid probe and PCR assays (reviewed in Teles 2006).

There are three main categories of molecular microbial analyses: (1) polymerase chain reaction (PCR)-based methods, including single target PCR, multiplex PCR and quantitative PCR; (2) DNA-DNA hybridization methods, such as *in situ* hybridization, checkerboard hybridization and 16S ribosomal RNA-based microarrays; (3) sequencing methods, such as pysequencing, real-time single molecule DNA sequencing and nanopore-based sequencing (reviewed in Paster and Dewhirst, 2009). The conventional polymerase chain reaction is a specific and reasonably sensitive method; it provides presence or absence of data rather than levels or proportions. Quantitative PCR overcome the last concern, but number of samples and species that can be conveniently processed is limited. The DNA probe techniques (whole genomic DNA, 16S rRNA genes) entails segments of single-stranded nucleic acid, labeled with an enzyme or radioisotope, that can locate and bind to their complementary nucleic acid sequences with low cross-reactivity to non-target microorganisms. The whole genomic probes are more likely to cross-react with non-target organisms due to the presence of homologous sequences between different bacterial species. They have a high sensitivity and the ability to evaluate a wide range of species. The reverse capture hybridization methods, presence/absence data only, but can be extended to a very wide range of species. In the direct hybridization formats, DNA probes offer the advantage of quantifying the sample.

Human Oral Microbe Identification Microarray (HOMIM)

In their laboratory, Paster and Dewhirst have developed a molecular technique to detect the oral biofilm using a 16S rRNA-based microarray technology known as the HOMIM. It is a custom-designed, 16S rRNA-based oligonucleotide reverse capture microarray. A total of 421

probes, representing roughly 300 of the most predominant oral bacterial species initially identified from Sanger sequencing (http://mim.forsyth.org/). The probes are arranged phylogenetically and in replicate on each aldehyde-coated glass slide. HOMIM provides information on the nine most common bacterial phyla found in the oral cavity, including: *Bacteriodetes, Firmicutes, Proteobacteria, Synergistetes, Fusobacteria, Spirochaetes, Actinobacteria, SR-1*, and *TM-7* (Colombo et al,2009). Each array has a total of 24 cluster probes targeting more than two closely related species in addition to multiple positive and negative controls. The lower limit of detection is $>10^4$ bacterial cells.

The HOMIM method has been extensively validated by the following reports: investigating the diversity and site-specificity of the oral microflora in elderly; the microflora of root caries in elderly; microbial profiles of refractory periodontitis, severe periodontitis and periodontal health; specified bacterial species in the crevicular fluid; bacterial signature associate with poor oral health; association of mode of birth delivery and the oral microbiota in infants; microbiota of severe early childhood caries before and after therapy; alterations in diversity of the oral microbiome in pediatric inflammatory bowel disease; the association of variations of oral microbiota and with pancreatic diseases; new approaches for isolation of previously uncultivated species; correlation network analysis applied to complex biofilm communities; the subgingival microbiota of Papillon-Lefèvre Syndrome (Preza et al, 2009a; Preza et al, 2009b Colombo et al, 2009; Asikainen et al, 2010; Lif Holgerson et al 2011; Tanner et al, 2011; Docktor et al 2011; Farrell et al, 2011; Sizova et al, 2011; Duran-Pinedo et al 2011; Albandar et al, 2011). Ahn and coworkers (2011), when comparing the oral microbiome community profiles assessed by the broad 16S rRNA pyrosequencing and custom 16S rRNA hybridization (HOMIM) showed that they were highly correlated at the phylum level and also at the genus

level. They indicated that both methods are currently suitable for high-throughput epidemiologic investigations relating identified and more common oral microbial taxa to disease risk

The oral biofilm diversity

The comparative analyses of small-subunit rRNA (16S or 18S rRNA) and other gene sequences show that life falls into three primary domains, *Bacteria*, *Eucarya*, and *Archaea* (Woese et al, 1990). It is estimated that >99% of microorganisms observable in nature typically are not cultivated by using standard techniques (Amann et al, 1995). Culture-dependent studies indicate that representatives of some bacterial phylum are cosmopolitan in the environment, whereas others appear restricted to certain habitats. Culture-independent studies so far conducted reflect and expand this view. The phylogenetic differences between the bacterial divisions probably are reflected in substantial physiological differences (reviewed in Hugenholtz et al, 1998).

Oral microbiota studies using high-throughput sequencing estimated the number of species-level phylotypes between 540 and about 10,000 (Keijser et al, 2008; Lazarevic et al, 2009). However, these figures were obtained using different sequencing coverage, sampling different anatomical sites and analyzing samples pooled from different number of individuals. Therefore, not all of the identified taxa are expected to be present in the same subject and at the same time (Paster et al, 2006). The core oral microbiome appears to consist of <1000 species-level taxa (Dewhirst *et al.*, 2010; Griffen *et al.*, 2011), although any number of species may appear transiently in a site so open to the environment.

The oral biofilm and disease

The predominant species from diseased sites are different from those found in healthy sites, although the putative pathogens can often be detected in low numbers at normal sites. Over 400 species were estimated to inhabit the disease-associated periodontal ecosystem, and approximately half of the taxa have not been cultivated (Paster et al, 2001). Early studies to elucidate differences between periodontal health and disease based on cultivation were extremely difficult to perform and yielded confusing and contradictory results. Cultural and DNA probe analysis detected distinguished bacteria in health, gingivitis, and initial periodontitis (Tanner et al, 1998). Targeted approaches such as DNA hybridization and PCR-based assays provided more power to track variation in levels of individual species, but did not provide a comprehensive view of communities. Using whole-genomic hybridization, Socransky et al (1998) delineated a set of three species showing strong associations with periodontal disease and with each other: *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*. Subsequently, this list of potential pathogens was expanded using species-specific 16S primers for PCR amplification (Kumar et al, 2003). Associations with chronic periodontitis were observed for several new phylotypes, including uncultivated clones D084 and BH017 from the Deferribacteres phylum, AU126 from the Bacteroidetes phylum, Megasphaera clone BB166, clone X112 from the OP11 phylum, clone I025 from the TM7 phylum, and the named species *Eubacterium saphenum, Porphyromonas endodontalis, Prevotella denticola*, and *Cryptobacterium curtum*. Species more prevalent in periodontal health included two uncultivated phylotypes, clone W090 from the *Deferribacteres* phylum and clone BU063 from the *Bacteroidetes*, and named species *Atopobium rimae* and *Atopobium parvulum*.

Substantial microbial diversity has been observed among different sites in the oral cavity and/or people (Mager et al, 2003; Socransky & Haffajee, 2005). Aas and coworkers (2005) identified bacteria in five healthy subjects using 16S rRNA cloning and sequencing techniques from samples of various oral sites, including subgingival plaque. Overall, bacterial species representing six different bacterial phyla were detected, of which over 60% have not been cultivated. The six phyla included the *Firmicutes* (e.g. *Streptococcus*, *Gemella*, *Eubacterium*, *Selenomonas*, *Veillonella*), the *Actinobacteria* (e.g. *Actinomyces*, *Atopobium*, *Rothia*), the *Proteobacteria* (e.g., species of *Neisseria*, *Eikenella*, *Campylobacter)*, the *Bacteroidetes* (e.g. *Porphyromonas*, *Prevotella*, *Capnocytophaga*), the *Fusobacteria* (e.g. *Fusobacterium* and *Leptotrichia*), and the TM7 phylum, for which there are no cultivable representatives. In subgingival plaque, several species of *Streptococcus* and *Gemella* were often found. Many species specifically associated with periodontal disease, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, were not detected in any sites tested. Furthermore, Bik and coworkers (2010) determined the composition of the oral microbiota from 10 individuals with healthy oral tissues by using the 16S r-RNA gene amplification, and clone libraries. They found that 20% of their sequences belonged to the genus *Streptococcus*. In agreement with the previous study (Aas et al, 2005) the *Streptococcus, Gemella, Abiotrophia, Granulicatella, Rothia, Neisseria,* and *Prevotella* were the most predominant bacterial genera in the oral cavity, but, in addition, they found many Proteobacteria (e.g., *Haemophilus, Lautropia*) to be abundant. A bacterial species previously shown to be associated with periodontal health (*Veillonella parvula*, *Veillonella* X042) (Kumar et al, 2005) was found in all specimens in this study, and was the third most abundant OTU in their combined sequence dataset. Despite the evidence for a conserved healthy oral community at the genus level in all 10 healthy mouths,

their study confirms results by Nasidze et al suggesting high variability in the oral microbiome between individuals, although in the latter study, saliva was the only specimen type examined (Nasidze *et al.*, 2009). Moreover, in agreement with other studies (Ximenez-Fyvie et al, 2000) it was identified members of the "red complex" in healthy mouths. However, it was identified in low numbers and limited to three subjects.

The 16S rRNA gene amplification and sequencing has resulted in a major advance. However, studies using cloning and Sanger sequencing (Kumar et al, 2005) suffered from the superficiality of the sequencing effort; it wasn't economically possible to obtain enough sequence information to examine the complexity of the system. The advent of 454 pyrosequencing of 16S rRNA genes has allowed the collection of thousands of sequences per sample (Keijser *et al.*, 2008; Zaura *et al.*, 2009), and provides the power to comprehensively study bacterial community composition at the level of species. Lazarevic and coworkers (2010) analyzed the salivary microbiota from five adults at three time-points by means of the 454 pyrosequencing technology. Samples were dominated by seven major phyla: members of *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes* and candidate division *TM7* were identified in all samples; *Fusobacteria* and *Spirochaetes* were identified in all individuals, but not at all time-points. Among individual taxa, phylum Bacteroidetes and order Clostridiales (Firmicutes) were the best indicators of intraindividual similarity of the salivary flora over time. Another study using the pyrosequencing of 16S rRNA genes from the oral buccal mucosa, Contreras and coworkers (2010), showed that the oral mucosa was highly dominated by four phyla: *Firmicutes* (mostly the genera *Streptococcus* and *Veillonella*), *Proteobacteria* (mostly *Neisseria*), *Bacterioidetes* (*Prevotella*) and *Actinobacteria* (*Micrococcineae*). This population carried unidentified members of the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* and

their microbiota included soil bacteria Gp1 (*Acidobacteriaceae*) and *Xylanibacter* (*Prevotellaceae*), and the rare genus *Phocoenobacter* (*Pasteurellaceae*).

Griffen and coworkers (2011) used 454 sequencing of 16S rRNA genes to compare subgingival bacteria from periodontally healthy and chronic periodontitis subjects. Differences between health- and periodontitis-associated bacterial communities were observed at all phylogenetic levels, and distinct community profiles was showed in health and disease. *Spirochaetes, Synergistetes* and *Bacteroidetes* were more abundant in disease, whereas the *Proteobacteria* were found at higher levels in healthy controls. Within the phylum *Firmicutes*, the class *Bacilli* was health-associated, whereas the *Clostridia*, *Negativicutes* and *Erysipelotrichia* were associated with disease. The authors indicated that in contrast to an earlier view that the oral microbiome consists of large numbers of uncultivated species (Paster et al, 2001), the majority of sequences could be mapped to cultivated species (with a 98% identity threshold). Recently, using pyrosequencing of the 16S rDNA genes, Huang and co-workers (2011) determined the bacterial taxa in three healthy subjects and three with gingivitis. Eight predominant taxa were found associated with gingivitis: *TM7, Leptotrichia, Selenomonas, Streptococcus, Veillonella, Prevotella, Lautropia,* and *Haemophilus.*

It has been reported that approximately 35% of the species present in subgingival biofilms are as yet uncultivated, so their role in periodontal pathogenesis is unknown (Teles et al, 2010). Furthermore, in a recent study it was detected higher levels and proportions of Archaea in the subgingival biofilm of generalized aggressive periodontitis when compared to healthy individuals (Matarazzo et al, 2011).

The experimental gingivitis model

The experimental gingivitis model is extensively used to study the gingival events and microbiology that occur during a bacterially induced change from a state of health to inflammation as it provides a well-controlled environment to study the pathogenesis and treatment of gingivitis. This model was first described by Loe (1965) and Theilade (1966) who induced gingivitis in healthy gingivae patients by withdrawing oral hygiene and identified the sequence of changes in the microbial flora detected by microscopy. Briefly, after assessment of plaque and gingivitis on day zero the participants were told to abstain from all mechanical plaque control measures for approximately 3-week experimental period. Mechanical oral hygiene measures were reinstituted for approximately 10 days when the generalized gingivitis subsided. The biofilm composition changed from mainly gram + cocci and short rods at baseline to a biofilm consisted of 50% gram + cocci and short rods, 30% gram-negative cocci and small rods, 8% filaments, 8% fusobacteria, and 4% spirilla and spirochaetes. Loesche (1978) isolated the plaque flora from dentogingival sites during a human gingivitis experiment and they showed that *Actinomyces viscosus* and *Bacteroides melaninogenicus* increased significantly when the bleeding gingivitis developed. Their findings raised the possibility that proportional changes in the gingival plaque flora may uniquely contribute to the development of gingival inflammation in this experimental model. More recently, Burrell and Walters (2008) adapted the experimental gingivitis model to include the use of intraoral stents that cover only selected teeth to be worn only during routine oral hygiene to permit the study of localized changes in biofilm overgrowth and inflammation. By using localized stents unilaterally the effects of contralateral biofilm maintenance can be compared within individuals to study localized biofilm overgrowth and

cross-arch differences in GCF inflammatory response as a way to adjust for site-specific changes that determine clinical and inflammatory mediator status within an individual.

The effect of oral hygiene on the oral biofilm

In the literature there are a number of studies that have looked the effect of surgical and non-surgical periodontal therapy in the prevalence and abundance of oral biofilms. However, fewer studies have addressed the effect of mechanical removal of plaque in reducing supra and subgingival biofilm. In this section of the thesis we presented a couple studies investigating the effect of the presence and absence of oral hygiene on the oral microbiota.

In a study using reverse-capture checkerboard hybridization, Corby and coworkers (2008) assessed the effects of dental flossing on the microbial composition of interproximal plaque samples in matched twins. Fifty-one twin pairs were randomized to a 2-week supervised and unsupervised treatment regimen consisting of tongue brushing and toothbrushing or tongue brushing and toothbrushing plus flossing. After the 2-week study period, putative periodontal pathogens and cariogenic bacteria were overabundant in the group that did not floss compared to the group that performed flossing. Those included *Treponema denticola*, *Porphyromonas gingivalis*, *Tannerella forsythia* (previously *T. forsythensis*), *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, and *Streptococcus mutans*. In a more recent study Uzel and coworkers (2011) monitored microbial shifts during dental biofilm re-development. Supra- and subgingival plaque samples were taken separately from healthy and periodontitis subjects at baseline and immediately after tooth cleaning. Samples were taken again during 1, 2,

4 and 7 days of no oral hygiene and analysed using checkerboard DNA-DNA hybridization. Supragingival counts of *Veillonella parvula*, *Fusobacterium nucleatum ss vincentii* and *Neisseria* mucosa increased from 2 to 7 days. Subgingival counts were greater for *Actinomyces*, green and orange complex species. Significant differences between groups in supragingival counts occurred for 17 of 41 species at entry. Supragingival plaque re-development was similar in periodontitis and health, but subgingival species recolonization was more marked in periodontitis.

Chapter 3. Material and Methods

Study Design

This prospective cohort study involved the induction of experimental gingivitis in volunteers as originally described by Löe and coworkers (1965). Between 33 to 37 participants were enrolled in each of the following five groups (for a total enrollment of 175 participants); BGI-H (all PD<3mm, BOP<10%) , BGI-G (all PD<3mm, BOP≥10%), BGI-P1 (1+ site with PD>3mm, BOP≤10%), BGI-P2 (1+ site with PD>3mm, BOP>10% but BOP≤50%), and BGI-P3 (1+ site with PD>3mm, BOP>50%). These clinical groups have been shown to reflect different biological phenotypes. The BGI-P1 group is similar to a treated and stable periodontitis recall patient whereas, BGI-P2 and BGI-P3 represent more diseased periodontal conditions. After two weeks from the prophylaxis, participants abstained from brushing and flossing teeth in one maxillary and mandibular posterior sextant during a three week period, no hygiene phase via placement of acrylic stents. The sextants selected were the right maxillary and mandibular sextants, except when fewer than four teeth were present in one posterior sextant. If there are fewer than 4 teeth in a right posterior sextant then the opposing posterior sextant was used for the stent placement. After the induction of experimental gingivitis (21 days), participants reinstated normal full mouth oral hygiene and daily plaque control. Participants were followed for four weeks during gingivitis resolution. Prophylaxis with scaling and root planing were performed at

exit. The study was a single masked study. All laboratory measurements were performed without knowledge of the participants' periodontal status.

Inclusion Criteria

-Adult males or females between the ages of 18 and 75 years (inclusive).

-Participants who were able and willing to follow study procedures and instructions.

-Participants who have read, understood and signed an informed consent form.

-Participants who presented with at least 4 teeth in the functional dentition with a minimal of 3 adjacent teeth with interproximal papilla in each posterior sextant that will have the stent.

-Participants who were in good general health.

-Participants who presented with one of the following five categories to be considered for enrollment:

(1)BGI health (all PD<3mm, BOP<10%); (2) BGI-gingivitis (all PD<3mm, BOP≥10%); (3) BGI-P1 (1+ site with PD>3mm, BOP≤10%); (4) BGI-P2 (1+ site with PD>3mm, BOP>10% but BOP≤50%); (5)BGI-P3 (1+ site with PD>3mm, BOP>50%

Exclusion Criteria

-Individuals who have a chronic disease with oral manifestations and/or exhibit gross oral pathology.

-Individuals receiving treatment for periodontal disease

-Treatment with antibiotics for medical or dental condition within 1 month prior to the examination.

-Chronic treatment (i.e., two weeks or more) with any medication known to affect periodontal status (e.g., phenytoin, calcium antagonists, cyclosporin, anticoagulants, non-steroidal anti-inflammatory drugs, high dose aspirin) within one month of the screening examination.

-Ongoing medications initiated less than three months prior to enrollment (i.e., medications for chronic medical conditions must be initiated at least three months prior to enrollment).

-Participants with clinically significant organ disease including impaired renal function, heart murmur, history of rheumatic fever or valvular disease, or any bleeding disorder.

-Participants with active infectious diseases such as hepatitis, HIV or tuberculosis.

-Severe unrestored caries, or any condition that is likely to require antibiotic treatment during the study, including the need for prophylactic antibiotic.

-Individuals who were pregnant, or who were expecting to become pregnant within the next three months and individuals nursing.

Continuance Criterion

-Participants were excluded from the study or analysis if any of the following conditions applied:

-Changes in the participant's medical status or medications that is not negligible.

-Use of antibacterial rinses (e.g. chlorhexidine, Listerine®, or hydrogen peroxide products).

-Use of non-study dentifrices, toothbrush or dental floss (e.g., triclosan or 0.454% stannous fluoride products) during the no hygiene and resolution phases of the study, or any irrigation device.

-Participant's inability or noncompliance to wear their stents or shields over one mandibular and one maxillary sextant during daily brushing procedures.

-Use of oral antibiotics and non-steroidal anti-inflammatory drugs were not permitted during the trial. (Participants requiring treatment for an acute medical or dental condition during the study were withdrawn from the investigation).

Clinical Periodontal Assessments

Clinical examiners were calibrated prior to commencement of the study for training of study procedures and for documentation of acceptable intra- and inter-examiner measurement reliability. Clinical parameters included the Silness and Löe Plaque Index, Löe and Silness Gingival Index, pocket depth, clinical attachment level and bleeding on probing. Clinical parameters were measured using a manual University of North Carolina (UNC-15) periodontal probe on Day 0 (Baseline), Day 21 (Peak) and Day 49 (Resolution). These parameters were measured at six periodontal sites per tooth for all teeth. The non-stent sextants were used as control sextants during data analysis.

Laboratory Assessments

Subgingival Sample Taking

Subgingival plaque samples were collected on days 0, 21 and 49. Four samples per participant were collected from the mesial surface of the first molars using sterile paper points and four samples were collected from the deepest pocket in each posterior sextant. If the deepest pocket is the same as the mesial of the first molar, two samples were taken from the same site, and the deepest pocket (HOMIM sample) will be obtained first. Each paper point were inserted in the periodontal sulcus/pocket, held in place for 20 seconds and immediately transferred from the periodontal pocket into a cryovial and snap frozen. Thus, there were two paper points per

site and 2 paper points in each cryovial. Samples were kept at -80C for bacterial microbiological RNA-probe analysis.

DNA extraction

DNA Isolation was performed using the Charge Switch® gDNA Mini Bacteria Kit (Catalog No. CS11301), Invitrogen™ http://www.invitrogen.com. Briefly, bacteria were lysed (lysis buffer), bound and then eluted DNA from the magnetic beads. Extracted DNA was stored at -80 prior to shipping to Forsyth for analysis. Quality control guidelines specify that each plaque sample contain at least 200 ng of genomic DNA with a 260/280 ratio of 1.6-1.8.

Identification and Quantification of Bacteria

Individual subgingival biofilm samples were analyzed quantitatively and qualitatively for the presence of bacteria using the 16S rRNA-based microarray method, the Human Oral Microbe Identification Microarray (HOMIM), provided by Forsyth Institute. This high sample-throughput technology allows the simultaneous detection over 300 key and predominant bacterial species, including the uncultivables.

HOMIM protocol

16S rRNA-based oligonucleotide probes are synthesized with a 5'-(C6)-amine modified base, eight spacer thymidines and 18 to 20-nucleotides comprising the target sequence. Oligos

are printed on Quantifoil QMT Aldehyde Slides using a GeneMachine OmniGrid Arrayer and covalently linked. 16S rDNA amplicons from isolated sample DNA are produced by PCR using universal bacterial forward and reverse primers and are labeled with Cy3-dCTP. Five samples are run in duplicate on a single slide. Hybridization is performed in TeleChem Hybridization cassettes at 55°C for 16 h. The fluorescent spots on microarrays are detected using a GenePix 4000B microarray scanner and results analyzed using GenePix Pro software.

The current version of the HOMIM microarrays contains 456 unique oligonucleotide probes printed in duplicate on an array that contains a total of 960 printed spots. Hybridization spot intensities of each array profile are first subject to background justification with a minimal signal-to-background ratio of 2. The array profile is then generated by taking the log (base 2) and mean value for all the 456 duplicated probe signals. Between-the-array normalization is then done based on the signal intensities of the 16S universal probe available in each profile. Spot intensities are adjusted so that the level of the universal probe signals are equal in all profiles. Array profiles of the same sample are then combined to an average sample profile. The spot intensities of the sample profiles are then converted to one of the 6 integer signal levels ranging from 0 to 5, with 5 being the maximal intensity among all the profiles being compared. The integer profiles are then subject to cluster analysis. Pair-wise correlation coefficient distance matrix is computed and then used for building a hierarchical dendrogram based on the unweighted pair group method with arithmetic mean. The HOMIM online tool is in beta development stage and can be accessed online at http://bioinformatics.forsyth.org/homim.

Statistical Analysis

Statistical analyses and data management were performed using SAS (version 9.1.3, SAS Institute, Inc., Cary, NC, USA), statistical significance was set at $p < 0.05$, and the unit of analysis was the person. Frequency functions, and standard distributions, means, empirical distribution errors were determined to describe the data. Bivariate relationships were investigated using *t*-tests for continuous variables and Cochran Mantel-Haenszel chi-squared statistics and odds ratios and 95% confidence intervals (CI) for differences between categorical variables. Analysis of Variance was performed using SAS Proc GLM when the five BGI groups were compared.

Chapter 4. Results

Table 1. Baseline characteristics of all five BGI group subjects

	BGI-H	BGI-G	BGI-P1	BGI-P2	BGI-P3	p-value
Mean Age (StErr)	29.64(2.04)	30.28(2.1)	36.17(2.07)	36.75(2.1)	34.39(2.04)	0.051
Female **Male**	25(14.29%) 11(6.29%)	23(13.14%) 11(6.29%)	23(13.14%) 10(5.71%)	25(14.29%) 12(6.86%)	21(12%) 14(8%)	0.91
Mean BMI (StDev)	24.63(1.19)	27.60(1.23)	27.67(1.21)	29.86(1.23)	28.92(1.19)	0.033*
Caucasian **African American** **Other**	28(16.09%) 6(3.45%) 2(1.15%)	22(12.64%) 7(4.02%) 5(2.87%)	23(13.21%) 7(4.02%) 3(1.72%)	17(9.77%) 19(10.92%) 1(0.57%)	15(8.62%) 15(8.62%) 4(2.30%)	0.01*
Diabetic (Yes) **No**	0 36(20.57%)	3(1.71%) 31(17.71%)	1(0.57%) 32(18.29%)	1(0.57%) 36(20.57%)	2(1.14%) 33(18.86%)	0.4
Smoker (Yes) **No**	6(3.45) 30(17.24)	3(1.72) 31(17.82)	7(4.02) 26(14.94)	3(1.72) 34(19.54)	3(1.72) 31(17.82)	0.82

Continuous parameters were analyzed using Student's *t*-test. Categorical parameters were analyzed using the χ^2-test. *Statistically significant at $p<0.05$

Table 2. Baseline clinical parameters of all five BGI group subjects

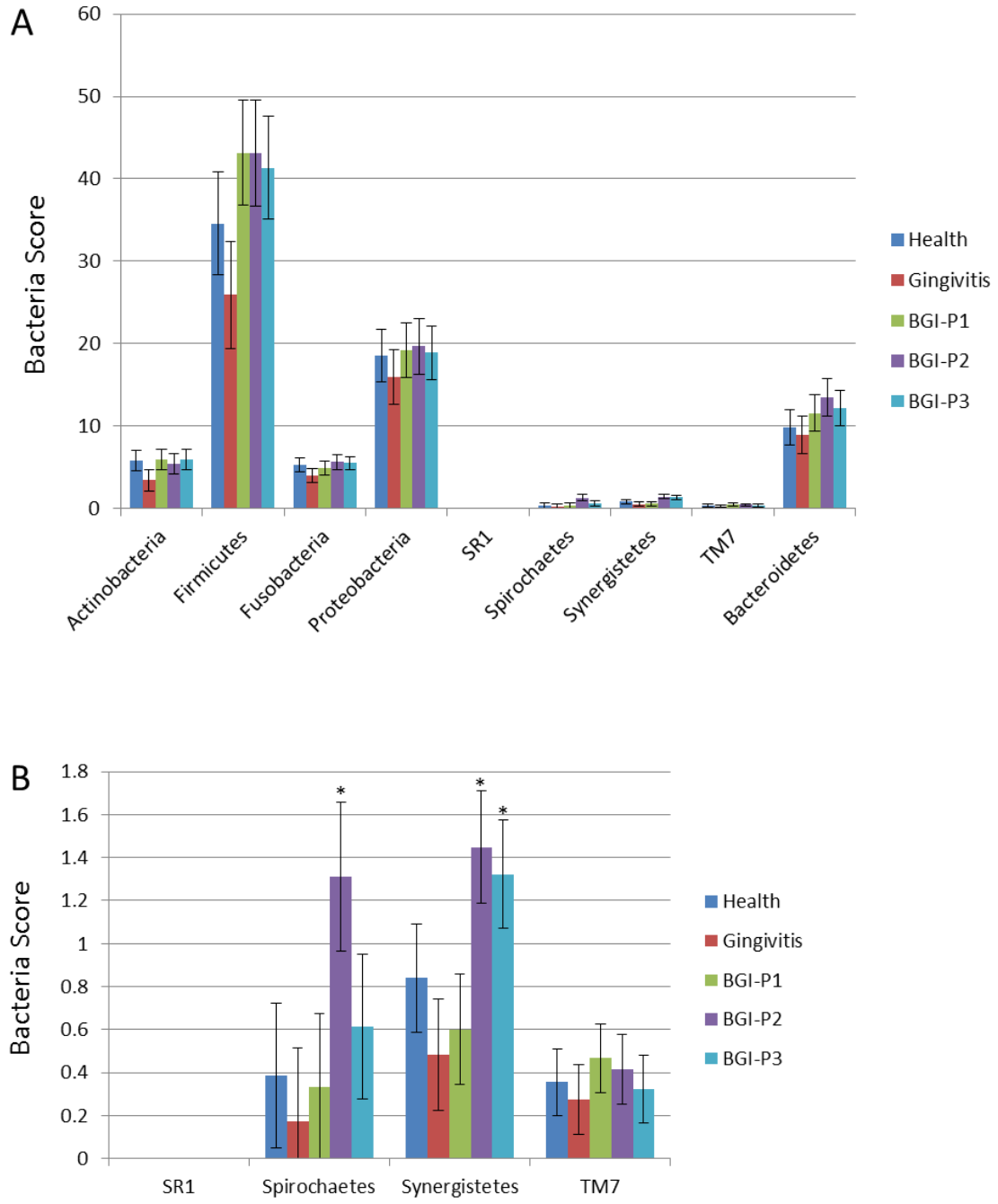
	BGI-H	BGI-G	BGI-P1	BGI-P2	BGI-P3	p-value
Mean Plaque score ≥1 (StErr)	47.7(3.7)	63(3.9)	52.2(4)	76.7(3.8)	79.2(3.9)	<0.0001*
Mean GI ≥1 (StErr)	65.7 (3.5)	74.7(3.7)	72.1(3.8)	94.3(3.6)	93.8(3.7)	<0.0001*
Mean BOP (StErr)	5.7(1.3)	22.6(1.4)	6.9(1.5)	30(1.4)	59.5(1.4)	<0.0001*
Mean AL≥3mm (StErr)	0.2(0.7)	1.3(0.8)	1.8(0.8)	2.3(0.8)	5.1(0.8)	<0.0001*
Mean PD≥4mm (StErr)	0	0	2.2(0.7)	4.1(0.6)	7.8(0.6)	<0.0001*

*Statistically significant at $p<0.0001$

A total of 299 subjects were screened for study eligibility; of these, one hundred and seventy five subjects (55%) met all study criteria. The mean age for participants did not differ significantly in between all five groups (p=0.051). However, the youngest individuals tended to be BGI (H) and the eldest were in BGI (P1 and P2) (Table 1). Sixty seven percent overall were female. Only subjects in BGI (H) were normal weight by body mass index (BMI<25kg/m^2), all the other BGI groups were overweight specially BGI (P2 and P3). Overall Caucasians were the most prevalent population. However in BGI (P2 and P3) African Americans were at increased numbers. Thirteen percent of the population was current cigarette smokers, and they were evenly distributed in all BGI groups. Only four percent of the population was diabetic and no significant difference was detected in their distribution in the BGI groups.

At baseline, plaque scores and BOP were significantly lower in BGI (H), and BGI (P1) than the other BGI groups. BGI (P2, P3) presented with significantly higher GI scores than BGI (P1) since this phenotype represents stable periodontits. As expected, BGI (P1,P2,P3) presented with increased mean AL≥3mm and PD≥4mm in comparison to BGI (H, G) (Table 2).

Figure 1. Baseline bacterial levels in all five BGI phenotypes



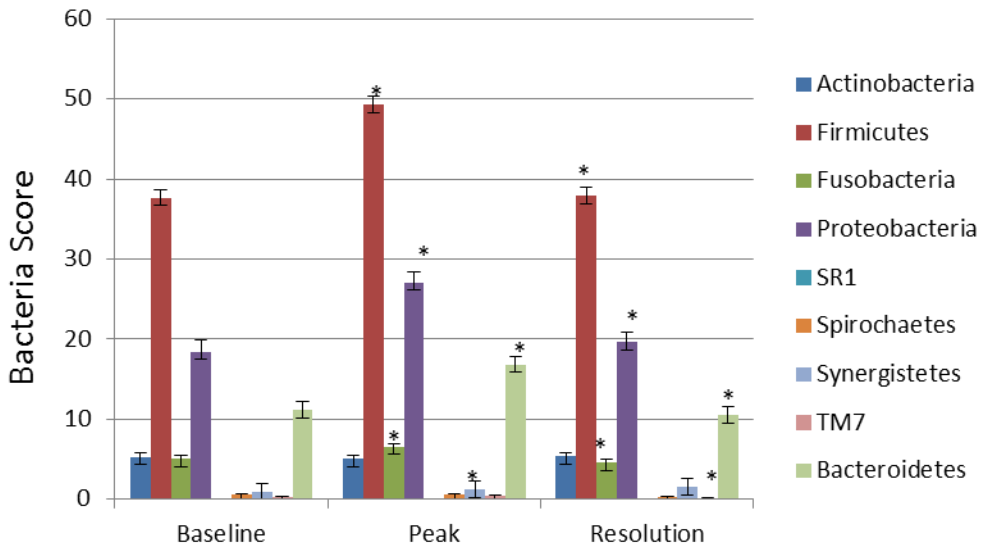
*Statistically significant at $p<0.05$

The "Bacteria Score" is a value that represents proportions of bacteria detected by HOMIM normalized to a positive control.

Bacterial levels detected by HOMIM were classified according to their phylum. The levels of bacteria were detected in all BGI groups. When comparing bacterial levels in each BGI group at baseline no significant differences were found except for the Spirochaetes and Synergistetes phylum (Figure 1A). At baseline the Spirochaetes phylum are detected in an increased level in the BGI-P2 group when compared to the BGI-P1 group (p=0.02) and BGI-G group (p=0.047). Also, the Synegystetes phylum are detected at increased levels in BGI-P3 when compared to BGI-P1 (p=0.047) and G (p=0.02). Furthermore, the BGI-P2 groups presented with increased levels of Synergistetes when compared to BGI-P1 (p=0.02) and BGI-G (p=0.01) (Figure 1B).

Figure 2. Bacterial phylum levels in all five BGI groups (baseline-peak-resolution)

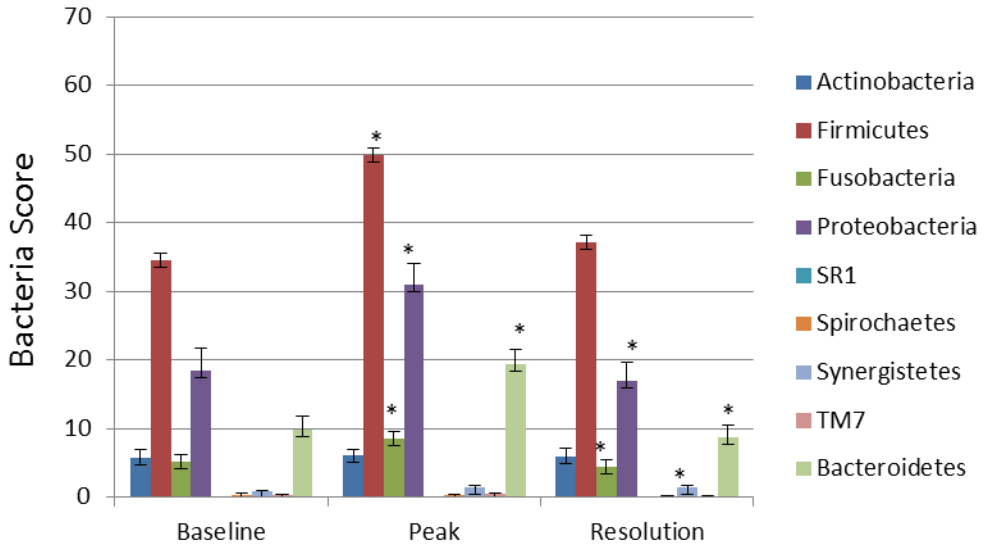
A. Overall



*Statistically significant at p<0.05

All the BGI groups were added together to be analyzed as an "overall" population. At the peak of induction a significant increase from the baseline is observed in the Firmicutes ($p \leq 0.001$), Fusobacteria ($p=0.003$), Proteobacteria ($p \leq 0.001$), Synergistetes ($p=0.04$), and Bacteroidetes ($p \leq 0.001$). At the resolution phase significant decrease to baseline levels were detected in the Firmicutes ($p=0.0038$), Fusobacteria ($p \leq 0.001$), Proteobacteria ($p \leq 0.001$), Bacteroidetes phylum ($p \leq 0.001$). The TM7 phylum had a significant decrease with oral hygiene ($p \leq 0.001$). However, in the Synergistetes phylum no changes were detected (Figure 2A).

B. BGI-Health

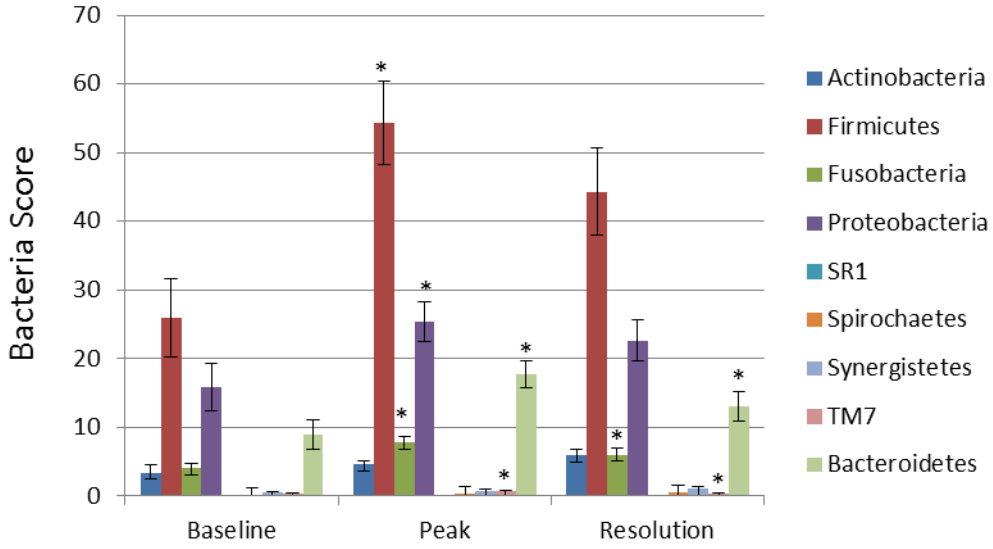


*Statistically significant at $p<0.05$

In the BGI-Health group, at the peak of induction a significant increase from the baseline is observed in the *Firmicutes* ($p=0.035$), *Fusobacteria* ($p=0.0072$), *Proteobacteria* ($p=0.0088$), and *Bacteroidetes* ($p=0.0053$). At the resolution phase significant decrease to baseline levels were detected in *Fusobacteria* ($p \leq 0.001$), *Proteobacteria* ($p \leq 0.001$), *Bacteroidetes* phylum

(p≤0.001). The TM7 phylum had a significant decrease with oral hygiene (p≤0.001). However, in the *Firmicutes* phylum no significant changes were detected in comparison to their level to the peak of induction (Figure 2B).

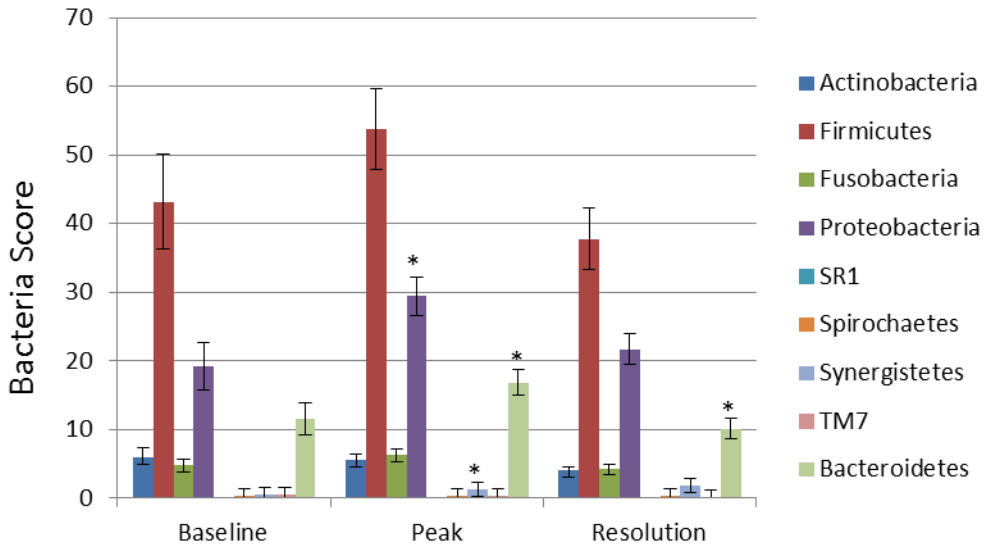
C. BGI-Gingivitis



*Statistically significant at p<0.05

In the BGI-Gingivitis group at the peak of induction a significant increase from the baseline is observed in the *Firmicutes* (p=0.0019), *Fusobacteria* (p=0.0017), *Proteobacteria* (p=0.034), TM7 (p=0.016) and *Bacteroidetes* (p=0.0053). At the resolution phase significant decrease were detected in *Fusobacteria* (p=0.037), *TM7* (p=0.047), and *Bacteroidetes* phylum (p=0.041). However, in the *Firmicutes* and the *Proteobacteria* phylum no significant changes were detected (Figure 2C).

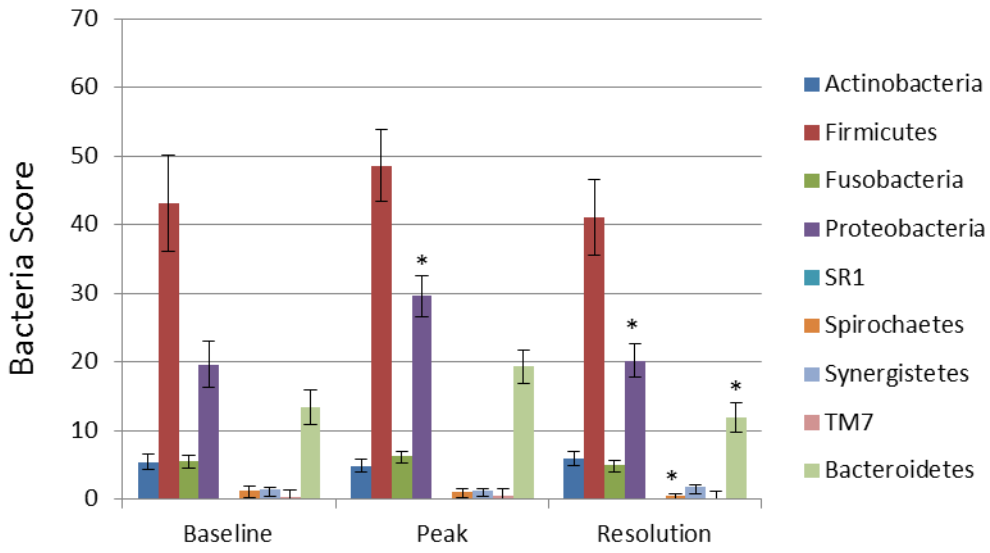
D. BGI-P1



*Statistically significant at $p<0.05$

In the BGI-P1 group, at the peak of induction a significant increase from the baseline is observed in the *Proteobacteria* ($p=0.014$), *Synergistetes* ($p=0.0045$), and the *Bacteroidetes* ($p=0.028$). At the resolution phase significant decrease were detected in *Bacteroidetes* ($p=0.0052$). Also, the *TM7* phylum had a significant decrease with oral hygiene ($p\leq0.001$). However, in the *Proteobacteria* and *Synergistetes* phylum no significant changes were detected. (Figure 2D).

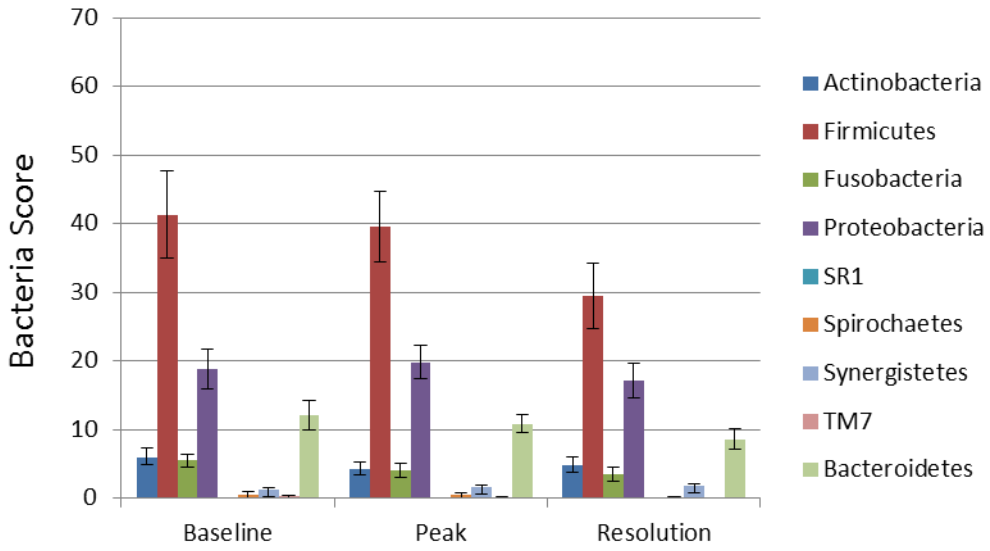
E. BGI-P2



*Statistically significant at $p<0.05$

In the BGI-P2 group, at the peak of induction a significant increase from the baseline is observed in the *Proteobacteria* phylum (p=0.039), which was significantly decreased (p=0.0094) at the resolution phase. Also, a decrease was detected in the *Bacteroidetes* (p=0.0052) and *Spirochaetes* (p=0.043) phylum at the resolution phase (Figure 2E).

F. BGI-P3



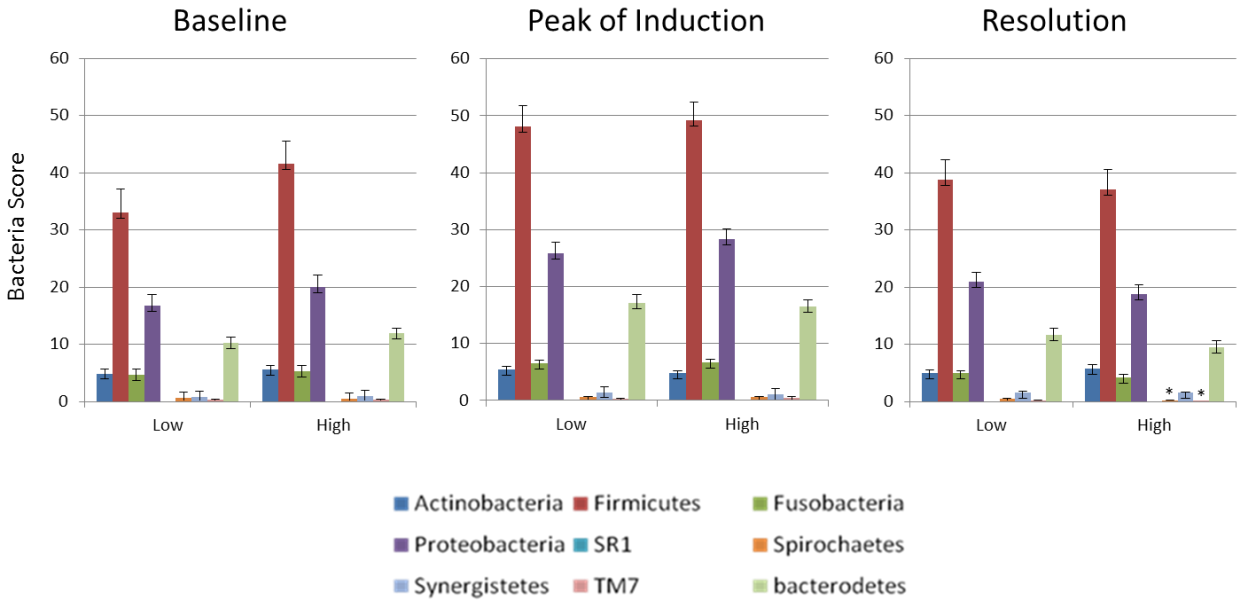
*Statistically significant at $p<0.05$

In the BGI-P3 group, at the peak of induction no changes from the baseline were detected. Also, despite the lower levels, no significant changes were detected in the resolution phase (Figure 2F).

Figure 3. The association of bacterial changes and clinical parameters (plaque, BOP, PD)

The median probing depth, pocket depth, BOP, and plaque score were calculated and the sample was classified as high or low phenotype. Therefore, a high phenotype to a certain clinical parameter was an individual who had high score throughout the period of the experiment.

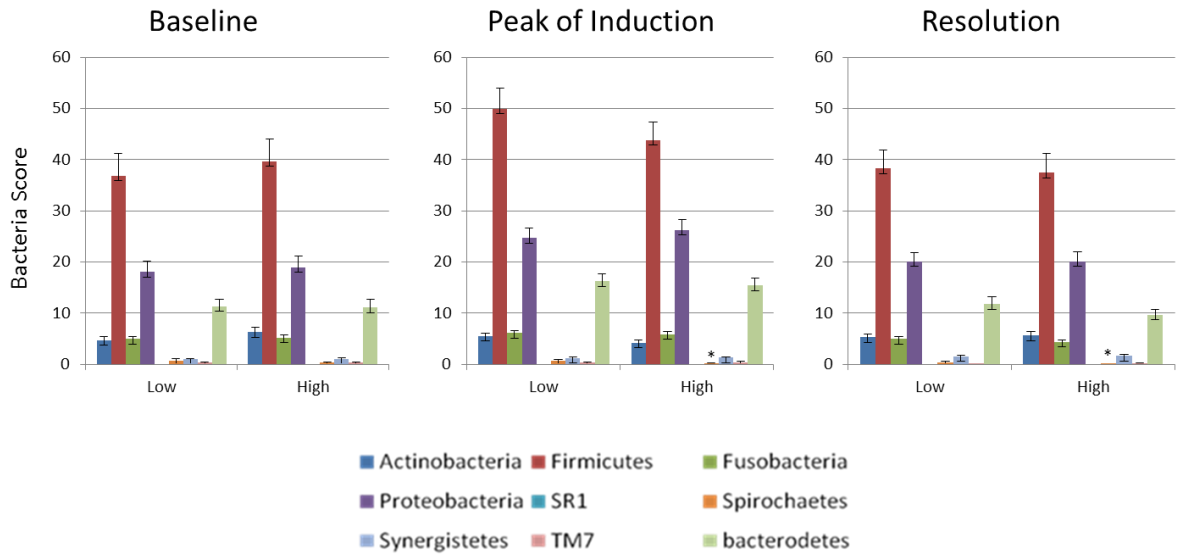
A. Plaque



*Statistically significant at $p<0.05$

At baseline and peak of induction, no significant difference was detected in between low and high plaque phenotype in the levels of all nine bacteria phylum. However, at the resolution Spirochaetes ($p=0.009$) and TM7 ($p=0.009$) presents at decreased levels in the high plaque phenotype in comparison to the low plaque responders (Figure 3A).

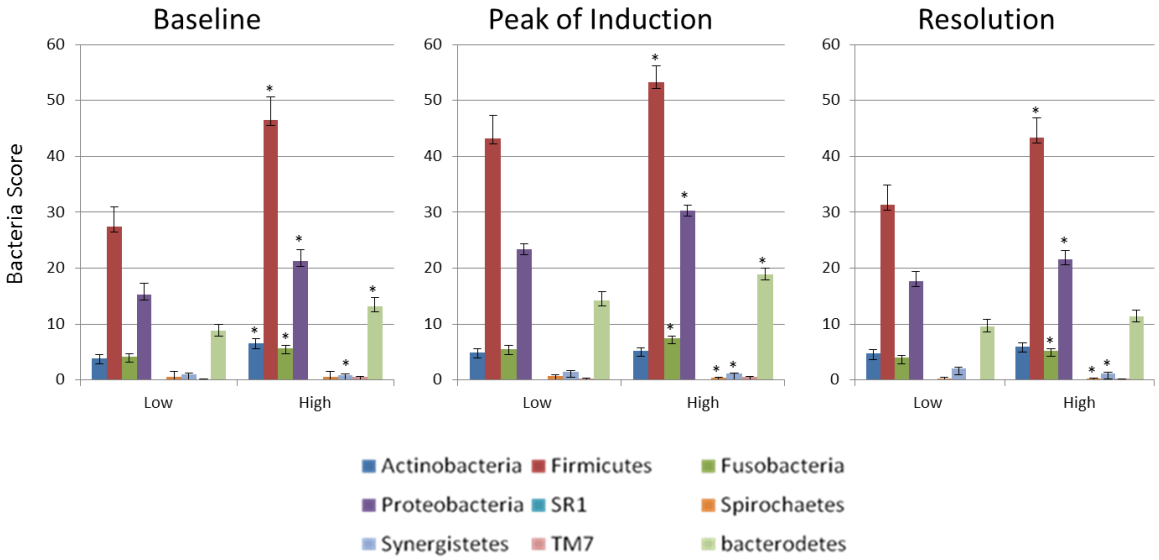
B. BOP



*Statistically significant at $p<0.05$

At the baseline, no significant difference was found at levels of all nine bacteria phylum in between low and high BOP phenotype. At the peak of induction and resolution, *Spirochaetes* present in significant lower levels in the high BOP phenotype in comparison with the low BOP phenotype ($p=0.02$; $p=0.01$) (Figure 3B).

C. PD



*Statistically significant at $p<0.05$

At baseline, *Actinobacteria, Firmicutes, Fusobacteria, Proteobacteria, Synergistetes and Bacteroidetes* present significant higher levels in the high pocket depth phenotype in comparison with the low pocket depth phenotype (p=0.003; p=0.0002; p=0.01; p=0.02; p=0.03; p=0.03). At the peak of induction*, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes* and *Bacteroidetes* presents significant higher levels in the high pocket depth phenoype in comparison with the low pocket depth phenotype (p=0.002; p=0.0002; p=0.0002, p=0.03; p<0.0001; p=0.0005). At resolution, *Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes* presents significant higher levels in the high pocket depth phenotype in comparison with the low pocket depth phenotype (p=0.0006; p=0.02; p=0.03; p=0.0007; p=0.002) (Figure 3C)

Chapter 5. Discussion

This study employed a 16S rRNA-based microarray technology, known as HOMIM, to assess and compare the diversity of microbiota associated with all five BGI groups (Offenbacher et al, 2007) at three instances during a plaque-induced gingival inflammatory model. At baseline, significant differences were detected in the levels of *Spirochaetes* and *Synergistetes*, which presented at increased levels in individuals from BGI-P2 and BGI-P3. These findings are in agreement with a recent study (Griffen et al, 2011), where they investigated the subgingival bacteria in health and chronic periodontitis individuals by 454 sequencing of 16S rRNA genes. It was reported that *Spirochaetes*, *Synergistetes* and *Bacteroidetes* were more abundant in disease, whereas the *Proteobacteria* were found at higher levels in healthy controls. In addition, Ahn et al (2011), when comparing the oral microbiome community profiles assessed by the broad 16S rRNA pyrosequencing and custom 16S rRNA hybridization (HOMIM), showed that they were highly correlated at the phylum level and genus level.

The HOMIM analysis of the subgingival plaque in all BGI groups detected changes in bacterial levels over the course of this experimental model of gingival inflammation. In the BGI-health individuals, bacteria in the *Firmicutes phylum* delay to rebound to baseline levels, whereas in the BGI-gingivitis subjects, in addition to *Firmicutes*, the phylum *Proteobacteria* also do not rebound during the resolution of gingival inflammation. Furthermore, beside *Proteobacteria, Firmicutes, Bacteroidetes*, and *Fusobacteria,* increased levels of the phylum*TM7* were detected at the peak of induction in the BGI-gingivitis when comparing with BGI-Health. Accordingly,

Huang and coworkers (2011) using sequence methods to detect bacteria in the subgingival plaque of a chinese population reported that the uncultivable *TM7* phylum were at increased levels in gingivitis sites when comparing to healthy sites. Interestingly, in this study, different from *Firmicutes* and *Proteobacteria*, the *TM7* levels were significantly reduced during the resolution phase.

In the periodontitis groups (BGI-P1), bacterial levels in the *Synergistetes and Proteobacteria* do not recover to baseline levels with the removal of the supragingival plaque in this study period, whereas in BGI-P2, *Spirochaetes* which at baseline presented at increased levels significantly decreased during the resolution phase. In BGI-P3 no significant changes were observed which may indicate that the subgingival biofilm in these individuals is stable and not affected by plaque induced inflammation and its resolution with manual removal of plaque.

In addition, subjects of all BGIs were grouped together and were differentiated in terms of low or high response to clinical parameters such as plaque, bleeding upon probing and probing depths. The clinical parameter that was mostly associated with changes in bacterial levels was the pocket depth. High phenotype to pocket depth presented with significant increased levels of bacteria in all phases of the experiment when compared to low phenotype. At baseline, *Actinobacteria* and *Firmicutes* are the phylum mostly linked to high pocket depth phenotype. In other words, subgingival plaque of individuals who initially presents with significantly increased levels of *Actinobacteria* and *Firmicutes* are more likely to increase their pocket depths when challenged with plaque-induced gingival inflammation. At the peak of induction, *Fusobacteria, Proteobacteria, Synergistetes* and *Bacteroidetes* were significantly associated with the high pocket depth phenotype, and at the resolution, it was *Firmicutes, Spirochaetes and Synergistetes.* These findings are in agreement with a previous investigation (Zijnge et al, 2010), who used

fluorescent in situ hybridization to localize in vivo the most abundant species from different phyla and species associated with periodontitis on seven embedded teeth obtained from four different subjects. They reported that *actinomyces* from the *Actinobacteria* phyla are presumably the first colonizers since they were detected in the basal layer of the subgingival biofilm. They suggested that *Actinomyces* sp. might survive, maybe due to their capacity to store intracellular glycogen (Takahashi and Yamada, 1999) or their capacity to scavenge on biofilm material like extracellular polymeric substances and on compounds from dead bacterial cells. In addition, in the intermediate layer, it was detected *fusobacteria* and members of the *bacteroidetes* phyla, and in the top layer beside members of the bacteroidetes, it was detected Synegistetes. They were in close contact to eukaryotic cells resembling polymorphonuclear leukocytes (PMN's). Increasing numbers of studies has been associating subgingival plaque *Synergistetes* with periodontitis (Hugenholtz et al, 2009; Vartoukian et al, 2009). It has been reported that *Synergistetes* is detected in significantly more subjects with periodontitis than healthy controls, and is more abundant in subgingival plaque at diseased sites than at healthy sites in subjects with periodontitis or healthy controls (Vartoukian et al, 2009).

The phylum *Synergistetes* includes a wide variety of genera, including *Aminobacterium*, *Aminomonas*, *Aminiphilus*, *Anaerobaculum*, *Cloacibacillus*, *Dethiosulfovibrio*, *Jonquetella*, *Pyramidobacter*, *Synergistes*, *Thermanaerovibrio*, and *Thermovirga* (Downes et al, 2009; Vartoukian et al, 2007). "*Synergistetes*" are widely distributed in the environment, form part of the normal microbiota of animals (Godon et al, 2005), and have also been isolated from a variety of sites in humans, including the oral cavity. Despite the frequent detection of "*Synergistetes*" taxa at oral disease sites, they are almost never encountered in healthy individuals (Aas et al, 2005; Kumar et al, 2005; Paster et al, 2001), suggesting that they have a pathogenic role. Human

oral "*Synergistetes*" can be divided into two main groups: cluster B, which comprises the only two species that have been cultured to date, *Jonquetella anthropi* (Jumas-Bilak et al, 2007) and *Pyramidobacter piscolens* (Downes et al, 2009), and cluster A, which includes more than 20 other taxa for which no cultivable representatives are available (Vartoukian et al, 2007). Unculturable "*Synergistetes*" in oral samples have been detected only by 16S rRNA gene sequence analysis, and the morphology of the cells remains unknown. The "*Synergistetes*" species that have been cultured to date are straight or curved gram-negative bacilli, and some of them are motile (Vartoukian et al, 2007). Furthermore, the "*Synergistetes*" species characterized to date have been found to be proteolytic and strictly anaerobic, a profile matched by the typical periodontal "pathogen."

In conclusion, plaque induced inflammation prompted substantial changes in subgingival biofilm composition in most of the BGI groups except BGI-P3 which the subgingival biofilm seems to be established. Removal of plaque for four weeks significantly reduced to baseline levels most bacteria in all BGI groups. However few bacterial phylum did not reduce to baseline levels, such as *Sinergistetes* in BGI-P1, which were also detected in increased levels in the high pocket depth responders. This indicates that this phylum may be highly involved in the progression of periodontal disease. There is still little information on these as-yet-uncultivated bacteria besides their cell morphology and the implication that they are subject to recombination events. One "*Synergistetes*" OTU has been identified as a disease marker for periodontitis (Vartoukian et al, 2009). It would be of value to attempt to isolate this novel species and ultimately to begin the search for possible virulence mechanisms possessed by this putative periodontal pathogen.

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