ROLE OF GENETICS IN SUBGINGIVAL AND SUPRAGINGIVAL BACTERIAL COLONIZATION

THESIS
Presented in Partial Fulfillment of the Requirements for the Degree Master of Science
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By
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*****
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The gingival sulcus and tooth consist of complex bacterial ecosystems, the majority of which are uncultivated. Although the sequence of oral bacterial colonization has been investigated, factors affecting this process, especially host genetics, are poorly understood. Twin studies provide a powerful model to study the contribution of genetics to diseases. Open-ended molecular methods are accurate and comprehensive approaches to compare complex microbial communities. The purpose of this study was to compare the subgingival and supragingival microbial profiles of periodontally healthy twins using a molecular method for bacterial profiling.

95 pairs of periodontally and systemically healthy twins, 18 to 36 years old, were selected. Zygosity information was elicited by questionnaire. Whole mouth subgingival and supragingival plaque samples were collected and pooled from each subject. Bacterial DNA was isolated from these samples and the 16S rRNA gene was amplified using polymerase chain reaction with fluorescent labeled broad-range eubacterial primers. The amplicons were restriction-digested using MspI and HhaI and Terminal Restriction Fragment Length Polymorphism Analysis (t-RFLP) was used to examine the composition of the microbial community in each individual. Similarity of microbial profiles between each twin pair was assessed using Bray-
Curtis similarity index based on fragment peak areas. The number of shared species was also computed for each twin pair and compared between groups.

59% of the twin pairs were monozygotic, while 41% were dizygotic. Differences in race, smoking status and gender were not statistically significant between the twin groups. 29% of the subjects were current or former smokers. The supragingival community exhibited an average of 45% of similarity between all twin pairs and the subgingival biofilm 34% among all twin pairs; however, there was no significant difference between the two twin groups by Kruskal Wallis Analysis. No differences were observed after current and former smokers were excluded. Our findings also suggested that smoking or age were not contributing factors in the bacterial acquisition.

The low number of fragments generated from the restriction digestion suggests that the diversity of a health-associated subgingival microbial community is low, with few dominant species. The microbial profiles of monozygotic twins were not significantly different from that of dizygotic twins, suggesting that the effects of host genetics on microbial colonization of supragingival plaque or subgingival biofilm are not evident in an established, health-compatible community. Further studies are required to elucidate the role of host genetics in early microbial colonization of the subgingival and supragingival plaque formation.
DEDICATION

To my family

Αφιερωμένο στην οικογένεια μου για όλη την υποστήριξη και αγάπη που μου ἔδειξε
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CHAPTER 1
INTRODUCTION

Periodontal disease is a bacterially-induced inflammation of the supporting structures of the teeth, leading to progressive loss of bone, periodontal ligament and soft tissue. The first reports on the disease were by the Chinese 3500 years ago, and its prevalence, although improved over the last years through preventive and treatment strategies, is still high, designating it as one of the most frequent diseases of the modern world. Albandar\textsuperscript{1} reported the prevalence of periodontitis in the United States to be 35%, in people age 30-90 years old. Mild form of the disease was presented in 21.8% of the population and 12% with moderate to severe form, when severity of the disease is calculated according to probing depths. In children and younger population, the prevalence is significantly lower, estimated at 2 to 5\%\textsuperscript{2}.

Apart from periodontal disease, dental caries is considered the most common disease of the oral cavity in our times. Dental caries is the localized destruction of susceptible hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates\textsuperscript{3}. According to National Health and Nutrition Survey 1999-2002, the prevalence of caries on permanent teeth for children and adolescent 6 to 19 years old was approximately at 41\%\textsuperscript{4}. The bacterial mediated etiology of dental caries was first suggested at the end of 19\textsuperscript{th} century\textsuperscript{5}. \textit{Streptococcus mutans} and \textit{Lactobacillus} species are normally found in low concentrations in a healthy
plaque\textsuperscript{5,6}. *Streptococcus mutans* is present in the oral cavity even prior to tooth eruption\textsuperscript{7} and transmission of the organism occurs both vertically (parent-offspring) and horizontally (spouses) from human reservoirs\textsuperscript{5}. Once the cariogenic organisms colonize the tooth surface they begin producing acid, leading to drop of the pH of the oral environment\textsuperscript{3}. When the area reaches an ecological niche, the bacteria adapt to the lower pH and tooth demineralization begins leading tooth to cavitation and inability of execution of proper oral hygiene, resulting to dental caries\textsuperscript{3}.

Genetic susceptibility to dental caries has been confirmed through numerous studies\textsuperscript{8-11}. Although the exact mechanism is unknown, it is speculated that the effect of genetics is on bacterial level, the density and structural integrity of enamel and the composition of secretions of salivary glands\textsuperscript{12}. Therefore, the presence of biofilm in a susceptible host, similar to periodontal disease, is essential for the initiation of a carious lesion.

The presence of a biofilm in combination with a susceptible host is the etiology of periodontal diseases\textsuperscript{13,14}. Microbiota colonizes the oral cavity and forms biofilm soon after birth. Transmission of the bacteria occurs through the mother’s genital tract, skin and oral cavity\textsuperscript{15}. Dental plaque evolves and changes through the years. Numerous factors have influential role in bacterial colonization, including puberty\textsuperscript{16}, smoking\textsuperscript{17}, bacterial characteristics\textsuperscript{18} and host factors such as pH and oxygen concentration in the oral cavity.

Bacterial colonization has been examined for over 100 years and although it was believed that the prevalence and severity of the disease correspond to the amount of plaque present (nonspecific plaque hypothesis), it is now believed that
certain bacterial species are responsible for the initiation and progression of the disease (specific plaque hypothesis). The transition from periodontal health to disease is associated with not only increase in the total microbial load from $10^2$-$10^3$ to $10^5$-$10^8$ isolates, but also with the decrease of gram-positive bacteria, *Streptococci* and *Actinomyces*. Gram-negative species are only 15% at periodontal health but an increase up to 50% is present when periodontal disease is established. *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia* are all considered primary pathogens and therefore indicators for future attachment loss. In disease active sites, these bacteria are present in abundance and treatment of the disease is associated with reduction of their level.

Biofilm is an ecosystem with a distinct bacterial profile and is a result of the equilibrium between bacterial adhesion, crevice and saliva flow, host immunity and oral hygiene. Bacterial colonization on tooth surfaces or sulcular epithelium requires specific adhesion molecules such as fimbriae and extracellular proteolytic enzymes, cell-associated proteins as well as tissue receptors, for example proline-rich proteins and type I and IV collagen. Bacteria can also attach to other bacterial species. Several coaggregation mechanisms have been proposed in the literature either by direct adhesion mediated by receptor-adhesin interaction or by mediation of cellular components by third species present in the community. Once the bacteria colonize the oral cavity and certain environmental conditions are met (temperature, pH, nutrients), their multiplication begins. Therefore, there is formation of a community with primitive circulatory and metabolic cooperation that may or may not overcome the host immune defense mechanisms by expression of
virulent factors such as lipopolysaccharide, heat shock proteins, fimbriae and extracellular proteolytic enzymes\textsuperscript{29}. Flow of saliva and crevice fluid, mastication forces, desquamation and host antibodies are some of the components that prevent plaque growth and tissue invasion\textsuperscript{30}. 60\% of this biofilm remains uncultivated due to its complexity\textsuperscript{31,32}.

Degree of susceptibility to periodontal disease is not similar for all individuals, after adjustment for several factors such as race, gender, smoking and/or oral hygiene\textsuperscript{1,13,33}. Increased susceptibility has been attributed to genetic factors such as IL-1 polymorphism\textsuperscript{13,34}, with 50\% heritability of chronic periodontitis\textsuperscript{35} and 70\% of aggressive periodontitis\textsuperscript{36} attributed to genetics.

The effect of host traits on periodontal disease prevalence and severity is obvious after examination of certain genetic diseases. Down syndrome, Chediak-Higashi syndrome, Papillon-Lefevre syndrome and cyclic neutropenia are only a few of the genetic diseases that have been associated with periodontal disease. These diseases affect the host immune mediators through different pathways resulting in compromised response to infectious diseases.

Furthermore, factors such as diabetes and smoking have a modifying role not only in bacterial acquisition but also on immune response. Diabetics are twice more likely to present with periodontal disease than non-diabetics\textsuperscript{37}. This has been attributed to production of advanced glycation end products (AGEs) that alter the function of endothelial cells and monocytes resulting in increased inflammatory response and production of inflammatory cytokines\textsuperscript{38}. Smoking increases the probability and severity of periodontal disease through alteration of
polymorphonuclear leukocyte functions, humoral and cellular immune responses, fibroblast function and vascularity\textsuperscript{29,39}.

Galton, in 1875, was a pioneer in twin model study, for estimation of the effect of host traits and environment on disease presence and progression. Ever since, the twin model is used extensively in medical research, for the examination of the etiology of complex diseases such as ankylosing spondylitis, multiple sclerosis, inflammatory bowel disease and schizophrenia\textsuperscript{40-43}. More recently, the dental field has adopted the twin model, to study genetic contribution to caries and periodontal disease\textsuperscript{44-47}. Twin studies are based on the principle that monozygotic twins have identical genomes whereas dizygotic twins share up to half of their DNA\textsuperscript{48}. Differences in periodontal disease prevalence and severity between dizygotic twins, can be attributed to both environmental and genetic influences\textsuperscript{49}. On the contrary, differences between monozygotic twins are attributed to environmental factors only, since monozygotic twins have similar phenotypic characteristics compared to dizygotics. Tooth morphology and occlusion are some of the phenotypic traits in the oral cavity that have been examined using the twin model design\textsuperscript{8,44,50}.

Bray-Curtis similarity index which is used as a measure of community, is probably the most widely used similarity index\textsuperscript{51}. This index has been shown to accurately reflect true similarity\textsuperscript{52} and is very effective in comparing natural bacterial communities\textsuperscript{53-55}. The index is not influenced by the community examined and whether the species are rare or abundant\textsuperscript{51}. Yet, it has been criticized due its large bias since it does not take into account abundance of species and abundant and rare species are treated equally. Therefore, the index presents with some bias in situations
where the sample size is not large enough to observe all species or when the community under examination has a lot of rare species.

Several methods have been used for identification of complex bacterial communities since there is no single one technique that can characterize all the organisms present in a community. The earliest among them were cultivation and microscopy. Cultivation can detect unrecognized species but less than 1% of any natural environment can be cultivated\textsuperscript{56}, it is extremely time consuming, expensive, and it is also difficult to speciate cultures\textsuperscript{30}. Also the fact that it is performed under selective conditions, affects the structural composition of the community in vitro\textsuperscript{57}. Microscopy, like culturing, has limited precision in identification of bacterial species and can only be performed in a limited number of species\textsuperscript{30}. Light and electron microscopy, as well as cultural techniques were later replaced with immunofluorescence methods\textsuperscript{30}. These techniques were based on the antibody development to specific taxa, therefore they were used for limited species for which reagents were developed\textsuperscript{30}. Thereafter, targeted molecular approaches which include polymerase chain reaction (PCR), real time PCR and DNA-DNA hybridization were used extensively for identification of pre-selected specific species with low to modest abundance\textsuperscript{30}.

Open ended molecular methods, which are the most commonly used techniques in recent years, include 16S cloning and sequencing and terminal restriction fragment length polymorphism analysis (t-RFLP). These methods circumvent the need for cultivation of bacterial species and allow the examination of all species in an established community, including as-yet-uncultivated species and
bacteria that have not been previously described. The concept behind these methods is phylogenetic analysis of the 16S gene, which is a critical component of cell functions and presents with genomic similarity above the species level. Stability of the specific gene is prerequisite, leading to minimal tolerance of possible mutations and allows the comparison among all bacteria and the 18S rRNA gene of eucaryotes.

t-RFLP analysis measures the size polymorphism of terminal restriction fragments from a PCR amplified product. t-RFLP has been used successfully for the assessment of the diversity of several bacterial communities such as coastal water, plant species and soil. It provides a rapid and sensitive technique for assessing community diversity. Underestimation of the bacterial diversity is still possible, since closely related bacterial species may have common restriction sites thus making it difficult to distinguish them. In the oral environment, t-RFLP has been used for comparison of bacterial species in the saliva of healthy and patients with periodontitis and for evaluation of subgingival microflora after periodontal treatment.

Our central hypothesis was based on the principle that monozygotic twins have identical genomes whereas dizygotc twins share up to half of their genes, therefore monozygotic twins may have more similar supragingival and subgingival microbial profiles than dizygotc twins at periodontal health.

The purpose of our study was to examine the contribution of host traits on both supragingival and subgingival microbial acquisition in a stable, health-associated microbial community in periodontally and systemically healthy twins,
using an open-ended molecular method to examine the composition of the community.

The significance of the study is that it could provide new insights regarding periodontal disease susceptibility and lead to better means of identifying subjects at risk, provide the basis for future studies to identify genes controlling bacterial colonization, and result, in long term, in the development of more targeted and efficient preventive strategies and improved regenerative therapies.
CHAPTER 2
MATERIALS AND METHODS

Subject selection and study design

The research design was approved by The Ohio State University Office of Responsible Research Practices. The sample consisted of monozygotic and dizygotic twins, age range 18 to 36 years, recruited from The Ohio State University over a 2-year period and the Twins Festival in Twinsburg Ohio, held in August 2006.

All participants gave informed consent in accordance to procedures established by The Ohio State University Review Board. Subjects completed a questionnaire providing information regarding date of birth, sex, ethnicity, race, educational level, medical history, oral hygiene habits, zygosity and smoking habits. Inclusion criteria consisted of systemic health, presence of 20 natural teeth and healthy periodontium. Subjects requiring antibiotic prophylaxis prior to dental treatment, diagnosed with diabetes or HIV infection, pregnant women or subjects currently on immunosuppressive medication were excluded from the study.
Zygosity Assessment

Zygosity was assessed based on the responses to the following questions:

1. “When you and your twin were children (ages 6-13), were you “as alike as two peas in a pod?” (Options: as alike as two peas in a pod, normal family likeness - no more alike physically than ordinary sisters or brothers, don’t know)

2. “Were you and your twin mixed up as children?” (Options: Yes, very often, now and then, never)

3. “In that case, by who were you mixed up?” (Options: Parents, teachers, others, nobody)

4. “Did you and your twin have the same eye color?” (Options: Yes, no, don’t know)

5. “Did you and your twin have the same natural hair color?” (Options: Yes, no, don’t know)

6. “Did you and your twin have the same complexion (skin) color?” (Options: Yes, no, don’t know)

7. “In your opinion, you and your twin are: definitely identical, probably identical, probably fraternal, definitely fraternal or not sure”

The answers to the above questions determined zygosity. When the answers to questions 1 to 6 was “yes” and to question 7 “definitely identical” or “probably identical”, the twin pair was classified as monozygotic. When one or more answers to questions 1 to 6 was “no” or “don’t know” and the answer to question
7 “probably fraternal”, “definitely fraternal” or “not sure”, then the twin pair was classified as dizygotic.

**Sample collection**

Subgingival plaque was collected and pooled from each twin by inserting sterile endodontic paper points (Caulk-Denstply) for 10 seconds in the gingival crevice of 12-15 randomly selected teeth. Supragingival plaque was collected from the same teeth of each twin using a sterile microbrush. Paper points and brushes were separately stored in microcentrifuge tubes at -20°C until further analysis.

**DNA Isolation**

**Supragingival plaque**

200μl of phosphate-buffered saline (PBS) was added to the brushes in the tubes and soaked for 2 hours with vortexing. The sampling device was removed; the solution was transferred into a tube with 0.25g of glass beads and homogenized in a bead beater for 60 seconds at 5000rpm. Vials were centrifuged for 2 minutes and the liquid was carefully aspirated without pulling off the beads.

DNA was isolated with a Qiagen DNA MiniAmp kit (Qiagen, Valencia, CA) using the tissue protocol according to the manufacturer’s instructions. Briefly, 180μl of Tissue Lysis Buffer ATL and 40μl of Proteinase K were added and incubated at 56°C in a shaking water bath for 2 hours. 200μl of Lysis Buffer was added and incubated at 70°C for 10 minutes. 200μl of 100% ethanol was added and the mixture was applied to the QIAamp Spin Column and centrifuged. Impurities were removed
by sequential washes with 500ml of two wash buffers. Elution buffer of 50 μl was added in the column, incubated at room temperature for 5 minutes and centrifuged at 8000rpm for 1 minute to elute DNA.

**Subgingival plaque**

200μl of phosphate-buffered solution (PBS) was added to the paper points and soaked for 20-30 minutes. The paper points were removed and DNA was isolated using the Qiagen DNA MiniAmp kit as described above.

**t-RFLP analysis.**

Bacterial 16S rRNA genes were amplified using 22 cycles of PCR with fluorescent-labeled broad range bacterial primers A18-FAM (5’- TT TGA TCC TGG CTC AG–VIC-3’) and 317-HEX (5’- FAM-AAG GAG GTG ATC CAG GC -3’) in a 50μl reactor (Applied Biosystems, Foster City, CA). The cycling conditions included denaturation at 94°C for 1 minute, annealing at 42°C for 2 minutes and elongation at 72°C for 3 minutes. A final, 10 minute elongation at 72°C followed 22 cycles of amplification (Figure 1).

The amplicons were purified using a Qiaquick kit (Qiagen, Valencia, CA). Restriction digestion was carried out with 10μl of purified PCR product and 10 U of *MspI* or *HhaI* in a total volume of 20μl at 37°C for three hours. 20μl of the restriction digestion product was purified by AMPure beads (Agencourt Bioscience Corporation, Beverly, MA) according to the manufacturer’s protocol and eluted with 50μl water. 5μl of the purified product was denatured with 10μl of deionized
formamide and mixed with 0.2μl GeneScan 1200 LIZ size standard (Applied Biosystems, Foster City, CA). Fragment lengths were determined on an AB 3730 DNA Analyzer in GeneScan mode. The number of peaks as well as the height and area of each peak; reflecting the sizes and intensities of the terminal fragments were determined using the GeneMapper 4.0 Software.

**Data analysis**

Fragments with a peak height of less than 25 fluorescence units were excluded from analysis. Peak areas were standardized by converting the raw values to a proportion of the total area as previously described\textsuperscript{64}. Peaks representing less than 1% of the total area were assigned a value of zero and the areas of the remaining peaks recalculated as a proportion of the new total peak area.

The total numbers of peaks as well as the number of shared peaks were compared between each twin pair. A Bray Curtis similarity index was computed for each twin pair using the formula

\[
1- \frac{\sum_{n=95} (\text{Peak Area}_A - \text{Peak Area}_B)}{\sum_{n=95} (\text{Peak Area}_A + \text{Peak Area}_B)}
\]

Statistical analysis was carried out with JMP (SAS Institute Inc., Cary, NC). Non-parametric tests were used for comparison of bacterial parameters.
Step 1. DNA Isolation from paper points and microbrushes

Step 2. Amplification of 16S gene with PCR

Step 3. Restriction digestion with *HhaI* and *MspI*

Step 4. t-RFLP Analysis

Figure 1. DNA isolation and t-RFLP method
CHAPTER 3

RESULTS

Subjects were divided into monozygotic and dizygotic twins based on the analysis of the questionnaire, as previously described. 59% of the twin pairs were classified as monozygotic and 41% as dizygotic, a difference which was not statistically significant (p=0.11, t-test).

All subjects were diagnosed with periodontal health upon clinical examination with a Gingival Index (GI) (Loe & Silness 1963) <1 and Plaque Index (PI) (Silness & Loe 1964) <1.

There were no statistically significant differences between the twin groups with regard to age, gender, race or smoking status at baseline (Table 1).

Subgingival Plaque

Figures 2A and 2B show the degree of similarity in the subgingival microbial community between monozygotic and dizygotic twins. Figure 2A shows the Bray Curtis similarity index in all subjects and Figure 2B the similarity index after current and former smokers were excluded. Mean microbial similarity for monozygotic twins was not statistically different from dizygotic twins (p=0.4, t-test). No differences were evident between the twin groups when smokers were excluded (p=0.9, t-test).
Figures 3A and 3B represent the number of shared peaks between each twin pair. Figure 3A represents all the subjects and 3B represents only non-smokers. No differences were observed in the number of shared species in each twin pair between monozygotic and dizygotic groups (p=0.7, t-test), even after the exclusion of former and current smokers (p=0.5, t-test).

The correlation between age and the microbial similarity is shown in figures 4A and 4B. Figure 4A shows the correlation between age and similarity index. Figure 4B shows the correlation between age and the number of species shared by each twin pair. Both graphs indicate that the influence of age on bacterial colonization is similar for monozygotic and dizygotic twins.

The contribution of race in bacterial acquisition could not be analyzed since the majority of the subjects were Caucasian (Figures 5A, 5B).

**Supragingival Plaque**

The microbial community similarity between monozygotic and dizygotic twins is shown in Figure 6A, with the exclusion of smokers in Figure 6B. The Bray-Curtis similarity index was not statistical significant different between the twin groups (p=0.6, t-test), even after exclusion of smokers (p=0.8, t-test).

The number of species common to each pair, was not significantly different between groups (p=0.2, t-test) (Figure 7A). No significant differences were noted when non-smokers were analyzed independently (p=0.1, t-test) (Figure 7B).
The correlation between age and the microbial similarity is shown in Figures 8A and 8B. Figure 8A shows the correlation between age and similarity index. Figure 8B shows the association between age and the number of species shared by each twin pair. No indication of contributing effect of age on bacterial colonization is evident in both graphs.

The contribution of race to supragingival bacterial acquisition could not be evaluated since most of the subjects were Caucasian (Figures 9A and 9B).
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Table 1. Demographic data (n=95)
Figure 2. The Percent Total Number of subgingival species among monozygotic and dizygotic twins. A. All samples. B. Excluding former and current smokers. Mean diamonds and 95% confidence intervals are shown.
Figure 3. The Number of Shared Peaks in subgingival plaque among monozygotic and dizygotic twins. A. All samples. B. Excluding former and current smokers. Mean diamonds and 95% confidence intervals are shown.
Figure 4. Influence of age on community similarity in subgingival plaque. A. Effect on Similarity Index. B. Effect on Shared Peaks. Monozygotic twins are presented in red and dizygotic twins in blue.
Figure 5. Influence of race on community similarity in subgingival plaque. A. Effect on Similarity Index. B. Effect on Shared Peaks. Monozygotic twins are presented in red and dizygotic twins in blue.
Figure 6. The Percent Total Number of supragingival species among monozygotic and dizygotic twins. A. All samples. B. Excluding former and current smokers. Mean diamonds and 95% confidence intervals are shown.
Figure 7. The number of Shared Peaks in supragingival plaque among monozygotic and dizygotic twins. A. All samples. B. Excluding former and current smokers. Mean diamonds and 95% confidence intervals are shown.
Figure 8. Influence of age on community similarity in supragingival plaque. A. Effect on Similarity Index. B. Effect on Shared Peaks. Monozygotic twins are presented in blue and dizygotic twins in red.
Figure 9. Influence of race on community similarity in supragingival plaque. 
A. Effect on Similarity Index. B. Effect on Shared Peaks. Monozygotic twins are presented in blue and dizygotic twins in red.
CHAPTER 4
DISCUSSION

Periodontal disease and caries, two of the most common diseases to affect humans, occur only in the presence of microbial biofilms. Acquisition and colonization of bacteria are affected by several factors, both bacterial and host related. Evidence from several ecosystems within the human body indicates that the host genotype influences the composition of each microbiome\textsuperscript{65-67}. For example, variations in host-secreted bacterial adhesins, immune response mediators and antimicrobial peptides can influence the presence or levels of a particular species in an ecosystem. In the oral cavity there are very few studies examining the influence of host genetics on the composition of the supragingival or subgingival biofilm. The purpose of this investigation was to use a molecular approach to compare the supragingival and subgingival microbial communities of periodontally healthy monozygotic and dizygotic twin pairs.

**Twin study design**

A twin study is a simple but powerful way to study the contributions of genetics to a certain trait or disease. The twin model is based on the principle that monozygotic twins have identical genomes whereas dizygotic twins share only half of their genes\textsuperscript{48}. 
Thus, differences in disease experience between dizygotic twins can be attributed to both environmental and genetic differences whereas between monozygotic twins are necessarily attributable to environmental factors. In the oral cavity, the twin model has been used to study the effects of several phenotypic traits such as sucrose tolerance, second premolar morphology, dental caries, occlusion and tooth morphology. Since several of these tooth-related factors, along with host immune mechanisms, can affect bacterial colonization, our central hypothesis was that monozygotic twins would demonstrate greater similarity in supragingival and subgingival microbial profiles than dizygotic twins.

**Zygosity assessment**

The use of questionnaire is considered a reliable, simple and practical method for examining and determining zygosity in twins. Validity of the questionnaire approaches 95-96% in comparison to genotyping for zygosity determination, with misclassification tendency towards monozygotic twins.

**Terminal restriction fragment length polymorphism (t-RFLP)**

The present investigation used terminal restriction fragment length polymorphism (t-RFLP) for bacterial community profiling. Several methods have been used to examine bacterial communities through the years. These include cultivation, microscopy and targeted molecular methods such as immunoassays, polymerase chain reaction (PCR), real time PCR and DNA-DNA checkerboard. Culturing has significant limitations arising from the selective nature of the growth
medium and culture conditions. Less than <1% of any natural environment can be cultivated, and greater than 50% of oral bacterial organisms are as-yet-uncultivated. Microscopy does not allow an accurate distinction between different bacterial species since it uses phenotypic characteristics such as morphology, size, colony structure and staining characteristics for bacterial identification. PCR and DNA-DNA checkerboard circumvent the limitations of cultivation, since they utilize bacterial DNA. They allow the examination for selected species even when they are at very low levels, however, their use is limited to species that have been previously identified and characterized.

The underlying principle of t-RFLP is the use of restriction enzymes that will cut DNA at specific sites. Several restriction enzymes are available that have specific sequence recognition sites. The present investigation used MspI and HhaI for digestion. Investigations have shown that the greatest diversity of oral microbial communities is obtained with these enzymes. The bacterial community DNA was amplified using fluorescent-labeled primers using PCR. 22 cycles of PCR were used to ensure representational amplification of the community DNA. It has been shown that greater than 25 cycles of amplification can result in a plateau effect leading to masking of relative levels of organisms within a community. The restriction digestion results in fluorescent labeled fragments of different lengths. The length of each fragment as well as the fluorescent signal from each is measured and the number of species as well as the levels of each species is computed. Fluorescent signals of less than 25 units were excluded from analysis (noise) based on data from Sakamoto et al.
t-RFLP offers several advantages over traditional methods, since it is an open-ended approach that requires no \textit{a priori} knowledge of the bacterial community under investigation. It also uses bacterial DNA to examine the composition of a community and therefore overcomes the limitations of cultivation-based approaches. Restriction fragment digestion of DNA is sequence-specific since the presence of restriction sites is dictated by the nucleotide sequences in the gene. Therefore, this method provides a highly specific method to examine the composition of a community. The data can be combined with sequence information, allowing for accurate identification of bacterial species. Yet, underestimation of the bacterial diversity is possible, since closely related bacterial species may have common restriction sites thus making it difficult to distinguish them.

The target of our investigation was the 16S ribosomal RNA gene. This gene is a housekeeping gene and therefore, is ubiquitous among all living organisms. The 16S gene has several regions of homologous sequences, making it possible to design universal primers targeted to all oral bacteria. Interspersed within these homologous sequences are tracts of highly variable sequences. These heterogeneous sequences provide unique bacterial signatures, which allow for accurate bacterial profiling.

**Shared species**

A peak on the t-RFLP profile represents a fragment length unique for a species; therefore the number of shared peaks is a measure of the bacterial species common to each twin pair. Greater number of shared peaks in monozygotic twin pairs as compared to the dizygotic twin group would be indicative of genetic
influence on the type of species acquired by each individual. The results of our study demonstrated similar concordance in the shared organisms between the twin groups; suggesting that the effect of genetics was not evident in this acquisition (Figures 3A, 3B, 6A, 6B). Our results are in concordance with previous study by Michalowicz et al\textsuperscript{46}, where genetic influence was not evident on subgingival disease-associated bacteria. In contrast, studies by Bretz\textsuperscript{16,44} and Corby\textsuperscript{47}, showed significant genetic contribution on the prevalence of certain non-pathogenic bacteria as well as bacteria associated with caries. Several factors could account for this difference. The present study examined similarity in the composition of microflora of the subgingival and supragingival microbial community as a whole while Bertz et al\textsuperscript{44} examined specific microbial species. Further, the number of shared peaks is an estimate of species prevalence and not abundance, which was the measure used in the Corby\textsuperscript{47} study. It has also been shown that t-RFLP may underestimate the diversity of a community containing several closely related species\textsuperscript{62}. Therefore, further studies using more robust open-ended molecular approaches for identification and enumeration of bacterial species are warranted.

**Comparison of bacterial community profiles**

The supragingival community exhibited an average of 45% of similarity and the subgingival biofilm 34% among all twin pairs; however, there was no evidence in the data to indicate a greater community similarity in monozygotic twins than in dizygotic twins (Figures 2A, 2B, 3A, 3B, 5A, 5B, 6A, 6B). This is contrast to studies on other host-associated ecosystems\textsuperscript{72,73}. Evidence in the literature reports that
carriage of specific bacterial organisms is determined by host traits. The populations of *Bifidobacteria* and *Lactobacillus* in human fecal samples are characteristic of a particular host. Also, studies in animals have shown that the major histocompatibility complex can influence the composition of fecal microflora. The formation of a stable supragingival and subgingival community is determined not only by host-bacterial interaction but by inter-bacterial communication. These inter-bacterial relationships can either be synergistic or antagonistic. Extracellular enzymes produced by certain bacteria may open binding sites or they can provide growth conditions favorable for other bacterial species. Therefore, microbial colonization may be influenced by genetics at the initial stages of plaque formation but that might not be the case once the community has reached a niche, where the inter-bacterial relationships may have the predominant role. It is important to examine the role of genetics in early bacterial colonization using the twin model.

Bacteria colonize the gingival sulcus soon after tooth eruption and several factors affect this acquisition, such as temperature, pH, nutrients and oxygen concentration. Supragingival and subgingival bacterial communities are dynamic entities, which evolve through the years. However, there was no evidence in our data to indicate a modifying role of age on genetic influence. Our results are in contrast with the study done by Moore et al, where subgingival flora composition was examined in 11, 12.5 and 14 years old twin pairs. Through culture, they reported that certain bacterial species were specific and unique for each age group among twin pairs and concluded that age and puberty had a modifying role on the effect of genetics. This
discrepancy between the two studies could be attributed to a masking effect, due to pre-selection of the participants for age and adulthood.

In the present study, smoking did not appear to be an environmental modifier of both supragingival and subgingival microbial colonization, which may be attributed to the fact that the majority of our sample population was non-smokers. 42% of periodontal disease in the USA is attributed to smoking with significant clinical, vascular and microbiological implications. Smokers present with limited gingival bleeding, increased bone and attachment loss. On the microbiological level, non-treated smokers have been characterized with increased subgingival levels of periodontopathogenic bacteria such as Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Tannerella forsythia compared to non-smokers, therefore making them more susceptible to periodontal disease.

In summary, in a cross-sectional examination, the contribution if any, of host traits on supragingival and subgingival microbial colonization in a stable, health-associated microbial community is not apparent. Further studies are needed to examine the role of genetics in bacterial colonization during early plaque development.
REFERENCES


