J Clin Periodontol 2011; 38: 517–524 doi: 10.1111/j.1600-051X.2011.01718.x

Journal of Clinical Periodontology

Contribution of host genotype to the composition of health-associated supragingival and subgingival microbiomes

Anastasia Papapostolou[1], Brandon Kroffke[1], Dimitris N. Tatakis[1], Haikady N. Nagaraja[2] and Purnima S. Kumar[1]

[1]Division of Periodontology, College of Dentistry, The Ohio State University, Columbus, OH, USA; [2]Division of Biostatistics, College of Public Health, The Ohio State University, Columbus, OH, USA

Papapostolou A, Kroffke B, Tatakis DN, Nagaraja HN, Kumar PS: Contribution of host genotype to the composition of health-associated supragingival and subgingival microbiomes. J Clin Periodontol 2011; 38: 517–524. doi: 10.1111/j.1600-051X.2011.01718.x.

Abstract

Aim: Periodontitis and caries are two of the most prevalent diseases to affect humans, however; the individual susceptibility to these diseases varies significantly in the population. The aim of this investigation, therefore, was to examine the influence of host genotype on the composition of health-associated supragingival and subgingival microbiomes.

Materials and methods: Subgingival and supragingival plaque was collected from orally and systemically healthy adult monozygotic and dizygotic twin pairs. Zygosity was determined by multiplexed PCR amplification of 13 short tandem repeats. Terminal restriction fragment length polymorphism was used for bacterial community profiling. The number of species shared by the twin pairs as well as the similarity of the microbial communities between the twins was computed and compared using two-sample *t*-test

Results: There was no difference in the number of species shared by the twin pairs as well as the similarity of the microbial communities between the twin dyads. Age was not a modifier of genetic influence on these microbial parameters. There was no difference between monozygotic and dizygotic twin pairs in the correlation between supragingival and subgingival community similarity.

Conclusion: The contributory role of host genotype, if any, is not apparent on an established, health-associated oral microbial community.

Key words: bacteria; genotype; 16S; subgingival; supragingival

Accepted for publication 14 February 2011

Bacteria in dental plaque play an important role in the aetiology of periodontal diseases and caries. Although both diseases are highly prevalent, susceptibility to these diseases is extremely variable among individuals. It is known that genetic factors are critical determinants of disease susceptibility (Tatakis & Kumar 2005); however, the exact mechanisms by which host genotype contributes to caries and periodontal disease are not well elucidated. Bacteria colonize the supragingival and subgingival biofilms soon after tooth eruption and form complex yet stable communities associated with periodontal and dental health. Evidence from other host-associated ecosystems indicates that the host genotype significantly influences the composition of the microbial community qualitatively (Stewart et al. 2005) as well as quantitatively (McCartney et al. 1996, Kimura et al. 1997). It has been shown that bacterial acquisition and colonization in dental plaque is influenced by several host-associated colonization factors, for example, tooth structure, pH and host immune mediators (Quirynen et al. 1991, Quirynen 1994, Skopek & Liljemark 1994). As the host genotype is a critical determinant of these colonization factors, it is important to examine its potential

Conflict of interest and source of funding statement

None of the authors have any conflicts of interest.

This study was funded by a start-up grant awarded to the senior author (PS Kumar) by the College of Dentistry, The Ohio State University. Additional support was provided by the Division of Periodontology, The Ohio State University.

contribution to the composition of the health-associated subgingival and supragingival microbial communities.

A twin study is a simple but powerful way to examine 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 (Petronis 2006). Thus, differences in the traits between dizygotic twins can be attributed to both environmental and genetic differences, whereas differences between monozygotic twins are necessarily attributable to environmental factors alone (Kinane & Hart 2003). In the oral cavity, the twin model has been used to study the effects of genetics on several phenotypic traits, for example, sucrose tolerance, second premolar morphology, occlusion, and tooth morphology (Wood & Green 1969, Boraas et al. 1988, Bretz et al. 2006).

Previous investigations on the influence of host genotype on oral bacterial colonization have used the twin model to examine the microbiota associated with gingivitis and periodontitis (Moore et al. 1993, Michalowicz et al. 1999) and caries (Corby et al. 2005a, b, 2007). The evidence is inconclusive, especially in the case of periodontal diseases, as these studies were limited to examining selected species, most of which have been cultivated and characterized previously. Oral microbial communities are complex, with several uncultivated members. In order to examine the contributions of host genotype to bacterial composition, it is important to use a comprehensive, quantitative molecular approach that is capable of enumerating all species within a community, including as yet uncultivated organisms and previously unknown and unsuspected species. Terminal restriction fragment length polymorphism (t-RFLP) of the 16S rRNA gene is a molecular approach that has been used to examine the profiles of several naturally occurring, complex microbial communities (Liu et al. 1997, Sakamoto et al. 2004, Smith et al. 2007, Dicksved et al. 2009, Fullmer et al. 2009). t-RFLP generates a unique ''fingerprint'' of each microbial community by exploiting the variations that exist within the 16S gene sequence between different bacterial species, providing quantitative information on the compositional differences between communities.

The purpose of the present investigation was to examine the contributions of host genotype to the composition of health-associated supragingival and subgingival microbiomes using t-RFLP for bacterial community profiling.

Material and Methods

Subject selection and study design

Approval for this study was obtained from the Office of Responsible Research Practices at The Ohio State University. Ninety-five pairs of periodontally and systemically healthy twins were recruited from The Ohio State University and the Twins Days Festival in Twinsburg, Ohio. Informed consent was obtained from all subjects in accordance with the procedures established by The Ohio State University Review Board. Demographic information and zygosity were assessed using a questionnaire. All subjects were examined by two examiners calibrated to a ''gold standard''.

Exclusion criteria included pregnancy, diabetes, HIV infection, use of immunosuppressant medications, bisphosphonates or steroids, antibiotic therapy or oral prophylactic procedures within the last 3 months and <20 teeth in the dentition. Exclusion criteria also included Loe and Silness gingival index >1 (Silness & Loe 1964) and probe depths >3 mm.

Sample collection

Sites selected for sampling were isolated with cotton rolls and gently dried. Supragingival plaque was collected and pooled from 15 randomly selected mesial interproximal spaces in the anterior dentition (mesial of the first right premolar to mesial of the first left premolar), using sterile microbrushes (DENTSPLY-Caulk, Milford, DE, USA). Following supragingival plaque removal, subgingival plaque samples were collected and pooled from the same mesial sulci by inserting one sterile endodontic paper point (DENTSPLY-Caulk) into each sulcus for 10 s. Paper points and brushes were separately stored in microcentrifuge tubes at -20°C until further analysis. Unstimulated saliva was collected by continuously expectorating into a tube for 1 min.

DNA Isolation and Molecular Analysis

Supragingival plaque

Bacteria were separated from the microbrushes by adding 200 µl of phosphate-buffered saline (PBS) to the tubes and vortexing. The sampling device was removed; the solution was transferred into a tube with 0.25 g of glass beads and homogenized in a bead beater for 60 s at 5000 rpm. Vials were centrifuged for 2 min. and the liquid was carefully aspirated without pulling off the beads. DNA was isolated using a Qiagen DNA MiniAmp kit (Qiagen, Valencia, CA, USA) using the tissue protocol according to the manufacturer's instructions.

Subgingival plaque

Bacteria were separated from the paper points by adding 200 µl of PBS to the tubes and vortexing. The points were then removed and DNA was isolated as described above.

t-RFLP analysis

t-RFLP analysis was carried out as described earlier (Fullmer et al. 2009). Briefly, bacterial 16S rRNA genes were amplified by PCR with fluorescent-labelled broad-range bacterial primers. The cycling conditions included denaturation at 94°C for 1 min., annealing at 42°C for 2 min. and elongation at 72°C for 3 min. A final, 10-min. elongation at 72°C followed 22 cycles of amplification. The amplicons were purified using a Qiaquick kit (Qiagen). Restriction digestion was carried out with 10 μl of purified PCR product and 10 U of *Msp*I or *Hha*I in a total volume of 20 μl at 37°C for 3 h. Ten microlitre of the restriction digestion product was purified by AMPure beads (Agencourt Bioscience Corporation, Beverly, MA, USA) according to the manufacturer's protocol. Five microlitre of the purified product was denatured with 10 μl of deionized formamide and mixed with 0.2 μl GeneScan 1200 LIZ size standard (Applied Biosystems, Foster City, CA, USA). Fragment lengths were determined on an AB 3730 DNA Analyzer (Applied Biosystems) in the 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. All reactions were duplicated.

Zygosity assessment

Zygosity was assessed both by questionnaire and by genotyping. Zygosity determination was made based on responses to questions relating to eye and hair colour as well as similarity and

confusion during childhood as described in the Danish Twin Registry (Kaprio et al. 1978). Genotyping was performed by PCR amplification of 13 short tandem repeats using a commercially available kit (AmpFISTR Profiler Plus, Applied Biosystems, Carlsbad, CA, USA). Briefly, DNA was isolated from 50 μl of saliva using a QiaAmp kit (Qiagen). PCR was performed using 2 μl of DNA as a template and fluorescently labelled primers for each of the 13 loci in a 50 μl reaction. Amplicon size was determined on a 3730 DNA analyzer in the GeneScan mode. The presence of a peak was recorded as positive for the marker on each allele. Twins dyads were considered monozygotic when they were concordant for the presence of at least 12 markers.

Data analysis

Terminal fragments (t-RFs) with a peak height of <50 fluorescence units were excluded from analysis. Peak areas were standardized by converting the raw values to a proportion of the total area as described previously (Fullmer et al. 2009). Peaks representing $<1\%$ of the total area were assigned a value of 0 and the areas of the remaining peaks were recalculated as a proportion of the new total peak area.

Peak data from the two fluorophores (FAM and HEX) were averaged and the total number of peaks, as well as the number of shared peaks, was compared between each twin pair. The total number of t-RFs as well as the area of each t-RF was used to compute the Bray Curtis similarity index. EstimateS (version 7.5, R. K. Colwell, http://purl.oclc.org/estimates) was used to compute the similarity index. Statistical analyses were carried out using JMP (SAS Institute Inc., Cary, NC, USA). A two-sample *t*-test was used to compare the total and shared peaks as well as the community similarity index between monozygotic and dizygotic twins. Regression analysis with interaction term was used to examine the effect of age on shared species and community similarity. Reported *p*-values correspond to two-tailed tests. Regression analysis was also used to examine the effect of zygosity on similarity and shared species between supragingival and subgingival plaque. Demographic variables (in Table 1) were compared using two-sample *t*-tests, the Fisher exact test or the χ^2-test as appropriate.

Table 1. Demographic data of study population

	Monozygotic ($n = 61$)	Dizygotic ($n = 34$)	*p*-value
Mean age (years)	26.0 ± 5.1	25.5 ± 3.5	0.20
Gender – female (per cent)	87.5	56.4	0.18
Race (per cent)			0.09
Caucasian	88.5	76.5	
African-American	6.6	5.9	
Asian	3.3	17.7	
Other	1.7	0.0	
Education (average years/individual)	13 ± 3	14 ± 2	0.25
Dental prophylaxis (number of annual visits/individual)	0.96	0.97	0.23
Brushing frequency (times per day/individual)	1.87	1.78	0.23
Frequency of flossing (times per day)	1.0	1.0	0.23
Tobacco exposure (per cent)			0.15
Current	13.1	23.5	
Former	0.0	2.9	
Never	86.9	73.5	

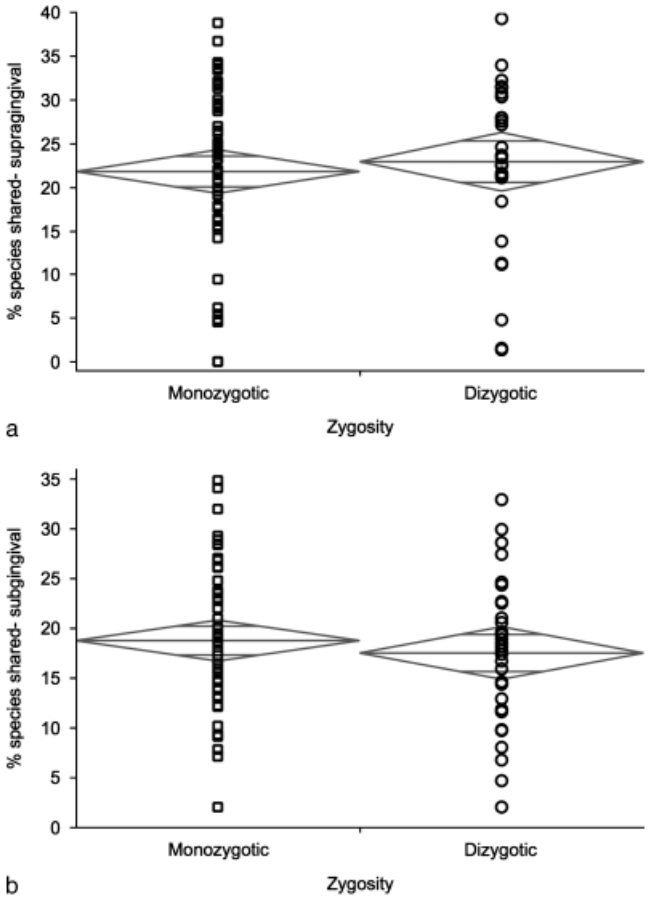

Fig. 1. Percentage of species shared by 61 monozygotic and 34 dizygotic twin pairs in supragingival plaque (a) and subgingival plaque (b). Group means (central lines), standard deviation (small lines) and 95% confidence intervals (height of diamonds) are shown. There were no differences in the percentage of species shared by monozygotic and dizygotic twin pairs ($p > 0.05$, two-sample *t*-test).

Results

Study population

A total of 95 twin pairs were sampled for this study. DNA zygosity revealed 61 pairs to be monozygotic and 34 to be dizygotic. The DNA-based and questionnaire-derived zygosities were concordant in 70 out of the 95 dyads, with a κ-statistic of 0.44 ± 0.09 (standard error) for the degree of agreement. Ten pairs of monozygotic twins and 15 pairs

of dizygotic twins were misclassified using the questionnaire-derived zygosity assessment. Therefore, all analyses were carried out using the DNA-based zygosity assessment.

The demographic characteristics of the sample population are shown in Table 1. There were no differences in gender, race, tobacco exposure or oral hygiene parameters between the two groups ($p > 0.05$, χ^2-test, two-sample *t*-test).

Microbial profiles

In supragingival plaque, an average of 30 and 20 species were detected using *Msp*I and *Hha*I, respectively. In subgingival plaque, an average of 49 and 57 species were detected using the same restriction enzymes. Figure 1 shows the mean percentage of species that were shared by each monozygotic and dizygotic twin pair along with 95% confidence intervals. Supragingival samples are shown in Fig. 1a and subgingival in Fig. 1b. Monozygotic twins shared (mean ± SD) 21.7 ± 10% of species in supragingival plaque and 18.7 ± 7.3% of species in subgingival plaque. The corresponding values for dizygotic dyads were 22.9 ± 9.0% and 17.5 ± 7.3%, respectively. The differences were not statistically significant ($p > 0.05$, two-sample *t*-test).

Figure 2 shows the similarity of the supragingival and subgingival microbial communities between monozygotic and dizygotic twin pairs. The supragingival microbial communities exhibited a mean ± SD similarity of 42.4 ± 22.5% in monozygotic twins and 45.6 ± 23.5% in dizygotic twins. The subgingival communities demonstrated a mean similarity of 36.7 ± 17.4% between monozygotic twin pairs and 32.2 ± 11.6% between dizygotic twins. These differences were not statistically significant ($p > 0.05$, two-sample *t*-test).

Figure 3 shows the effect of age as a modifier of microbial similarity. In the supragingival microbiome, younger individuals shared a higher percentage of species (Fig. 3a) and demonstrated greater similarity in microbial profiles (Fig. 3b) when compared with older individuals ($p = 0.003$ and $p = 0.04$, respectively, regression analysis). However, there was no difference between monozygotic and dizygotic twin pairs ($p > 0.05$). The effect of age was not evident in the subgingival microbial community (Fig. 3c and 3d).

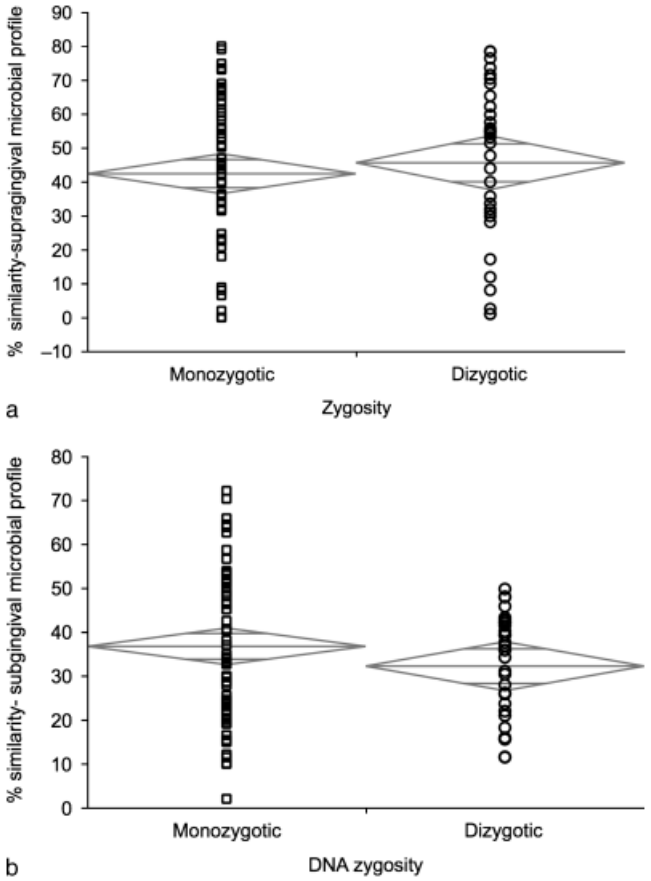

Fig. 2. Per cent similarity of the microbial community between 61 monozygotic and 34 dizygotic twin pairs. The supragingival community is shown in (a) and the subgingival community in (b). Group means (central lines), standard deviation (small lines) and 95% confidence intervals (height of diamonds) are shown. There were no differences in the community similarity between monozygotic and dizygotic twin pairs ($p > 0.05$, two-sample *t*-test).

Figure 4 shows the effect of zygosity on the correlations between supragingival and subgingival plaque. The correlation coefficient between supragingival and subgingival plaque was 0.07 for microbial similarity and 0.12 for shared species. There was no difference in the correlation coefficients between monozygotic and dizygotic twins ($p > 0.05$, regression analysis).

Discussion

In contrast to previous investigations of subgingival bacteria in twins (Bergstrom & Floderus-Myrhed 1983, Moore et al. 1993, Michalowicz et al. 1999), the present investigation examined the influence of host genotype on the composition of *health-compatible* oral bacterial communities. It is known that bacteria form complex, yet stable, health-associated communities in the supragingival and subgingival biofilms (Aas et al. 2005, Kumar et al. 2006). Evidence from host-associated microbial ecosystems in the body suggests that genetics influences the composition of these communities both qualitatively and quantitatively (Stewart et al. 2005, Dicksved et al. 2008, Turnbaugh et al. 2009, Willing et al. 2009). Therefore, the present investigation used an open-ended, quantitative molecular approach to comprehensively examine the supragingival and subgingival microbial communities in periodontally healthy twin pairs. This allowed us to identify a unique microbial profile for each individual that included both cultivated and as-yet-uncultivated organisms in the health-associated oral microbiome, and the results suggest that the contributions of host genotype to the composition of this community are not significant.

Sequence-specific digestion of the 16S rRNA gene results in terminal fragments of varying sizes, as the location of

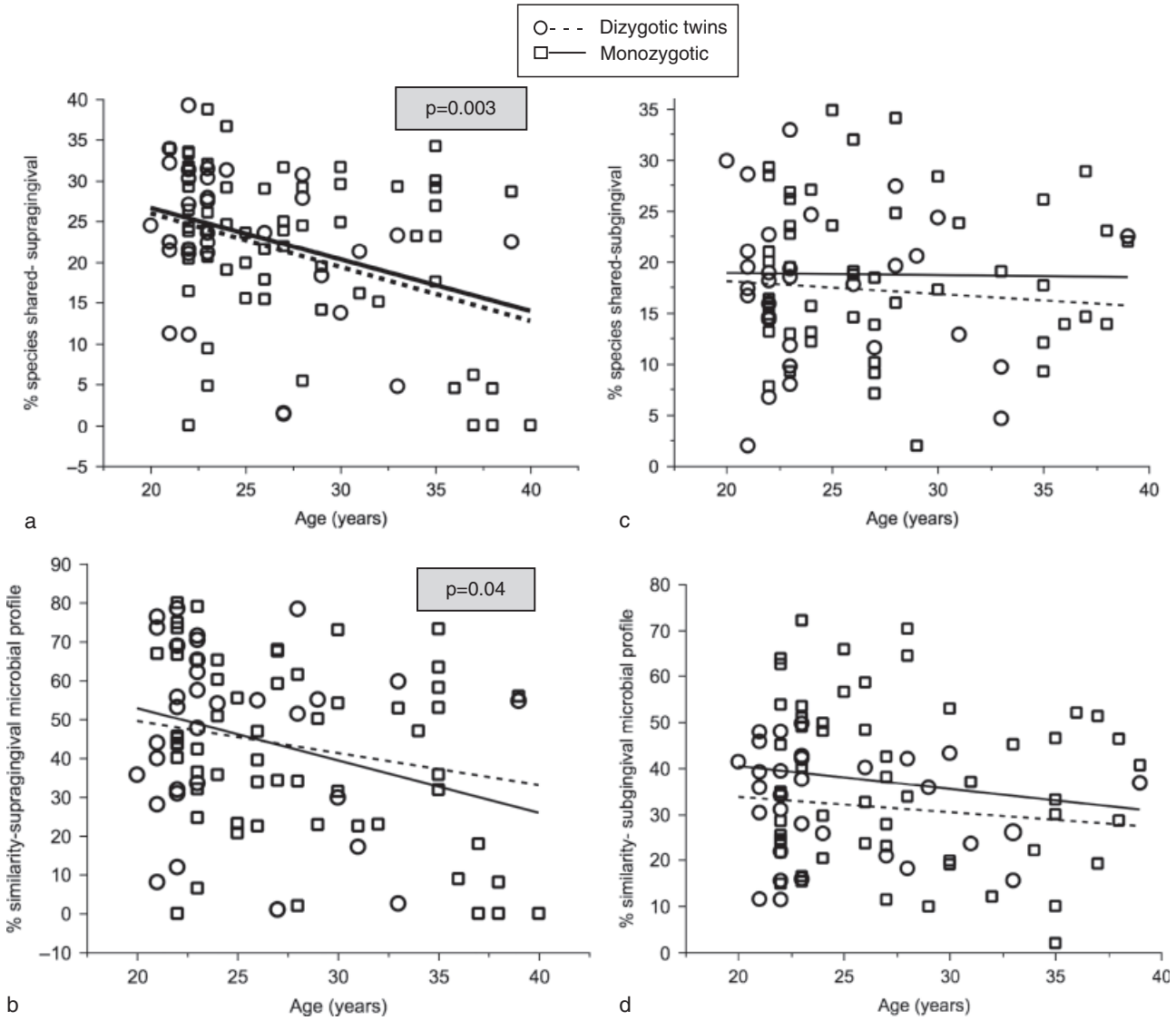

Fig. 3. Age as a modifier of shared species and community similarity. For supragingival plaque, shared species are shown in (a) and community similarity in (b). For subgingival plaque, shared species are shown in (c) and community similarity in (d). Both twin groups exhibited similar changes in shared species and community similarity with age ($p > 0.05$, regression analysis with the interaction term).

the restriction site is dictated by the nucleotide sequence of the gene for each species. Thus, the total number of t-RFs represents the number of unique species in a community, while the area of a terminal fragment measures the level of each species in the community. The average number of species in supragingival and subgingival plaque ranged from 27 to 60 in each individual. This is in concordance with previous investigations on health-associated oral microbial communities (Kroes et al. 1999, Paster et al. 2001, Kumar et al. 2006). An average of 32% of species were shared among all related as well as unrelated individuals (data not shown), indicating that, in a state of health, a core microbiome exists in all individuals. This finding corroborates a previous investigation indicating the presence of a health-associated core microbiome (Zaura et al. 2009). There were no differences in the total number of species shared by monozygotic and dizygotic twins in either supragingival or subgingival plaque (Fig. 1), suggesting that the effect of genetics on the type of bacteria that colonize dental plaque is not evident in a mature, stable bacterial community. Further, age did not appear to modify the effect of genetics on the number of species shared (Fig. 3), and correlations between shared species in supragingival and subgingival plaque were not different between the twin dyads (Fig. 4). However, age was consistently associated with a decrease in the number of shared supragingival species, suggesting that with increasing age, each individual may acquire a more unique supragingival flora, a finding that is congruent with reported age-related differences in the health-associated supragingival oral flora (Percival et al. 1991). Further studies are warranted to corroborate this finding. Environmental factors such as smoking also did not play a modifying role in the genetic influence (data not shown). Taken together, the data indicate that the qualitative effects of host genotype on supragingival and subgingival microbial communities cannot be discerned

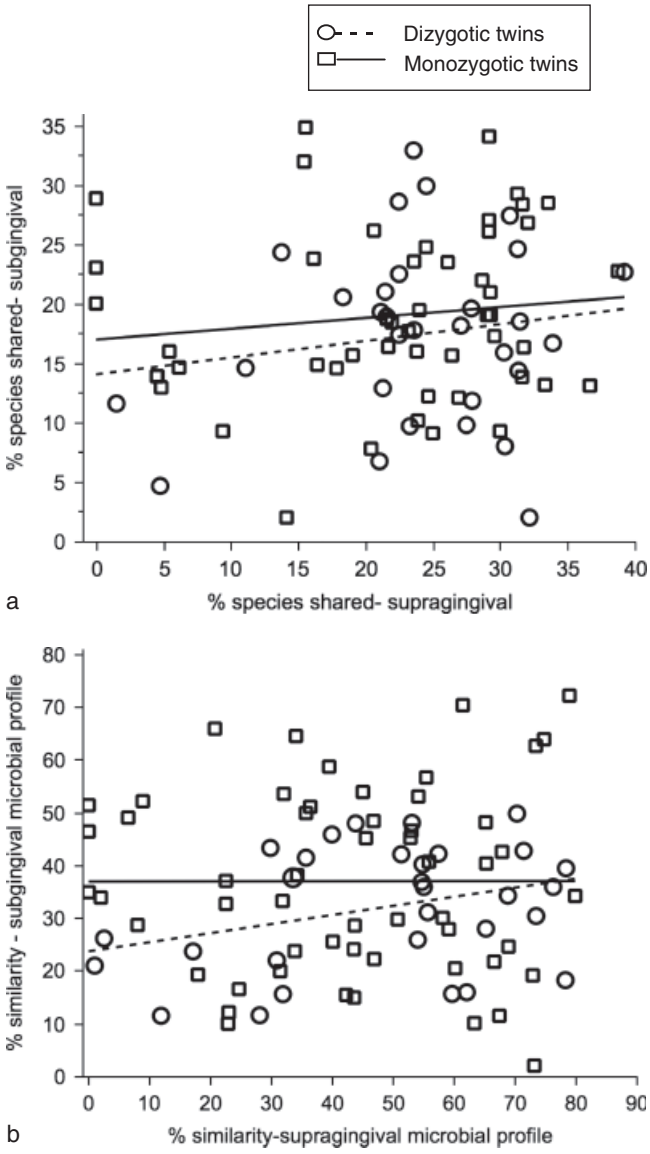

Fig. 4. Correlation between supragingival and subgingival microbial profiles in 95 twin pairs. Correlations between shared species in supragingival and subgingival plaque are shown in (a) and community similarity in (b). There were no differences between the twin groups in correlations between supragingival and subgingival microbial profiles ($p > 0.05$, regression analysis with the interaction term).

following the formation of a climax community.

A recent evaluation of existing genetic studies in the periodontal literature concluded that the main drawbacks of these studies were small sample sizes, as well as examinations of selected response variables, for example, single genetic variants or haplotype blocks (Schafer et al. 2011). Therefore, the goal of the present study was to investigate the influence of host genotype on health-compatible supragingival and subgingival communities as a whole without an emphasis on selected microbial species. t-RFLP has been used to examine the microbial profiles of complex communities, however, underestimation of the number of species is possible by fragment analysis, as closely related species may share common restriction sites (Liu et al. 1997). Further, species of low abundance may not be consistently represented in the t-RF profile (Liu et al. 1997). Thus, any possible effect of genetics on colonization by numerically minor species may have been masked using this approach. Therefore, it is important to corroborate these results using an open-ended molecular approach to characterize and quantify numerically dominant and minor species in the community.

The Bray–Curtis similarity index estimates both the number of species as well as the levels of species common to two communities, thereby providing a composite similarity profile based on species evenness and richness. The supragingival and subgingival microbial communities were equally similar between monozygotic and dizygotic twin pairs, indicating little or no role for genetics in the composition of the apex community. Again, age did not appear to modify the effect of genetics and the correlation between microbial similarities in the two microbiomes was not influenced by zygosity. A similar lack of genetic influence on selected bacterial species in subgingival plaque has been reported in adolescent and adult twins with periodontal disease (Moore et al. 1993, Michalowicz et al. 1999). However, previous investigations on the supragingival microbiome in children have indicated that the levels of certain bacteria are heritable (Bretz et al. 2005, 2006, Corby et al. 2007). It has been shown that inter-bacterial interactions play a critical role in the development and formation of a stable biofilm community (Kolenbrander et al. 1989, Kolenbrander & Palmer 2004). While it is possible that genetics mediates early plaque colonization, this effect may be superceded by bacterial and environmental factors as the biofilm community matures. Further studies are warranted to examine the extent of genetic influence on bacterial acquisition during early plaque formation.

Questionnaire-based zygosity assessments have been extensively used since the inception of the Danish twin registry in the 1970s and it has been reported that these assessments have a validity of 95% or greater (Kaprio et al. 1978, Chen et al. 1999, Jackson et al. 2001, Christiansen et al. 2003). However, our results reveal approximately 75% agreement between the questionnaire and the DNA-based zygosity assessments, with the greatest misclassification occurring for dizygotic twins. This suggests that DNA-based zygosity assessments are important for accurate classification, and forms the rationale for using this assessment in the present study.

In summary, a cross-sectional open-ended molecular analysis of the subgingival and supragingival microbiomes of periodontally healthy twins reveals no

evidence of a genetic contribution to the composition of these communities. Further studies are indicated to examine the effect of genetics on bacterial acquisition during early plaque colonization as well as during the development of the microbiome in early childhood.

Acknowledgements

The authors are grateful for the assistance of Dr. Huei-Ling Chang in the clinical assessment of the recruited subjects, and the Plant Microbe Genomics Facility for zygosity assessments. The authors thank the Twins Days Festival in Twinsburg, Ohio, for facilitating the recruitment of subjects.

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Address:
Purnima Kumar
4111 Postle Hall
305, W 12th Avenue
Columbus
Ohio 43210
USA
E-mail: kumar.83@osu.edu

Clinical Relevance

Scientific rationale for the study: It is known that the host genotype contributes to susceptibility to periodontal diseases and caries; however, the extent to which genetics influences colonization by oral bacteria, which are the primary aetiological agents of these diseases, is not well understood.

Principal Findings: The profiles of the supragingival and subgingival microbiomes were similar between monozygotic and dizygotic twin pairs.

Practical implications: Host genotype does not appear to influence the composition of health-associated supragingival and subgingival microbiomes in a qualitative or a quantitative manner, suggesting that environmental factors play a greater role in determining the composition of these bacterial communities.