The Dental Plaque Microbiome in Health and Disease

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Abstract

Dental decay is one of the most prevalent chronic diseases worldwide. A variety of factors, including microbial, genetic, immunological, behavioral and environmental, interact to contribute to dental caries onset and development. Previous studies focused on the microbial basis for dental caries have identified species associated with both dental health and disease. The purpose of the current study was to improve our knowledge of the microbial species involved in dental caries and health by performing a comprehensive 16S rDNA profiling of the dental plaque microbiome of both caries-free and caries-active subjects. Analysis of over 50,000 nearly full-length 16S rDNA clones allowed the identification of 1,372 operational taxonomic units (OTUs) in the dental plaque microbiome. Approximately half of the OTUs were common to both caries-free and caries-active microbiomes and present at similar abundance. The majority of differences in OTU’s reflected very low abundance phylotypes. This survey allowed us to define the population structure of the dental plaque microbiome and to identify the microbial signatures associated with dental health and disease. The deep profiling of dental plaque allowed the identification of 87 phylotypes that are over-represented in either caries-free or caries-active subjects. Among these signatures, those associated with dental health outnumbered those associated with dental caries by nearly two-fold. A comparison of this data to other published studies indicate significant heterogeneity in study outcomes and suggest that novel approaches may be required to further define the signatures of dental caries onset and progression.


Editor: Sarah K. Highlander, Baylor College of Medicine, United States of America

Received October 9, 2012; Accepted February 5, 2013; Published March 8, 2013

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Funding: This project has been supported with funds from the J. Craig Venter Institute and with Federal funds from the National Institute of Dentistry and Craniofacial Research (NIDCR), National Institutes of Health (NIH), Department of Health and Human Services, under Grant No. NIH-1R01DE019665-01 and partially funded by NIDCR-NIH grant DE15351. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Dental caries is the single most common disease in childhood with a prevalence rate five times higher than the next most prevalent disease, asthma [1]. Dental caries remains a significant public health issue in children worldwide. Over 50% of children in the United States 5 to 9-years-old have at least one cavity or filling, and that increases to 78% among 17-year-olds [2]. These adverse outcomes are disproportionately present in disadvantaged segments of the United States population including children, adults and the elderly. It has been shown that dental care is the most common unmet need among children in the United States [3,4].

Microbiological strategies to identify associations related to dental caries are limited due to the lack of highly selective media formulations and the uncultivability of many oral species. These approaches have implicated mutans streptococci and Lactobacillus spp., as signatures of childhood dental caries [5–10]. The more recent use of culture-free methods has alleviated many of these barriers. Surveys of microbial diversity in the human oral microbiota based on culture-free, cloned analysis of 16S rDNA gene sequence and phylogeny have estimated that greater than 700 microbial species inhabit the oral cavity [11–16]. The amount of 16S rDNA profiling data pertaining to the dental caries disease model is limited but growing rapidly [3,10,17–19]. Becker and colleagues [18] and Aas et al., [17] used 16S rDNA analysis of caries-free and caries-active children and adults to reveal species associated with dental health such as Streptococcus sanguinis and genera associated with dental caries in both primary and permanent dentitions such as Streptococcus mutans and low-pH, non-mutans streptococci, Veillonella spp., Actinomyces spp., Bifidobacterium spp., Lactobacillus spp., Propionibacterium spp., and Atopobium spp. Munson et al., [10] and Chhour et al., [5] reported that the predominant microbes in a small number of adults with advancing, deep caries lesions were S. mutans and Lactobacillus spp. but also included the genera Prevotella, Selenomonas, Dialister, Fusobacterium, Bifidobacterium, and Pseudoribobacter [5,10]. Corby and colleagues employed 16S rDNA profiling of dental caries-associated flora in infants and children who were caries-active (n = 86) and caries-free (n = 118) [19]. S. mutans, Actinomyces spp., and Lactobacillus spp. were over-abundant in caries-active subjects whereas beneficial species associated with dental health included Streptococcus parasanguinis, Alistipes defectivus, Streptococcus mitis, Streptococcus oralis, and S. sanguinis. Taken together, the number of species/OTUs identified in these studies ranged from 75 to 197.

More recent studies have surveyed various domains in the oral environment in greater depth [15,20,21]. These surveys have shed light on the high-level of species diversity present in oral bacterial communities associated with both health and disease. A study focused on establishing associations of severe early childhood caries (ECC) using the 454 sequencing platform identified taxa...
associated with caries including *Granulicatella elegans, Veillonella atypica, Veillonella spp.*, *Streptococcus cristatus, Capnocytophaga gingivalis, Eubacterium
diseases. The role of these communities in maintaining oral health, disease onset and progression.
how these communities contribute to pH homeostasis, maintaining oral health and disease.
signatures may ultimately be needed to define a C-F and C-A
dynamic range of individual species and genome abundance.
or the depth of clonal analysis. We elected to conduct a deep survey using
Sanger sequencing of nearly full-length 16S rDNAs in order to
to better understand the distinctions between microbial biofilms derived from caries-free (C-F) and caries-active (C-A)
dental plaque. We employed a normalization strategy to enable the observation of less abundant members of the dental plaque community. Our study examined dental plaque derived from children 5–7 years of age from a single geographical region. It remains to be seen to what extent the dental plaque microbiome
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accurate characterization of dental caries phenotypes in C-F and C-A subjects. These included: 1) Clinical examination of dental caries in all teeth, assessed with the aid of artificial light and a dental mirror according to NIDCR criteria [24] to include white spot lesions and cavitated lesions; 2) Digital imaging fiberoptic trans-illumination (DIFOTI) recorded images of dental lesions (incipient and frank lesions) to complement the caries clinical examination [25]; 3) Quantitative light fluorescence (QLF) profiled images of dental lesions [26] similar to the DIFOTI procedure that are not readily captured by visual examinations and complemented the caries clinical examination. C-A subjects had an average of 12 (range = 6–17) decayed tooth surfaces whereas C-F subjects presented with a decay component = 0 (Figure 1).

**Materials and Methods**

**Dental Caries Phenotype Determination**

Dental caries examinations were performed on 8 subjects (5 female, 3 male) that were C-A (n = 4) and C-F (n = 4) respectively. These subjects (5–7 years old) were medically healthy and presented with only primary dentition. This group of children resides in the suburbs of the city of Montes Claros, State of Minas Gerais, Brazil. Water fluoride levels in this city are less than
doctoral (0.7 ppm) and dental check-ups for this group were negligible.

**Ethics Statement**

Parents signed informed consent approved by New York University and UNIMONTES (State University of Montes Claros) institutional review boards after the children assented.

**Dental Caries Examinations**

We used a combination of three dental caries exams for accurate characterization of dental caries phenotypes in C-F and C-A subjects. These included: 1) Clinical examination of dental caries in all teeth, assessed with the aid of artificial light and a dental mirror according to NIDCR criteria [24] to include white spot lesions and cavitated lesions; 2) Digital imaging fiberoptic trans-illumination (DIFOTI) recorded images of dental lesions (incipient and frank lesions) to complement the caries clinical examination [25]; 3) Quantitative light fluorescence (QLF) profiled images of dental lesions [26] similar to the DIFOTI procedure that are not readily captured by visual examinations and complemented the caries clinical examination. C-A subjects had an average of 12 (range = 6–17) decayed tooth surfaces whereas C-F subjects presented with a decay component = 0 (Figure 1).
Dental Plaque Biofilm Sampling

Subjects were instructed to refrain from brushing or eating prior to sampling. Dental plaque samples were obtained using a sterile toothbrush passed slowly across all tooth surfaces in order to maximize sample collection. We elected to collect an overall plaque sample of the entire dentition rather than sampling site-specific surfaces that are associated with health or disease to enable characterizations that would otherwise be biomass limited. Moreover, our previous studies demonstrate that the dental microbiota associated with localized healthy tooth surfaces and caries lesions are similar within the same oral cavity [19]. Dental plaque was dislodged from the toothbrush by agitation for 1 minute into tubes containing 8 mL of sterile reduced transport fluid (RTF) [27] held at 4°C prior to storage at −80°C.

Genomic DNA Isolation from Dental Plaque

Dental plaque samples were thawed and 1 mL of the cell mixture was used for DNA isolation as described by Turnbaugh et al., [28]. In order to achieve uniform lysis of cells we used mechanical disruption with a bead beater (BioSpec Products, Bartlesville, OK) in the presence of 2% SDS, phenol:chloroform and 0.1-mm-diameter zirconia/silica beads (BioSpec Products). The lysate was extracted one additional time and genomic DNAs were precipitated with isopropanol. The genomic DNAs were further purified using the MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). The genomic DNA was maintained at 65°C for 5 minutes, NaCl was added to 0.5 M, and then held at 65°C. Hydroxyapatite (HA) columns were prepared using ~20 cm³ of resin (BioRad, Irvine CA) and were maintained at 65°C using a water jacket for optimal binding and nucleic acid migration. The columns were equilibrated successively on agarose gels and quantitated using a UV spectrophotometer. The average yield of genomic DNA from plaque samples was ~100 μg/subject. The yields of DNA from each dental plaque were non-limiting and similar in C-F and C-A subjects.

Re-association Kinetics Enrichment (RAKE) of Moderate and Low Abundance Genomic DNAs

We combined genomic DNAs (10 μg each) isolated from 4 C-A and 4 C-F subjects to create two reference pools (C-A and C-F). The C-A and C-F genomic DNA pools in re-association buffer (50 mM HEPES, pH 7.5, 200 μM EDTA) were sheared to 3–8 Kb by nebulization. The samples were heat denatured in a volume of 10 μl at 95°C for 5 minutes, NaCl was added to 0.5 M, and then held at 65°C. Hydroxyapatite (HA) columns were prepared using ~20 cm³ of resin (BioRad, Irvine CA) and were maintained at 65°C using a water jacket for optimal binding and nucleic acid migration. The columns were equilibrated sequentially with 10 mL of sodium phosphate buffer (SPB); Wash 1 (480 mM SPB), followed by wash 2 (120 mM SPB) and wash 3 (30 mM SPB) all at 65°C. At various timepoints (0–60 hours), the DNAs were diluted 100-fold in 30 mM SPB and loaded onto hydroxyapatite columns to separate and recover ssDNA and dsDNA. The ssDNA was eluted using 120 mM SPB followed by elution of dsDNA with 480 mM SPB. The DNAs were then precipitated, washed, resuspended in TE, and quantitated using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). This procedure resulted in four distinct genomic DNA fractions from both C-F and C-A genomic DNA pools: 1. non-enriched, 2. 64-fold enriched (4 hours re-association), 3. 1,000-fold enriched (24 hours re-association) and 4. 10,000-fold enriched (60 hours re-association). These determinations were made by performing qPCR and comparing the number of cycles required for ssDNA and dsDNA fractions to cross the fluorescence threshold.

16S rDNA Amplicon and DNA Sequence Generation

The recovered ssDNA fractions were used as a template in PCR reactions using the universal primers 8F/1492R to generate nearly full-length 16S rDNA amplicons. The 16S rDNA amplicons were cloned into a TOPO cloning vector, p2EZeo (Invitrogen Inc, Carlsbad, CA) to generate 8 libraries for random shotgun sequencing as follows: non-enriched C-F and C-A, 64-fold enriched C-F and C-A, 1,000-fold enriched C-F and C-A, and 10,000-fold enriched C-F and C-A. All clones were sequenced using the Sanger method run on an ABI 3730 XL sequencing instrument. The average read length of clones (paired-end reads) was 992 bases. Eight libraries, two of which were sequenced in two independent sequence runs were submitted to the trace archive division at GenBank with the following accessions: C-F unenriched: 2334489092–2334499005, C-A unenriched: 2334532826–2334550317, 269128 C-F unenriched: 2334458738–2334468399, CA unenriched: 2334512867, CF 64-fold enriched: 2331272350–2331277318, CA 64-fold enriched: 2331279180–2331284211, CF 1000-fold enriched: 2334460400–2334489001, CA 1000-fold enriched: 2334512868–2334521646, CF 10,000-fold enriched: 2331270338–2331272349, CA 10,000-fold enriched: 2331277319–2331279179.

16S rDNA Phylogenetic Analysis and OTU Determination

The 16S rDNA paired-end sequences were processed using the JCVI internal 16S rDNA data analysis pipeline. Briefly, short reads, vector sequences, and sequences of poor quality were filtered. Sequences were then screened for possible chimeras using Chimera Slayer [29] and were removed from further analysis. We next clustered the sequences using the cd-hit-est based clustering method [30]. Operational taxonomic units (OTUs) were defined using a 97% sequence similarity cutoff, roughly corresponding to species-level groupings. Sequences were grouped into distinct OTUs using Felsenstein-corrected similarity matrices such that the least similar pair within an OTU shared at least 97% similarity across its length. The Ribosomal Database Project (RDP) Classifier [31] was used to classify the 16S rDNA sequences into the the most detailed taxonomic category by aligning 16S rDNA sequences to a curated database of taxonomically annotated sequences at the resolution of genus-level assignments. In order to achieve taxonomic assignments to the species level, all of the 16S rDNA sequences were mapped to the curated 16S rDNA database (RDP v10.0) using BLASTN. Sequences with less than 97% similarity to sequences in the RDP database were considered novel reads, whereas those with greater than 97% identity were used to associate a group of OTUs to specific species. An OTU is considered to be a novel OTU only if all members belonging to that OTU failed to be assigned to a known OTU.

Statistical Analysis

The relative abundance of each OTU was computed by dividing the counts of individual OTUs associated with each pool by the total number of OTUs identified from the 16S rDNA profiling. We thus established a relative species frequency for the C-F and C-A pools. A standard z-test of the equality of frequencies was used to determine the significance of the difference in relative OTU frequencies between the C-A and C-F pools. Given that multiple OTUs were assessed for frequency differences, we accommodated the resulting multiple comparisons by computing false discovery rates [32,33] (http://cran.r-project.org/web/packages/qvalue/).
Results

We performed a deep survey of the microbial composition of the dental plaque microbiome derived from children (ages 5–7) that were either C-F or C-A as determined by clinical examination, DIFOTI and QLF for accurate phenotype characterization to establish its population structure and to enable a comprehensive comparison of the species/OTUs present in C-F and C-A subjects (Figure 1). In total we sequenced 53,038 16S rDNAs derived from C-F and C-A pooled samples yielding 47,198 clone sequences (C-F = 21,705 and C-A = 25,493) (Table 1). The majority of clones analyzed (66%) were from unnormalized libraries, whereas additional sequencing was performed on clones derived from normalized libraries using a procedure we refer to as Re-Association Kinetic Enrichment (RAKE), a normalization procedure that is based on C_{ot} analysis, wherein the re-association rate of denatured double stranded DNAs is dependent on the concentration of DNA sequences in the mixture (see Materials and Methods). This allowed us to perform a more comprehensive analysis of dental plaque microbiota.

The combined (C-F and C-A) results of the 16S rDNA analysis indicate several interesting points regarding the diversity and community structure of the dental plaque microbiota. In total, we identified 1,372 OTUs, 984 OTUs in the C-F microbiome and 1,014 OTUs in the C-A microbiome. This is by far the largest number of OTUs identified in the dental plaque microbiome using full-length 16S rDNA sequence data. The data indicate that the species present in the dental plaque microbiome span at least four orders of magnitude in abundance. A total of 626 OTUs were common to both microbiomes. A total of 375 OTUs were observed in ten or more clone sequences. The OTUs derived exclusively from C-F (358 OTUs) or C-A (388 OTUs) microbiomes were generated from 790 and 750 16S rDNA clones respectively, indicating that these OTUs correspond to very low abundance phylotypes. This observation indicates that the primary difference between C-F and C-A subjects is not related to the "appearance" of unique phylotypes, but rather the altered difference between C-F and C-A subjects is not related to the abundance phylotypes. This observation indicates that the primary difference between C-F and C-A subjects is not related to the abundance phylotypes.

Taxonomic Characterization of the Dental Plaque Community

The microbial diversity present in dental plaque is not randomly distributed among bacterial taxa. Among the ~70 previously defined phyla, only ten were observed in our survey. Moreover only four of these phyla are present in abundance greater than 1% of the total. The dominance of the Firmicutes is striking and comprises more than 82% of the surveyed community. Nearly 66% of the Firmicutes represent Streptococcus spp., corresponding to a large number of named and uncharacterized phylotypes. This observation reflects the massive expansion of this genus in dental plaque biofilms and emphasizes its overall importance with respect to the biochemical flux and maintenance of homeostasis of the biofilm community. The next most abundant phylum is Proteobacteria (11%) followed by Bacteroidetes (3%) and Fusobacteria (2%). Additional phyla observed at less than 1% included: Actinobacteria, Cyanobacteria, Deferribacteres, Spirochaetes, SR1 and TM7. Despite the large number of OTUs identified, these belonged to a limited number of only 36 genera (5 or more clones, ~0.01%). Only 10 genera were observed at high abundance (>1%). These included: Streptococcus (52%), Veillonella (8.5%), Granulicatella (7.2%), Fusobacterium (5.6%), Neisseria (5.5%), Campylobacter (4.7%), Gemella (3.7%), Abiotrophia (2.6%), Selenomonas (1.3%) and finally Capnocytophaga (1%). It is potentially interesting to note that excepting the Streptococcus, the most abundant genera display a narrow range of relative abundance (2.1–4%).

Population Structure of the Dental Plaque Microbiota

The 1,372 OTUs observed corresponded to 205 named species and 44 genus level assignments (Figure S1). We observed two dominant species present in the dental plaque samples profiled here, S. mitis (25.5%) and S. sanguinis (9.1%). There were 14 additional species present at abundance levels greater than 1% in the combined data. These included: Veillonella paracasei (7.5%), Streptococcus oris (6.1%), Neisseria subflava (3.0%), Gemella haemolysans (2.8%), Granulicatella elegans (2.6%), Streptococcus gordoni (2.6%), Abiotrophia defectiva (2.5%), S. cristatus (1.9%), C. gracilis (1.8%), C. shoaeae (1.4%), S. infantis (1.3%), S. constelatus (1.3%), S. mutans (1.2%) and C. concisus (1.1%). This composition is consistent with data generated as part of the HMP consortium surveys [34–36]. Collectively, these 16 species comprise approximately 71% of the bacterial biomass of the dental plaque biofilms surveyed here. The biochemical flux occurring in dental biofilms is strongly influenced by the metabolic activities of these species. We examined an additional 129 OTUs representing moderately abundant OTUs (0.1–1%) and found that it reflected a similarly high representation of Streptococcus spp. and included a disproportionately large number of Proteobacteria, namely Neisseria spp. and Campylobacter spp. We observed ~400 OTUs that we classify as low abundance phylotypes (0.01–0.1%). The remaining ~800 OTUs observed represent very low abundance phylotypes, present at <0.01% of the total microbiome. The depth of our sequencing efforts limit the extent that we can project the true size and diversity of the very low abundance species constituting the long tail of the population structure. The population structure of the dental plaque microbiome therefore appears to represent a very small group of dominant species that display only modest phylogenetic diversity (seven genera) followed by approximately one hundred moderately abundant species with increased phylogenetic diversity. Finally, the less abundant phylotypes define the highest level of phylogenetic diversity in the population.

Differential Abundance of Genera Associated With C-F and C-A Microbiota

We examined the similarities and differences of genera present in C-F and C-A subject pools as depicted in Figure 2. Nearly two-thirds of the observed genera are over-represented in the C-F microbiome, however the four most abundant genera including Streptococcus spp., Veillonella spp., Campylobacter spp. and Neisseria spp. are all over-represented in the C-A subject pool. The observed differential representation of these genera is small in each case but potentially meaningful given the high abundance of these genera in the community. Among the moderately abundant genera, virtually all are over-represented in the C-F microbiota except for the Actinomyces spp. and Eubacterium spp. The last group of genera,

Table 1. 16S rDNA Libraries for C-F and C-A Pools.

<table>
<thead>
<tr>
<th>Library</th>
<th>C-F</th>
<th>C-A</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-enriched</td>
<td>13,530</td>
<td>18,295</td>
<td>31,825</td>
</tr>
<tr>
<td>64-fold enriched</td>
<td>1,618</td>
<td>1,667</td>
<td>3,285</td>
</tr>
<tr>
<td>1,000-fold enriched</td>
<td>6,052</td>
<td>4,991</td>
<td>11,043</td>
</tr>
<tr>
<td>10,000-fold enriched</td>
<td>505</td>
<td>540</td>
<td>1,045</td>
</tr>
<tr>
<td>Total</td>
<td>21,705</td>
<td>25,493</td>
<td>47,198</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0058487.t001

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representing those of low abundance display greater variability when comparing C-F and C-A microbiota.

**Taxonomic Analysis of Differentially Abundant Phylotypes in C-F and C-A Microbiota**

We compared the species profile of the C-F and C-A subject pools to identify differentially abundant phylotypes. The most compelling observation when comparing the OTUs derived from C-F and C-A dental plaque is the remarkable uniformity in abundance of the vast majority of represented phylotypes. This uniformity is likely to reflect a strong selective pressure that has been applied over millions of years of co-evolution that acts to maintain the species homeostasis of the dental plaque microbiome. We used standard z-tests of equality of phylotype frequencies between C-A and C-F subject pools to identify those exhibiting the greatest differences. False discovery rate calculations suggested that 87 phylotypes were strongly associated with either caries health or disease and these species had less than a 1% chance of being a false positive result despite the multiple comparisons ($p < 0.05$).

**Figure 3** provides a Volcano plot depicting the probabilistic significance of the frequency differences (-log p-values from the z-tests) as a function of the standardized difference in phylotype abundances. It can be seen that for some phylotypes, the abundance differences are very large and have a very low probability of having occurred by chance.

We analyzed the relative abundance of phylotypes present in C-F and C-A subject pools. In total we identified 87 differentially represented phylotypes comparing C-F and C-A 16S rDNA profiles (Table 2 and Table S1). It is of note that among the most abundant species in dental plaque biofilms, all (16 phylotypes >1% abundance) were differentially abundant in C-F compared to C-A microbiomes (Figure 4). Moreover, 11 of the 16 species were over-represented in the C-A subject pool. This observation is in contrast to the moderately abundant phylotypes (0.1–1%) where the number of species over-represented in the C-F subject pool is 26 compared to 10 phylotypes over-represented in the C-A subject pool and 14 phylotypes that displayed no significant difference in abundance.

**Actinobacteria.** Four members of this taxon were differentially abundant in our survey. *Propionibacterium propionicum* was exclusively observed in the C-F subject pool, whereas *Corynebacterium matruchotii* was exclusively observed in the C-A subject pool. Two *Actinomyces*, *A. naeslundii* and an uncharacterized species were both over-represented in the C-A subject pool.
Bacteroidetes. Among the twelve members of this phylum that were differentially represented, all but two were over-represented in the C-F subject pool, namely Prevotella auricular and Prevotella salivae. The remaining members of this group include: two Porphyromonas spp.: one uncharacterized species and P. catoniae, seven Prevotella spp. including P. tannae, one uncharacterized phylotype, P. nanceiensis, P. saccharolytica and P. intermedia and finally three Capnocytophaga spp. C. leadbetteri, C. spinitoga and C. granulosa.

Firmicutes: Gemella, Abiotrophia, Granulicatella. Four Gemella phylotypes were differentially abundant and all were over-represented in the C-F subject pool, including: G. haemolysans, G. sanguinis, G. morbillionum and G. bergeri. Two Abiotrophia spp. were both over-represented in the C-F subject pool including A. defectiva and an uncharacterized Abiotrophia spp. Two Granulicatella spp. were differentially abundant in our survey, G. elegans was over-represented in the C-F subject pool, whereas G. adjacens was over-represented in the C-A subject pool.

Firmicutes: Veillonella, Eubacterium, Parvimonas, Mogibacterium, Catonella, Peptostreptococcus, Centipeda, Dialister, Megaplasma, Mitsuokella, Selenomonas, Solobacterium. Among the four differentially abundant Veillonella spp. observed, two were over-represented in the C-F subject pool including: V. criceti and an uncharacterized phylotype. The remaining two phylotypes, V. parvula and an uncharacterized phylotype were over-represented in the C-A subject pool. Among the four differentially abundant Eubacterium spp. three were over-represented in the C-F subject pool including: E. brachy, E. yurii and an uncharacterized Eubacterium spp. E. saburreum was over-represented in the C-A subject pool. Parvimonas micro, Mogibacterium timidum, Catonella morbi, Peptostreptococcus stomatis, Centipeda periodontii, Dialister pneumosintes and Solobacterium moorei were all over-represented in the C-F subject pool. Megaplasma micro-nucleiformis and an uncharacterized Mitsuokella spp. were over-represented in the C-A subject pool. Finally, three Selenomonas spp. were differentially abundant, S. infelix and S. dianae were over-represented in the C-F subject pool, and an uncharacterized Selenomonas spp. was over-represented in the C-A subject pool.

Firmicutes: Streptococcus. Among the 18 differentially abundant phylotypes in this genus only four were over-represented in the C-F subject pool including: S. infantis, S. australis, S. angigerfermentans, and S. svis. The remaining 14 phylotypes were over-represented in the C-A subject pool including: S. sanguinis, S. mutans, S. sbovis, S. mitis, S. intermedius, S. gordoni, S. parasanguinis, S.
**Discussion**

We developed a strategy (RAKE) to enrich 16S rDNA sequences through sequential removal of the most abundant nucleic acid species. We exploited the re-association kinetics of the dental plaque microbiome to sub-divide the re-associating DNAs into separate fractions prior to application of 16S rDNA sequence profiling. Compared to more traditional strategies that apply brute force sequencing to native metagenomic samples, the application of RAKE allowed a deeper and therefore more comprehensive characterization of the bacterial community. This method may also be applied to metagenomic DNAs where 10–100 fold normalization can be achieved (SNP, unpublished observation).

The application of RAKE resulted in a near doubling of the number of OTUs identified.

**Genera of Dental Plaque Biofilms: Differential Abundance in C-F and C-A Subjects**

The data summarized in Figure 2 indicate that the majority (24 of 36) of genera present in dental plaque biofilms are over-represented in the C-F subject pool, whereas only 12 are over-represented in the C-A subject pool. Members of the genus *Streptococcus* are dominant in dental plaque biofilms, ten times more prevalent than the next most abundant genera. The modest over-representation of this genus in the C-A subject pool, is biochemically significant given the numerical dominance of this genera and its fermentative lifestyle and directly impinge on acid production. The next most highly represented genera included two Proteobacteria, *Campylobacter* and *Neisseria*, along with *Gemella* and the *Veillonella*. All but the *Veillonella* were over-represented in the C-F subject pool. Taken collectively, these five genera constitute a contributing component of the interpersonal variability of microbial communities.

**Figure 4. Comparison of C-F and C-A Species Abundance.** The 16 most abundant species and their relative abundance in C-F and C-A subject pools.

doi:10.1371/journal.pone.0058487.g004
~75% of the biofilm’s microbial biomass. The metabolic activities of species over-represented in the C-F subject pool are of potential interest as they are likely to play an important role in maintaining microbial composition and pH homeostasis. The biochemical basis underlying the caries phenotype is related to the balance of the metabolic pathways related to acid production, acid re-mediation via metabolism of strong acids (lactate) to weaker acids and finally, base production, generally in the form of microbially produced ammonium ions, carbonate or arginine [29,37,38]. The Veillonella are known to metabolize lactic acid to weaker acids. The fact that our survey indicates that Veillonella spp. are generally associated with heightened abundance in C-A subject pool suggests that they may serve as an “acid sink” in the ecosystem, allowing acidogenic species to continue to produce additional acid at a high rate through sugar metabolism and defines a significant example of caries-related mutualism. The remaining genera are mixed in their over-representation but the majority are over-represented in the C-F subject pool.

Differential Species Abundance as Signatures of Dental Health and Disease

Despite the overall uniformity of phylotype representation in the C-F and C-A microbiota, we observed 87 phylotypes that were differentially represented in a statistically significant manner. Fourteen of the 87 differentially abundant phylotypes observed were found exclusively in either the C-F or C-A microbiome, however each instance corresponded to very low abundance phylotypes (<0.01%). We suggest that shifts in the community structure involving abundant species are more likely to be of biological significance with respect to caries. The significance to the observed differences in abundance of low abundance species from a biochemical perspective is not clear. The phylotypes under-represented in C-A microbiomes may reflect their acid sensitivity and therefore represent useful diagnostic indicators of the pH trends in the dental plaque environment. The remaining 73 statistically significant differentially abundant phylotypes corresponded to a broad range of abundance. Slightly more than 50% of the differentially abundant phylotypes (52) were present at an abundance of 0.1% or higher.

The differentially represented Actinobacteria were predominantly (75%) over-represented in the C-A subject pool. These findings are consistent with a number of previously published findings [17–19]. Corynebacterium matruchotii is capable of both sugar utilization and the metabolism of organic acids like lactate and acetate [39]. The latter capability may explain their over-representation in the C-A microbiota. The relative balance of these activities in the context of dental biofilms has not been reported. This species has been previously described as a health-associated species, in contrast to our observations [23]. The differential representation of Bacteroidetes included a larger number of species. Ten of the twelve representative species were over-represented in the C-F subject pool. A group of three moderate-abundance (0.2–0.4%) Capnocytophaga spp. were over-represented in the C-F subject pool and may represent candidate signatures of dental health, consistent with a previous report associating C. gingivalis with dental health.

The high relative abundance of Streptococcus in our survey (52%) is somewhat higher than observed in other studies. One possible explanation for this disparity is that the dental biofilm communities were derived from subjects with limited fluoride exposure. We have observed similar results (48% Streptococcus) during an analysis of an adolescent cohort from Puerto Rico where fluoride was also absent in the drinking water (SNP, unpublished data). The differential representation of species belonging to the phylum Firmicutes were by far the most diverse, including 16 member genera. All four of the differentially represented Gemella spp. were over-represented in the C-F subject pool. G. hamolytica is an abundant species (2.7%) and G. morbillorum is moderately abundant (0.9%). The Gemella have not previously been associated with caries status. Both of the differentially represented Abiotrophia spp. were over-represented in the C-F subject pool. A. defectus is an abundant species (2.5%). Both of the differentially represented Granulicatella spp. are abundant. G. elegans (2.6%) is over-represented in the C-A subject pool, and G. adjacens (0.9%) is over-represented in the C-F subject pool. In total, 18 Streptococcus spp. were differentially abundant and 13 of those were over-represented in the C-A subject pool. More significant than the number is the fact that together these 13 species comprise nearly 50% of the dental plaque biomass. The distinction between dental health and disease may have less to do with the overall abundance of Streptococcus spp. but rather the relative balance between strongly acidogenic species and less acidogenic species and their relative rates (velocity) of acid production. It is well established that S. mutans and S. sobrinus are among the most acidogenic species present in dental plaque. Likewise S. mitis isolates, while variable in their acid production, include highly acidogenic strains [40]. It is unclear why S. sanguinis and S. mitis, species normally associated with dental health, are over-represented in the C-A subject pool. Similarly, S. cristatus and S. gordonii have previously been associated with dental health [23], whereas we observe associations of these species with caries activity. These conflicting results may reflect strain level phenotypic variation that serves to elevate the acidogenicity and/or aciduricity of the represented species. The validity of this speculation however will require experimentation. The strong over-representation of S. mutans in C-A subjects is consistent with numerous reports but also illustrates that despite the sampling of dental plaque from all tooth surfaces, the S. mutans signature remains apparent. The Veillonella are asaccharolytic and metabolizes the products generated by primary fermentation for energy. Their ability to metabolize lactate to weaker acids suggests that its metabolic activities may be protective and associated with caries health. Our results suggest that V. parvula, an abundant species through its lactate metabolism may serve as an acid sink in dental biofilms and further enable sugar metabolism of acidogenic and aciduric species. These findings are consistent with those recently reported by Gross and colleagues and with in vitro cultivation experiments demonstrating that S. mutans produced more acid when co-cultivated with V. parvula compared to when S. mutans was grown as a mono-culture [41].

Among the Fusobacteria, all three differentially abundant phylotypes were over-represented in the C-F subject pool. Among the differentially abundant Proteobacteria were six Neisseria spp. Two-thirds of these were over-represented in the C-F subject pool, although the most abundant species, N. subflava was over-represented in the C-A subject pool. Finally six Campylobacter spp. were differentially represented. Among the three most prevalent, C. showae is over-represented in the C-F subject pool, whereas both C. gracilis and C. concisus were over-represented in the C-A subject pool. It is known that individual species within both the Neisseria and Campylobacter are capable of metabolizing sugar to lactate and many members of these genera are also capable of metabolizing lactate to lower pKa acids [42]. As additional reference genome sequences pertaining to the oral cavity continue to accumulate [43], we will be able to relate metabolic coding potential of genomes to the dental caries phenotype.

The association of S. mutans and lactobacilli in the development and progression of dental caries is strong, however there are several documented cases of caries in human subjects that are
independent of S. mutans and Lactobacilli [5–7,9,10,18]. Recently, Kanasi et al., reported that Bifidobacterium spp. were associated with severe caries in children [22]. We failed to identify any significant Lactobacilli or Bifidobacterium spp. in our survey. When we compared the sequences of available 16S rRNAs corresponding to these phyla we conclude that the primer pairs used here to amplify 16S rDNA should have resulted in amplification of Lactobacilli but may not have efficiently amplified Bifidobacterium spp. due to several mismatches noted in the priming sites.

Despite the growing number of published studies examining the species profiles associated with caries status there appears to be a limited agreement in many of the results obtained. Furthermore, the methods used and depth of characterization achieved in each study make effective data comparisons difficult to perform. The recent report by Gross et al., represents a significant study illustrating the interpersonal variability of dental biofilm microbiota [23]. We hypothesize that the human dental plaque microbiota and others contain a large number of member species/strains that are functionally inter-changeable components within their respective community. Our study emphasizes that it is the shift in abundance of groups of species rather than the “appearance” of novel cariogenic species or the pathogenicity of a single species that best describes the distinction between C-F and C-A microbiota. The large number of potentially “dispensable” species present in dental plaque biofilm may strongly confound association studies. Future efforts to enable the recognition of “synonymous species” in microbiomes may be critical for establishing an understanding of those that provide protective qualities to maintain dental health compared to those that can contribute to disease.

It should be emphasized that our preliminary profiling of C-A and C-F subjects, reflect a census of two microbiomes comprised of a small number of human subjects. In order to establish statistically rigorous species associations as diagnostic indicators of dental health or disease, the profiling of larger numbers of samples derived from individuals rather than pools will be required. The use of twins discordant for dental caries together with longitudinal studies may help to elucidate these intriguing concepts. In addition, we anticipate that metagenomic analysis of species and their associated gene and metabolite expression associated with dental health and disease will further hasten our ability to reduce oral health disparities in the general population.

Supporting Information
Figure S1 Population Structure of Dental plaque Biofilm Microbiome. The dental plaque microbiome is comprised of a small group of 16 highly abundant OTUs (>1% abundance, blue) followed by 117 OTUs representing moderately abundant phylotypes (>0.1% abundance, green), 500 OTUs representing low abundance phylotypes (>0.01% yellow) and finally a large and incompletely characterized group of very low abundance OTUs (>0.001%, red) constituting a long tail in the population structure. (TIF)

Table S1 Phyotypes Differentially Represented in C-F and C-A Dental Biofilms (continued). Differentially abundant phylotypes in C-F and C-A microbiota. (DOC)

Acknowledgments
The authors wish to thank Bryan Frank and Kenneth Shatzkes for editorial help on the manuscript preparation.

Author Contributions
Conceived and designed the experiments: SNP WAB. Performed the experiments: ES JL ACO. Analyzed the data: NJS JL MK. Contributed reagents/materials/analysis tools: MK. Wrote the paper: SNP WAB MK NJS.

References


