Metagenome sequencing-based strain-level and functional characterization of supragingival microbiome associated with dental caries in children

Nezar Noor Al-Hebshi* , Diivyashi Baraniya 1 , Tsute Chen 1 , Jennifer Hill 4 ; Sumant Puri 1 4 ; Marisol Tellez 2 ; Nur A. Hasan 2 4 ; Rita R. Colwell 1 4 and Amid Ismail 1

*Oral Microbiome Research Laboratory, Maurice H. Kornberg School of Dentistry, Temple University, Philadelphia, PA, USA; 1Department of Microbiology, Forsyth Institute, Cambridge, MA, USA; 2Department of Pediatric Dentistry and Community Oral Health Sciences, Maurice H. Kornberg School of Dentistry, Temple University, Philadelphia, PA, USA; 4CosmosID Inc., Rockville, MD, USA; 5University of Maryland Institute for Advanced Computer Studies, University of Maryland, College Park, MD, USA; 6Maryland Pathogen Research Institute, University of Maryland, College Park, MD, USA

ABSTRACT
Studies of the microbiome associated with dental caries have largely relied on 16S rRNA sequence analysis, which is associated with PCR biases, low taxonomic resolution, and inability to accurately study functions. Here, we employed whole metagenome shotgun sequencing, coupled with high-resolution analysis algorithm, to analyze supragingival microbiomes from 30 children with or without dental caries. A total of 726 bacterial strains belonging to 406 species, in addition to 34 bacteriophages were identified. A core bacteriome was identified at the species and strain levels. Species of Prevotella, Veillonella, as yet unnamed Actinomyces, and Atopobium showed strongest association with caries; Streptococcus sp. A514 and Leptotrichia sp. Oral taxon 225, among others, were overabundant in caries-free. For several species, the association was strain-specific. Furthermore, for some species, e.g. Streptococcus mitis and Streptococcus sanguinis, sister strains showed differential associations. Noteworthy, associations were also identified for phages: Streptococcus phage M102 with caries and Haemophilus phage HP1 with caries-free. Functionally, potentially relevant features were identified including urate, vitamin K2, and polyamine biosynthesis in association with caries; and three deiminases and lactate dehydrogenase with health. The results demonstrate new associations between the microbiome and dental caries at the strain and functional levels that need further investigation.

Introduction
Over the past 15 years, Sanger sequencing of 16S rRNA clones, frequently coupled with reverse-capture checkboard DNA-DNA hybridization, has been widely employed to study the microbial community associated with dental caries [1–10]. Results of these studies have revealed significant diversity, and many novel species/phylotypes have been identified. In addition to substantiating evidence for the role of mutants streptococci and lactobacilli, they quite consistently revealed an association between a number of microorganisms and dental caries, including Propionibacterium spp., Bifidobacterium spp., Veillonella spp., Actinomyces spp. and Atopobium spp., as well as acidogenic non-mutans streptococci, especially in those subjects for which Streptococcus mutans was not detectable. It has also been possible to identify candidate health-associated bacterial species, such as Streptococcus mitis. Notably, most of these studies focused on caries of primary teeth [2,4,5,7,9,10].

One drawback of Sanger sequencing of 16S rRNA clones, however, has been its high cost and the laborious laboratory work required, limiting the number of samples and clones that can be feasibly analyzed [11]. Fortunately, this limitation has been overcome by the advent of next generation sequencing (NGS). NGS allows analysis of microbial communities to unprecedented depth and breadth at relatively lower cost. Thus, it offers an invaluable tool for analysis of the oral microbiome in health and disease [11]. Typically, studies using NGS to characterize microbial communities target one or more regions of the 16S rRNA gene, which due to their hypervariability serve as good markers of bacterial taxa in samples. This approach has recently been used in a series of studies to explore microbiomes of dental caries [12–15], providing better insight into the diversity of the microbial community associated with dental caries. And despite methodological differences among these studies in terms of sampling (saliva vs. supragingival plaque vs. carious dentine), hypervariable regions selected for sequencing and the bioinformatic analysis pipeline used, a number of taxa consistently showed association with dental caries, including, S. mutans,
Lactobacillus spp., Propionibacterium spp., Veillonella spp., and Atopobium spp.

Targeted 16S rRNA gene sequencing, however, requires gene amplification by PCR, which is known to introduce errors such as nucleotide substitution, insertion and deletion as well as chimera formation, leading to detection of spurious species and inflating microbial diversity [16,17]. In addition, PCR is beset by a number of biases such as 1) limited primer coverage, which can result in failure to amplify some taxa, particularly novel ones [18], and 2) differential amplification of templates, which can alter the relative abundance of species and thus distort the original microbial community structure [19]. Shotgun whole metagenome sequencing (WMS) does not involve gene amplification by PCR and allows identification of microbial taxa comprising a community to a higher resolution than 16S rRNA-based sequencing; it also enables exploring microorganisms other than bacteria, e.g., viruses and fungi [20]. In addition, WMS data can be analyzed to characterize the functional potential of the microbial community (gene and pathway analysis).

In the pioneer study by Belda-Ferre et al. [21], WMS was employed to characterize the functional potential of the supragingival microbiome; in addition, 16S rRNA sequences extracted from the data were used to obtained taxonomic profiles. However, no differential abundance analysis was performed, probably because of the small sample size (four subjects with caries and two without). In a larger-scale study, Belström et al. combined metagenomics and metatranscriptomics to study the oral microbiome of 10 subjects with healthy mouth, 10 with dental caries and 10 with periodontitis [22]. Consistent with the literature, the study showed mutans streptococci, Lactobacillus spp. and Veillonella spp., and their transcripts, to be associated with dental caries. It is unfortunate, however, that saliva rather than supragingival plaque was used for the analysis, which may explain why functional analysis did not return relevant results.

So far, there has been no attempt to exploit WMS data to obtain strain-level taxonomic assignments, explore association with microorganisms other than bacteria or perform in depth functional analyses. The aim of this study, therefore, was to employ WMS to perform strain-level, multi-kingdom profiling as well as comprehensive functional characterization of the supragingival microbiome associated with dental caries in children.

Materials and methods

Study subject recruitment

The study was conducted in compliance with the Helsinki Declaration on medical research involving human subjects and was approved by the Temple University’s Institutional Review Board (protocol # 24355). An assent was obtained from each participating child and an informed written consent was obtained from his/her parent or guardian.

Study children were recruited from the Pediatric Dentistry Clinic at the Temple University Kornberg School of Dentistry. Each child had to fulfill the following criteria: 6–10 years old with all first permanent molars erupted (mixed dentition); no history of antibiotic, antifungal, or steroid intake or use of mouthwashes in the three months prior to sampling; no evidence of oral abscess or candidiasis; no history of diabetes, immunodeficiency, or dental prophylaxis in the previous 30 days. Supragingival plaque samples were obtained from eligible children, as described below, before full mouth prophylaxis, and clinical examination were performed. Caries status was assessed with clinical visual examination following the International Caries Classification and Management System (ICCMS) [23] as well as radiographic examination. Eventually, 10 caries-free children (defined as having no carious lesions, including white spots, and no previous fillings), 10 with early caries (defined as having at least one tooth with early, non-cavitated carious lesion), and 10 with advanced caries (defined as having at least one tooth with cavitiated carious lesion) were recruited. The characteristics of the study groups are presented in Supplementary Table 1.

Microbial sampling and DNA extraction

A whole-mouth, supragingival plaque sample was obtained from each study subject as follows: First, the sampling sites were isolated with cotton rolls and air-sprayed to minimize contamination with saliva. Then, a sterile curette was used to scrape dental plaque from the buccal surface of all teeth present. In subjects with active caries, cavities were avoided. As the sample was collected, the plaque was pooled by wiping it onto a single sterile gutta-percha point. Finally, the point with supragingival plaque sample was placed it into a tube containing sterile TE buffer and stored at −80°C.

At the time of DNA extraction, the samples were thawed and vortexed vigorously to dislodge plaque from the gutta-percha points into the buffer. Sterile forceps (a new pair for every sample) were used to remove the points from the tubes. Each plaque sample was pelleted by centrifugation at maximum speed, washed twice in 1 ml PBS, and digested by re-suspending in 250 μl PBS containing 25 μl Metapolyzme (Sigma, USA), and incubating at 37° for 3 h. The lysate was used for DNA extraction employing the ZymoBiotics miniprep kit (Zymo Research, Germany) according to the manufacturer’s instructions. Quantity and quality of the extracts were assessed using a Qubit 3.0 (ThermoFisher Scientific, USA).
**DNA sequencing and compositional analysis**

Fragment libraries were prepared from 200 ng DNA, using the IonXpress Plus Fragment Library kit (ThermoFisher Scientific, USA), according to the manufacturer’s instructions. The libraries were sequenced on an Ion S5XL sequencer (ThermoFisher Scientific) to generate 200 bp single-end sequence reads. Each sample was sequenced, with an average of 21 M sequence read depth. Unassembled sequencing reads were directly analyzed with the CosmosID (originally called GENIUS) metagenomic software (CosmosID Inc., Rockville, MD), as described elsewhere [24,25] for multi-kingdom microbiome composition analysis and quantification of relative abundances at all taxonomic levels. Briefly, the system utilizes a high performance data-mining k-mer algorithm and highly curated dynamic comparator databases (GenBook™) comprising over 150,000 microbial genomes and gene sequences representing over 10,000 bacterial, 5,000 viral, 250 protists and 1,500 fungal species. It constitutes both publicly available genomes or gene sequences through NCBI- RefSeq/WGS/SRA/nr, PATRIC, M5NR, IMG, ENA, DDBJ, CARD, ResFinder, ARDB, ARG-ANNOT, mvirdb, VFDB etc., in addition to a subset of genomes sequenced by CosmosID and its collaborators.

The pipeline has two separable comparators. The first consists of a pre-computation phase and a per-sample computation. The input to the pre-computation phase is a reference microbial database, and its output is a whole genome phylogenetic tree, together with sets of variable-length k-mer fingerprints (biomarkers) that are uniquely identified with distinct branches, nodes and leaves of the tree. The second per-sample, computational phase searches the hundreds of millions of sequence reads against the fingerprint sets in minutes. The resulting statistics are analyzed to give fine-grain composition and relative abundance estimates at all branches, nodes and leaves of the tree. The second comparator uses edit distance-scoring techniques to compare a target sample with a reference set. Overall classification precision is maintained through aggregation statistics. The first comparator finds reads for which there is an exact match with a k-mer uniquely identified in one or a set of reference strains; the second comparator then statistically scores the entire read against the reference to verify that the read is indeed uniquely identified with that set. For each sample the reads from a species are assigned to the strain with the highest aggregation statistics.

The resultant taxa abundance tables were used to calculate observed and expected species richness, alpha diversity indices, and beta diversity distance matrices. At each taxonomic level, microbial taxa which were present in all the subjects, irrespective of their relative abundances, were considered as core microbiome. Principle Coordinate Analysis (PCoA) was performed to cluster samples based on abundance Jaccard distance matrix (community structure). Differentially abundant taxa at the species and strain levels were identified using Linear discriminant analysis Effect Size (LEfSe) [26], with ‘caries’ and ‘caries-free’ as classes and ‘early-caries’ and ‘advanced caries’ as subclasses.

**Functional analysis**

Trimmomatic, v 0.36 [27] was used to quality-filter the raw reads: trimming with a quality sliding window of 4:8; cropping sequences to remove 20 bases from the start and bases beyond 220 from the end (based on results of analysis by FASTQC); and filtering out sequences less than 150 bp long. Reads were processed using Kneaddata (https://bitbucket.org/biobakery/kneaddata/wiki/Home) to remove human DNA sequences. Quality trimmed reads were processed through HUMANn2 (http://huttenhower.sph.harvard.edu/humann2) with default settings, which includes 1) screening with MetaPhAn2 for taxonomic identification using clade-specific markers [26]; 2) mapping reads to annotated pangenomes of the identified species in the ChocoPhlAn database using Bowtie2 [28] to obtain species-specific gene lists (nucleotide search); 3) translated search of unmapped reads against UniRef90 protein reference database [29] using DIAMOND [30]. The generated gene lists are then collapsed into protein families and enzyme classes/pathways using PFam [31] and MetCyc [32] databases, respectively. Species contribution is based on results obtained in step 2 above. The resulting gene families and pathway abundance files from all samples were joined and normalized to relative abundance. Merged data were unstratified and LEfSe was employed to identify differentially abundant features, as described above.

**Results**

**Sequencing and data processing statistics**

Ion torrent sequencing yielded an average of 21.1 million reads per sample (range 10–36 million reads). Raw reads (publically available at ftp://www.homd.org/publication_data/20180420/fastq/) were directly used for compositional analysis, but were quality-filtered for functional analysis. Use of Trimmomatic resulted in dropping an average of 31.41% of the reads from each sample. Kneaddata removed human DNA sequences, which accounted for 0.03–10.35% of the reads (average of 2.26%). Details of read counts for each sample, before and after each quality control step, are provided in Supplementary file 1. An average of 13.8 million
reads per sample (median 12.9 million; range 7.7–27.7 million reads) was obtained for downstream analysis. In compositional analysis, 51.26 % of the reads per sample (on average) hit the k-mer markers in the database, while in functional analysis (HUMAnN2) an average 44.93% could be assigned a function (the translated search stage in HUMAnN2 only reports proteins with >50% coverage by default).

**Overall microbial profile**

Bacterial sequences accounted for 99.6% of all reads that hit k-mer markers. The analysis pipeline resulted in identification of 726 bacterial strains belonging to 406 species, 94 genera, and 12 phyla, in addition to 34 bacteriophages, two protists and two fungi. The latter were identified in single samples, while further analysis of the protist sequences indicated potential false positives, so both fungi and protists are not discussed in additional detail.

Sample relative abundances and detection frequencies for all bacterial phyla, genera, species, and strains detected in this study are presented in Supplementary files 2–5. At the phylum level, the core bacteriome comprised Actinobacteria (46.7%), Firmicutes (22.5%), Bacteriodetes (14.5%), Proteobacteria (5.8%), Fusobacteria (5.8%), Saccharibacteria (4%), andSpirochetes (0.54%). At the genus level, 42–61 genera were detected per subject. Figure 1(a) illustrates relative abundances of the major 15 genera identified (those present at ≥ 1% relative abundance). Together, they comprised 87% of the average bacteriome. *Actinomyces* alone accounted for 36.05%, followed by *Streptococcus* (8.4%) and *Capnocytophaga* (6.1%). These 15 genera were identified in all samples and, together with an additional 19 genera, comprised the core bacteriome (Supplementary file 6).

The number of bacterial species/strains per sample ranged from 217 to 301. The 28 species and 23 strains with average relative abundance ≥1% are shown in Figure 1(b, c). In total, they comprised 53% and 48% of the average bacteriome at the species and strain levels, respectively. The majority of them were a part of the core bacteriome, comprising 133 species and 96 strains (Supplementary files 7 and 8). Named *Actinomyces* spp. and strains were the most prominent of the core taxa, in addition to *Actinobaculum* sp. oral taxon 183 str F0552, *Pseudopropionibacterium propionicum* str F0230a, *Corynebacterium matruchotii* str ATCC 14266, *Veillonella parvula* str ACS 068 V Sch12, *Veillonella dispar* str DORA 11, *Leptotrichia buccalis* str C1013b, TM7 single isolates, TM7a and TM7b, and corresponding species. *Streptococcus gordonii* and *Streptococcus sanguinis* were among the top

![Figure 1](image-url). The microbiome profile of supragingival plaque. DNA extracted from supragingival samples was shotgun-sequenced on an Ion S5XL sequencer. The generated reads (200 bp) where classified to the strain level using the CosmosID metagenomic pipeline that employs clade-specific k-mers derived from a comprehensive reference genome database (see text for details). (a) bacterial phyla, (b) bacterial species and (c) bacterial strains identified at average relative abundance ≥1%. (d) Relative abundance of major groups of bacterial phages detected.
core species, but no single strain of either species was detected in all samples.

In addition to bacteria, 34 strains of bacteriophages were also identified (Supplementary file 9). *Streptococcus* phages accounted for 93.6% of all phage sequences, while *Haemophilus* phages accounted for 3.18% (Figure 1(d)).

**Species richness and diversity**

Results of comparison of species richness and alpha diversity between groups are shown in Figure 2. The caries groups had significantly higher observed species richness and Chao1 index (expected species richness); differences in Shannon and Simpson indices were not significant. In PCoA, caries and caries-free subjects formed separate clusters at both species and strain levels, but clustering by caries subclass, i.e. early and advanced caries, was not observed (Figure 3(a,b)). Inter-sample distances, however, were significantly higher at the strain level (Figure 3(c)).

**Differentially abundant species and strains**

Differentially abundant species and strains observed for the caries and caries-free groups are shown in Figure 4. Fourteen *Prevotella* spp., prominently *Prevotella melaninogenica*, 10 *Veillonella* spp., primarily *Veillonella parvula*, six unnamed *Actinomyces* spp., three *Atopobium*, and two *Oribacterium* spp. were found to be associated with dental caries, while only six species, including *Streptococcus* sp. AS14 and *Leptotrichia* sp. Oral taxon 225, were more abundant in caries-free subjects. Detailed plots showing selected differentially abundant features are presented in Supplementary Figure 1, demonstrating an association (or inverse association) with disease severity, i.e. lowest average abundance in caries-free and highest in advanced caries (or vice versa). Although *S. mutans* was not detected by LEfSe analysis, a separate analysis with Kruskal-Wallis test followed by multiple Wilcoxon test for pairwise comparisons (the basic statistics of LEfSe), revealed a significant difference only between the advanced caries and no caries groups (Supplementary Figure 2).

At the strain level, four different scenarios were identified. In one scenario, only one strain of a species was detected across all samples and it, therefore, showed similar association to that of the corresponding species. In another scenario, a species was represented by more than one strain, but no particular strain accounted for the species association, such as in the case of *Atopobium parvulum* and *Veillonella atypica*; i.e. there were no significant differences between groups at the strain level, despite association at the species level. In the third scenario, a species was represented by more than one strain, but only a specific strain showed significant association, e.g. strains ASC 068 V Sch12 and OT 298 of *V. parvula* and *Prevotella maculosa*, respectively (Figure 5(a,b)). In the fourth and most interesting scenario, strains belonging to the same species showed a differential association with health and disease. For example, *S. mitis* bv 2 str SK95 and *Streptococcus parasanguinis* str FO449 were found to be associated with caries and caries-free groups, respectively, but sister strains showed opposite associations, as shown in Figure 5(c,d), with no association at the species level.

No significant differences in bacteriophage relative abundance was observed between groups. However, Chi-square analysis showed *Streptococcus* phage...
M102 to be significantly more prevalent – actually exclusively so – in the caries groups, while *Haemophilus* phage HP1 was detected at significantly higher rates in the caries-free group (Supplementary Figure 3).

Differentially abundant functional attributes

Biosynthesis of queuosine, urate, 1,4-dihydroxy-2-naphthoate, menaquinols, and polyamine comprised the most common pathways associated with caries, while L-lysine biosynthesis was strongly associated with caries-free (Figure 6(a)). Potentially relevant enzyme classes differentially abundant between groups included arginine, threonine, and dCTP deaminases, as well as lactate dehydrogenase in association with health and acyl-acyl carrier protein and 5′-nucleotidase with caries (Figure 6(b)). Sulfatase was the only protein family overrepresented in the caries groups (Supplementary Figure 4), while proteins

**Figure 3. β-Diversity analysis.** Inter-sample distances were calculated based on abundance Jaccard index employing standard QIME scripts. The samples were then clustered using Principle Coordinate Analysis (PCoA) to visualize distances at the species (a) and strain (b) levels. (c) Quantitative representation with boxplots of distances between the samples based on abundance Jaccard index at the species and strain levels. * P ≤ 0.001, Wilcoxon rank sum test.

**Figure 4. Differentially abundant taxa.** (a) species and (b) strains that showed significant differences in relative abundance between the caries and caries-free groups, as identified by linear discriminant analysis (LDA) effect size analysis (LEfSe), with ‘caries’ and ‘caries-free’ as classes and ‘early-caries’ and ‘advanced caries’ as subclasses. Note: many unnamed species, e.g. *Streptococcus* sp_AS14, are represented in the database by only one strain, and in this case the species and strain names are identical.
involved in signal transduction, transcriptional regulation and membrane transport were enriched in the caries-free group. Figure 7 shows those species linked with selected differentially abundant functional attributes. The Actinomyces spp. not associated with dental caries, including A. naeslundii, Actinomyces massiliensis, Actinomyces johnsonii, Actinomyces viscosus, and Actinomyces oris, in addition to S. mitis, S. sanguinis and C. matruchotii, were major contributors to the three deiminases. V. parvula and Veillonella sp. 6127 were the sole contributors to polyamine synthesis and, along with S. mitis and P. melaninogenica, also contributed to queuosine biosynthesis.

Discussion

To the best of our knowledge, this is the first study to characterize the supragingival microbiome associated with dental caries at the strain level using WMS. The CosmosID analysis pipeline employs clade-specific markers with strain-level resolution, generated from a large curated database (150,000+ genomes and gene sequences) [24,25]. In a recent study comparing 11 metagenomic analysis tools, BlastMegan was reported to provide the best overall performance [33]. Using the same training sets employed in that study, we found CosmosID to outperform BlastMegan, especially at the sub-species level (http://www.cosmosid.com/blog-cosmosid/benchmarking-genome-biology-2017). However, regardless of what analysis tool is used, false positives cannot be completely eliminated. In addition, the analysis assigns sequences of a species in a given sample to the most significant strain, resulting in identification of a single strain per species, and in turn, obscuring strain diversity within a sample, a limitation that is being addressed in the coming version of the analysis pipeline. Nevertheless, the identified strains are probably the predominant ones. In fact, a recent study of the gut microbiome showed that, in most cases, each species was either exclusively represented or dominated by a single strain [34]. Consistently, previous studies of dental caries have found that while children usually carry more than one genotype of S. mutans, a single genotype is generally predominant [35,36].
In the caries groups, we sampled the supragingival microbiome from surfaces unaffected by caries, with the assumption there would be a minimal effect of the disease process and that any alterations identified would represent early driver events that can serve as targets for prevention. In contrast, microbial changes within the dental caries lesions are late events and more likely a result rather than cause of disease. However, since sampling was performed prior to clinical examination, and despite extreme care, the possibility that plaque from surfaces with early carious lesions was accidentally collected cannot be totally excluded and is one limitation of this study. Nevertheless, since plaque was pooled from mostly...
intact surfaces, the effect of any contamination would have been diluted.

In contradiction with several previous reports [5–7,14], species richness and diversity, as observed in this study, were higher in the caries groups compared to the caries-free group. This is counter-intuitive since the cariogenic process probably selects for acidogenic and aciduric species, leading to reduced diversity. However, our results may be explained by the fact that we did not sample the carious lesion itself, the site that particularly shows statistically significant lower diversity in previous studies [5,6]. Indeed, Belstrøm et al. analyzing saliva samples with WMS, found no significant differences in richness and diversity between health subjects and those with dental caries [22].

The current study, however, substantiates existing evidence for the association between several species other than S. mutans and dental caries, including, Veillonella spp., Atopobium spp., and Actinomyces spp. For the latter, as yet unnamed species were found to be associated with caries, while recognized species such as A. naeslundii tended to be associated with health, consistent with previous studies [1,4].

We found that Prevotella spp. also showed significant association with caries, which substantiates results from a previous longitudinal study in which Prevotella, but not S. mutans was found to be the main predictor of early childhood caries [37]. In fact, S. mutans in the current study was identified in very low abundance and showed significant association with advanced caries only. Consistently, Simón-Soro et al. found that despite a significant increase in the proportion of S. mutans with progression of caries, non-mutans streptococci were far more abundant in the carious lesions [38]. These findings evoke the current debate that questions its primary role in dental caries [39,40]. There is increasing evidence to support that dental caries results from a dysbiosis involving different oral microbial taxa instead of the activity of a single taxon [41].

Streptococcus sp. AS14 and Leptotrichia sp. Oral taxon 225 showed the strongest association with health. Leptotrichia spp. were found to be overrepresented in heath in a previous study [1], although the association appears to vary from one species to another [9,10]. Streptococcus sp. AS14 is a human isolate, described here for the first time to occur in the oral cavity. Its potential role in dental caries merits further investigation. The most intriguing results involved S. mitis and S. parasanguinis, for which sister strains showed differential associations, which likely explains the controversy concerning the role of these species in the literature, with results of some studies showing association with caries and others reporting association with health [4,5,7,9]. In this study, Streptococcus phage M102 and Haemophilus phage HP1 correlated with caries and health, respectively, another novel finding. Interestingly, Streptococcus phage M102 is specific for S. mutans serotype C [42], the most common serotype of S. mutans. Given that the phage and its host were co-detected, the former may have occurred as prophage. Thus, Streptococcus phage M102 may act as a marker for S. mutans serotype C and could then be targeted to induce lysis of S. mutans, a potential prevention strategy of dental caries that is worth exploring.

The arginine, threonine and dCTP deaminases genes were found to be overrepresented in the caries-free group. Deaminase activity results in release of ammonia, which plays an important role in prevention against caries by neutralizing acids [43]. In line with this, a recent proteomic study found arginine deiminase to be enriched in dental plaque from caries-free individuals [44]. In this study, members of Actinomyces spp. especially A. naeslundii, A. johnsonii and A. massiliensis, in addition to S. mitis and S. sanguinis, were found to be major contributors to the three ammonia generating enzymes (Figure 7). Arginolytic activity of some of these species has previously been demonstrated [43]. However, threonine and dCTP deaminases have not been previously implicated in dental caries. The lactate dehydrogenase (LDH) gene was also enriched in caries-free samples, which again concurs with results from the same proteomic study referred to above. Interestingly, a historical study found LDH to delay onset and reduce severity of caries in rats [45]. L-lysine biosynthesis was also over-abundant in caries-free subjects. Interestingly, this amino acid was reported in a much earlier study to inhibit biofilm formation by S. mutans in vitro [46].

The pathways, enzymes, and protein families that were found to be overrepresented in the caries group are potentially relevant to the cariogenic process. Polyamines, for example, are known to play an important role in biofilm formation by many bacteria [47] and may thus have a similar role in formation of dental plaque. 1,4-dihydroxy-2-naphthoate and menaquinols are precursors of vitamin K2 (menaquinone) which, in a recent study, has been shown to enhance biofilm formation by Staphylococcus aureus [48]. Similarly, there is evidence to suggest uric acid boosts biofilm formation by Enterococcus faecalis [49]. 5′-nucleotidase is also potentially important as 5′-nucleotidase inhibitors have been found to inhibit growth and glucan formation by S. mutans [50]. It would be interesting to explore the role of these compounds in biofilm formation by oral bacteria.

Queuosine-mediated modification of tRNA is involved in many cellular processes including signaling pathways and virulence of bacteria [51] and may contribute to carcinogenicity of dental plaque by
downregulating arginine deiminase expression [52]. Sulfatases were also found to be enriched in the caries groups. Interestingly, according to an old theory, bacterial sulfatases hydrolyze sulfates of enamel and dentin, which in turn results in sulfuric acid production [53]. Although, this theory is not accepted today, it is possible that sulfatases are involved in a different way. Some oral streptococci, for example, have been reported to possess mucin-sulfatase activity, which may result in abating the protective action of salivary mucin [54]. It should be emphasized, however, that functional results obtained by analysis of WMS data are predictive and should thus be interpreted cautiously.

Two studies have previously employed WMS to assess the microbiome associated with dental caries [21,22]. In the study by Belstrøm et al. [22], saliva rather than supragingival plaque samples were analyzed so a direct comparison would not be meaningful. The study, in any case, substantiated evidence for the classical cariogens, i.e. mutans streptococci and lactobacilli. The study by Belda-Ferre et al. [21] performed compositional analysis based on analysis of 16S rRNA reads extracted from the WMS data. Without adjustment for 16S rRNA copy numbers (e.g. three for Actinomyces and five on average for streptococci) a reliable comparison of compositional results cannot be made.

Two general microbiological findings, irrespective of dental health status, are worth elaboration. One is that Actinomyces spp. and strains, hence phylum Actinobacteria, were the most abundant taxa in the children’s supragingival plaque samples. This finding is inconsistent with results from previous studies that used universal 16S rRNA amplification (sequencing or reverse-capture DNA-DNA hybridization), and in which Streptococcus and Veillonella spp. and, subsequently phylum Firmicutes, were found to dominate [2,5,6,9,55]. On the other hand, studies based on PCR-independent technologies, namely checkboard DNA-DNA hybridization, reported relative abundance of Actinomyces to be as high as 63% [10,56]. This is also consistent with early culture studies showing a high abundance of Actinomyces, especially in mature plaque [57]. In fact, direct comparison of culture and clonal analysis of 16S rRNA showed Actinobacteria to be underrepresented when the latter method was employed [8]. This observation strongly indicates that estimates of relative abundances using WMS are more reliable than those obtained using 16S rRNA sequencing. Choice of method for DNA extraction also must be considered in accounting for differences between studies, as it has been shown to significantly influence microbial profiling [58]. In the current study, an enzymatic mixture (metapolyzyme) and bead beating were used to achieve lysis for maximum recovery of DNA from the different bacterial species in the samples; the average DNA yield was 113 ng/μl.

Another noteworthy observation is that the viral sequences (predominantly phages) were the only non-bacterial microbial sequences consistently detected in all samples, a finding which is in agreement with previous reports that identified bacteriophages as a resident population of the oral cavity. However, their role remains poorly understood [59]. Although, a complex fungal community has been described in the oral cavity [60], fungal sequences were identified in only 2 of the 30 samples analyzed. This may be explained by two possible reasons. The first is that fungi have no affinity for supragingival plaque. The second is that they were present at such a low abundance that they were not detected at the sequencing depth used, another potential limitation of the current study.

In conclusion, this study demonstrates the potential of WMS, coupled with robust analysis tools, to characterize the oral microbiome to high taxonomic resolution and obtain reliable estimates of relative abundance of taxa with accurate prediction of microbial community function. It also highlights the importance of assessing the relationship of the microbiome with oral diseases to the level of the strain, by showing how different strains within the same species may differ in their association with dental caries. These inter-strain differences can be exploited for preventive strategies, such as replacement therapy. Similarly, functional analysis identified several microbial attributes with relevance to the cariogenic process and these represent potential targets for intervention, for example by boosting health-associated microbial activities and/or interfering with disease-associated activities. The potential role of phages represents an additional avenue for caries prevention research. It remains, however, important to confirm and validate results from this study in a larger-scale study using a real functional approach such as metatranscriptomics.

**Disclosure statement**

Rita Colwell and Nur Hasan are employees of CosmosID and hold stock in the company.

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**ORCID**

Sumant Puri http://orcid.org/0000-0002-5509-007X
References

[1] Aas JA, Griffen AL, Dardis SR, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol. 2008;46:1407–1417.

[2] Becker MR, Paster BJ, Leys EJ, et al. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol. 2002;40:1001–1009.

[3] Chhour KL, Wang SP, Leys EJ, et al. Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol. 2005;43:843–849.

[4] Cooper PM, Lyons-Weiler J, Bretz WA, et al. Microbial risk indicators of early childhood caries. J Clin Microbiol. 2005;43:5753–5759.

[5] Gross EL, Leys EJ, Gasparovich SR, et al. Bacterial 16S sequence analysis of severe caries in young permanent teeth. J Clin Microbiol. 2010;48:4121–4128.

[6] Munson MA, Banerjee A, Watson TF, et al. Molecular analysis of the microflora associated with dental caries. J Clin Microbiol. 2004;42:3023–3029.

[7] Petersen SN, Sorensen E, Liu J, et al. The dental plaque microbiome in health and disease. PLoS One. 2013;8:e58487.

[8] Kanase E, Johansson I, Lu SC, et al. Clonal analysis of the microbiota of severe early childhood caries. Caries Res. 2010;44:485–497.

[9] Jia T, Takeda T, Shibata Y, et al. Identification of the microflora associated with dental caries. J Dent Res. 2010;89:378–383.

[10] Munson MA, Banerjee A, Watson TF, et al. Molecular analysis of the microflora associated with dental caries. J Clin Microbiol. 2004;42:3023–3029.

[11] Petersen SN, Sorensen E, Liu J, et al. The dental plaque microbiome in health and disease. PLoS One. 2013;8:e58487.

[12] Kanase E, Johansson I, Lu SC, et al. Microbial risk markers for childhood caries in pediatricians’ offices. J Dent Res. 2010;89:378–383.

[13] Siqueira JF Jr., Foud AF, Rocha IN. Pyrosequencing as a tool for better understanding of human microbiomes. J Oral Microbiol. 2012;4. DOI:10.3402/jom.v4i0.10743.

[14] Jagathrakshakan SN, Sethumadhava RJ, Mehta DT, et al. 16S rRNA gene-based metagenomic analysis identifies a novel bacterial co-prevalence pattern in dental caries. Eur J Dent. 2015;9:127–132.

[15] Obata J, Takeshita T, Shibata Y, et al. Identification of the microflora associated with dental caries. J Dent Res. 2016;105:9:127–132.

[16] Xia C, Ran S, Huang Z, et al. Bacterial diversity and community structure of supragingival plaques in adults with dental health or caries revealed by 16S pyrosequencing. Front Microbiol. 2016;7:1145.

[17] Jiang S, Gao X, Jin L, et al. Salivary microbiome diversity in caries-free and caries-affected children. Int J Mol Sci. 2016;17:1978.

[18] Schloss PD, Gevers D, Westcott SL. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS One. 2011;6:e27310.

[19] Kunin V, Engelbrektson A, Ochman H, et al. Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. Environ Microbiol. 2010;12:118–123.

[20] Hong S, Bunge J, Leslin C, et al. Polymerase chain reaction primers miss half of rRNA microbial diversity. ISME J. 2009;3:1365–1373.

[21] Pinto AJ, Raskin L. PCR biases distort bacterial and archaeal community structure in pyrosequencing datasets. PLoS One. 2012;7:e43093.

[22] Ranjan R, Rani A, Metwally A, et al. Analysis of the microbiome: advantages of whole genome shotgun versus 16S amplicon sequencing. Biochem Biophys Res Commun. 2016;469:967–977.

[23] Belda-Ferre P, Alcaraz LD, Cabrera-Rubio R, et al. The oral metagenome in health and disease. ISME J. 2012;6:46–56.

[24] Belstrom D, Constancias F, Liu Y, et al. Metagenomic and metatranscriptomic analysis of saliva reveals disease-associated microbiota in patients with periodontitis and dental caries. NPJ Biofilms Microbiomes. 2017;3:23.

[25] Ismail AI, Sohn W, Tellez M, et al. The International Caries Detection and Assessment System (ICDAS): an integrated system for measuring dental caries. Community Dent Oral Epidemiol. 2007;35:170–178.

[26] Lax S, Smith DP, Hampton-Marcell J, et al. Longitudinal analysis of microbial interaction between humans and the indoor environment. Science. 2014;345:1048–1052.

[27] Hasan NA, Young BA, Minard-Smith AT, et al. Microbial community profiling of human saliva using shotgun metagenomic sequencing. PLoS One. 2014;9:e97699.

[28] Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12.R60.

[29] Bolger AM, Lohse M, Usadel B. Trimmmomatic: a flexible trimmer for Illumina sequence data. Bioinform. 2014;30:2114–2120.

[30] Langmead B, Silver LS. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357–359.

[31] Suzek BE, Wang Y, Huang H, et al. UniRef clusters: a comprehensive and scalable alternative for improving sequence similarity searches. Bioinformatics. 2015;31:926–932.

[32] Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2015;12:59–60.

[33] Finn RD, Bateman A, Clements J, et al. Pfam: the protein families database. Nucleic Acids Res. 2014;42:D222–D2230.

[34] Caspi R, Altman T, Billington R, et al. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome databases. Nucleic Acids Res. 2014;42:D459–D471.

[35] ABR M, Ounit R, Afshinnekoo E, et al. Comprehensive benchmarking and ensemble approaches for metagenomic classifiers. Genome Biol. 2017;18:182.

[36] Truong DT, Tett A, Pasolli E, et al. Microbial strain-level population structure and genetic diversity from metagenomes. Genome Res. 2017;27:626–638.

[37] Cheon K, Moser SA, Wiener HW, et al. Characteristics of Streptococcus mutans genotypes and dental caries in children. Eur J Oral Sci. 2013;121:148–155.

[38] Gilbert K, Joseph R, Vo A, et al. Children with severe early childhood caries: streptococci genetic strains within carious and white spot lesions. J Oral Microbiol. 2014;6:25805.

[39] Teng F, Yang F, Huang S, et al. Prediction of early childhood caries via spatial-temporal variations of oral microbiota. Cell Host Microbe. 2015;18:296–306.

[40] Simon-Soro A, Belda-Ferre P, Cabrera-Rubio R, et al. A tissue-dependent hypothesis of dental caries. Caries Res. 2013;47:591–600.

[41] Banas JA, Drake DR. Are the mutans streptococci still considered relevant to understanding the microbial etiology of dental caries? BMC Oral Health. 2018;18:129.
[40] Simon-Soro A, Mira A. Solving the etiology of dental caries. Trends Microbiol. 2015;23:76–82.

[41] Mira A, Simon-Soro A, Curtis MA. Role of microbial communities in the pathogenesis of periodontal diseases and caries. J Clin Periodontol. 2017;44(Suppl 18):S23–S38.

[42] van der Ploeg JR. Genome sequence of Streptococcus mutans bacteriophage M102. FEMS Microbiol Lett. 2007;275:130–138.

[43] Liu YL, Nascimento M, Burne RA. Progress toward understanding the contribution of alkali generation in dental biofilms to inhibition of dental caries. Int J Oral Sci. 2012;4:135–140.

[44] Belda-Ferre P, Williamson J, Simon-Soro A, et al. The human oral metaproteome reveals potential biomarkers for caries disease. Proteomics. 2015;15:3497–3507.

[45] Higham SM, Edgar WM. Effects of lactate dehydrogenase on fissure caries in rats. Caries Res. 1990;24:39–43.

[46] Malveaux FJ, Smith JJ. Inhibition of in vitro plaque formation by L-lysine. Infect Immun. 1972;5:267–268.

[47] Karatan E, Michael AJ. A wider role for polyamines in biofilm formation. Biotechnol Lett. 2013;35:1715–1717.

[48] Kirby DT, Savage JM, Plotkin BJ. Menaquinone (Vitamin K2) enhancement of Staphylococcus aureus biofilm formation. J Biosci Med. 2014;2:26–32.

[49] Srivastava M, Mallard C, Barke T, et al. A selenium-dependent xanthine dehydrogenase triggers biofilm proliferation in Enterococcus faecalis through oxidant production. J Bacteriol. 2011;193:1643–1652.

[50] Iwamoto M, Uchino K, Toukairin T, et al. The growth inhibition of Streptococcus mutans by 5'-nucleotidase inhibitors from Areca catechu L. Chem Pharm Bull (Tokyo). 1991;39:1323–1324.

[51] Vinayak M, Pathak C. Queuosine modification of tRNA: its divergent role in cellular machinery. Biosci Rep. 2009;30:135–148.

[52] Liu Y, Dong Y, Chen YY, et al. Environmental and growth phase regulation of the Streptococcus gordonii arginine deiminase genes. Appl Environ Microbiol. 2008;74:5023–5030.

[53] Candel A, Tronieri A. [Bacterial sulfatases and dental caries]. Boll Soc Ital Biol Sper. 1951;27:651–653.

[54] Smalley JW, Dwarakanath D, Rhodes JM, et al. Mucin-sulphatase activity of some oral streptococci. Caries Res. 1994;28:416–420.

[55] Keijser BJ, Zaura E, Huse SM, et al. Pyrosequencing analysis of the oral microbiota of healthy adults. J Dent Res. 2008;87:1016–1020.

[56] Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J Clin Periodontol. 2000;27:648–657.

[57] Syed SA, Loesche WJ. Bacteriology of human experimental gingivitis: effect of plaque age. Infect Immun. 1978;21:821–829.

[58] Abusleme L, Hong BY, Dupuy AK, et al. Influence of DNA extraction on oral microbial profiles obtained via 16S rRNA gene sequencing. J Oral Microbiol. 2014;6:23990.

[59] Pride DT, Salzman J, Haynes M, et al. Evidence of a robust resident bacteriophage population revealed through analysis of the human salivary virome. ISME J. 2012;6:915–926.

[60] Ghannoum MA, Jurevic RJ, Mukherjee PK, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog. 2010;6:e1000713.