THE ORAL MICROBIOME IN AN ECOLOGICAL PERSPECTIVE

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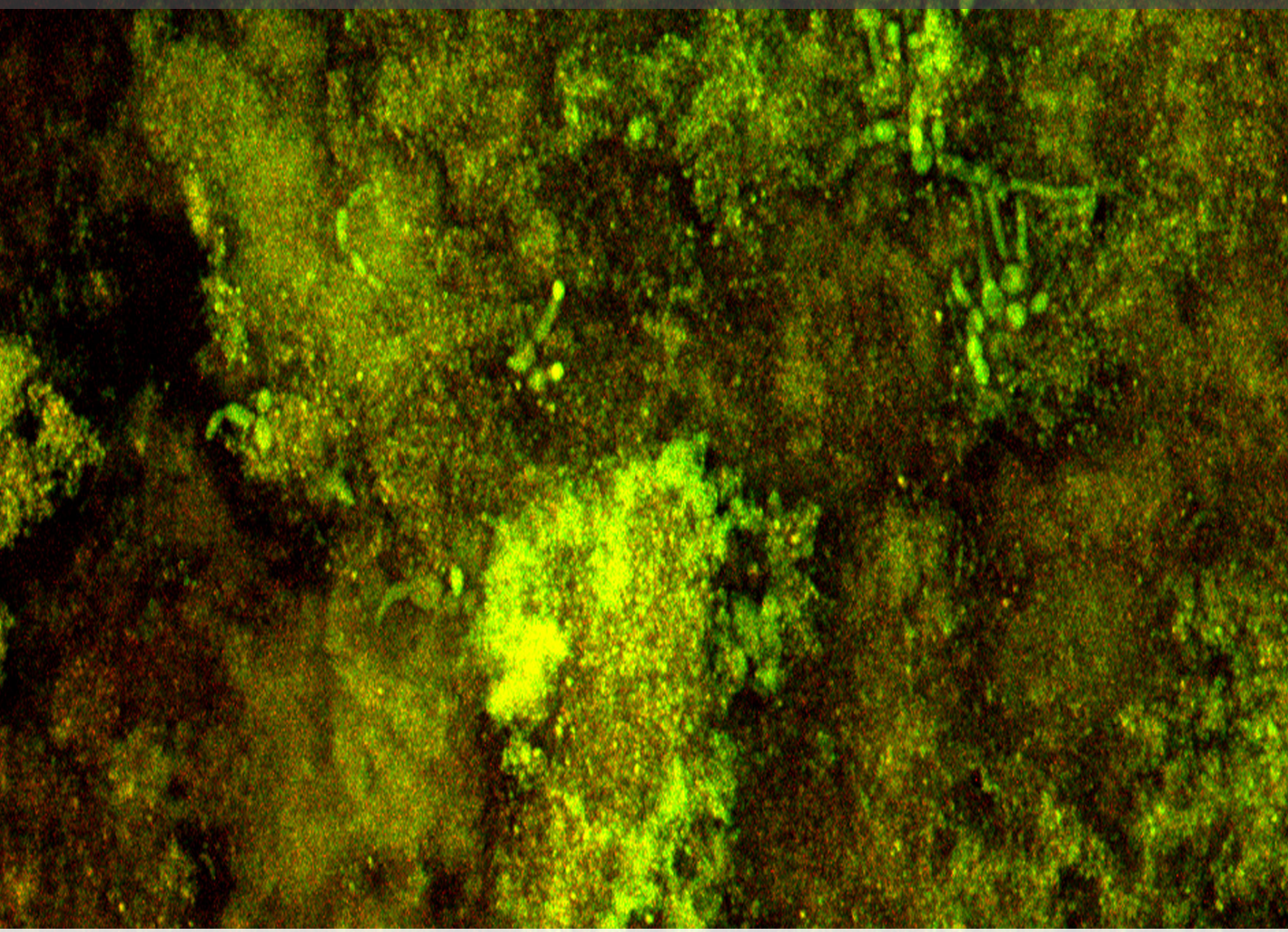

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THE ORAL MICROBIOME IN AN ECOLOGICAL PERSPECTIVE

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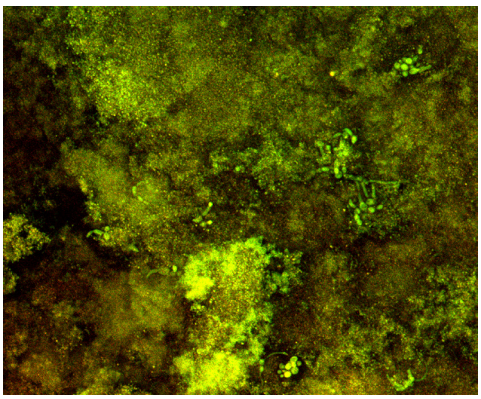

In vitro oral biofilm containing *Candida albicans*. The in vitro oral biofilm was inoculated with saliva supplemented with *C. albicans*. After 48 hours of growth at 37 degrees under aerobic conditions biofilm formation was imaged using Confocal Laser Scanning Microscopy. For this purpose biofilms were stained with Baclight fluorescent staining. *C. albicans* forms intimate contacts with oral bacteria within the biofilm, and both yeast and hyphal morphologies are common (Unal, Janus and Krom; unpublished data).

The oral cavity harbors an immense diversity of microorganisms, including bacteria, fungi, archaea, protozoa and viruses. At health, oral microbial community is thought to be in a state of homeostasis, even after numerous perturbations (e.g., toothbrushing, food intake) a day. The breach in this homeostasis can occur for instance if the perturbations become too excessive (e.g., frequent carbohydrate intake leading to acidification of the community) or the host is compromised (e.g., inadequate immune response resulting in persistent inflammation of periodontal tissue). Aggressive antimicrobial therapy (e.g., antibiotics in case of periodontal disease or preventive antibiotic therapy before and after dental extractions) is commonly applied with all the negative consequences of this approach. So far little is known on the interplay between the environmental, host and microbial factors in maintaining an ecological balance. What are the prerequisites for a healthy oral ecosystem? Can we restore an unbalanced oral microbiome? How stable is the oral microbiome through time and how robust it is to external perturbations? Gaining new insights in the ecological factors sustaining oral health will lead to conceptually new therapies and preventive programs.

Recent advances in high throughput technologies have brought microbiology as a science to a new era, allowing an open-ended approach instead of focusing on few opportunistic pathogens. With this topic we would like to integrate the current high-throughput 'omics' tools such as metagenomics, metatranscriptomics, metaproteomics or metabolomics with biochemical, physiological, genetic or clinical parameters within the oral microbial ecosystem.

We aim to address questions underlying the regulation of the ecological balance in the oral cavity by including the following areas:

- Ecology of oral microbiome at health
- Ecology of oral microbiome under oral diseases
- Ecology of oral microbiome during non-oral diseases
- Shifts in the oral microbiome by therapeutic approaches (e.g., antimicrobials, replacement therapy, pre- and probiotics)
- Modeling of oral ecological shifts (e.g., animal models, in vitro microcosm models)
- Complex inter- and intra-kingdom interactions (e.g., bacterial-fungal-host) related to oral ecology
- Environmental (e.g., diet, tobacco), host-related (e.g., immune response, saliva composition and flow) and biotic (e.g., bacterial competition) factors influencing oral ecology
- Geographic variation in oral microbial ecology and diversity

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EDITORIAL
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Editorial: The oral microbiome in an ecological perspective

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Keywords: metatranscriptomics, horizontal gene transfer, fungal–bacterial interactions, quorum sensing, immune system, dental plaque, biofilm, oral ecology

Pure cultures have been the basis for microbiology research over a century. However, although working with clonal lineages in the laboratory has allowed fundamental advances in microbial physiology and genetics, microorganisms are never alone. Even extreme environments like hypersaline waters or acidic ponds are not formed by single species. The human body is no exception, and the oral cavity contains hundreds of bacterial species, that together with fungal and viral inhabitants form highly complex communities where they interact with each other and with the host. These interactions include physical coaggregation, chemical signaling, transfer of genetic information, stimulation of the immune system, metabolic complementation, growth synergism and antagonism or pH buffering among others, and they are so intricate that the final contributing output of the whole community is much larger than the addition of the individual species forming it. This is why the use of holistic, metagenomic approaches to study oral microbial ecology becomes fundamental to understand the ecosystem in health and disease.

The advent of high throughput sequencing techniques has allowed gathering a wealth of data on the bacterial content of the oral cavity. However, most of this work has initially been focused on descriptive studies in which the general taxonomy composition of microbial communities was depicted. We must now enter a second phase in which more functional approaches are performed, including *bona fide* metagenomic and metatranscriptomic approaches in which the total gene repertoire and actively expressed genes in the community are identified under different circumstances, without the biases imposed by PCR or cloning procedures. Experimental approaches are also required to validate the correlations that may have been suggested by taxonomic studies and to describe the molecular basis for inter-species interactions. Finally, we cannot forget the physical environment where oral microbes thrive and where the immune system probably plays a crucial role in selecting for a given community. We believe that understanding the basis for these ecological interactions will provide formidable insights to diagnose oral diseases and to prevent its development, and hope that this special issue may contribute to that purpose.

In this topic two functional studies have been included, in which RNAseq strategy has allowed researchers to describe the mRNA populations of dental plaque in twins, in an attempt to normalize for genetic host factors (Peterson et al., 2014); and to focus on the gene expression patterns of different *Veillonella* species within caries lesions (Do et al., 2015). One of the consequences of the close physical interaction in oral biofilms is the possibility for horizontal genetic transfer (reviewed by Roberts and Kreth, 2014), with important consequences in relation to antibiotic resistance. As Bachtiar and colleagues show, inter-species interactions are not limited to closely related organisms but can actually cross kingdom borders, and the authors describe a surprising case of quorum sensing signals produced by a gram-negative bacterium that inhibits biofilm formation in *Candida* (Bachtiar et al., 2014). In fact, the ecology of fungal–bacterial interactions may be instrumental for development of oral biofilms. Microbiome studies have been severely biased toward the prokaryotic component, assuming that fungal species only play a role as opportunistic

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pathogens. This view is changing fast, and the potential relevance of bacterial–fungal interactions to oral health is now being evaluated as shown by the review by Diaz et al. (2014).

The new technical possibilities in unraveling the complexity of the oral ecosystem are brought forward by McLean, where advancements toward a systems level understanding of the human oral microbiome are presented (McLean, 2014). An applied aspect of ecological interactions is shown by studying the effect of an oxygenating agent on oral microorganisms *in vivo*, where twice daily exposure to this agent in the form of a mouthwash prevented plaque growth and induced shift in microbial composition (Fernandez y Mostajo et al., 2014).

The role of indigenous microbiome in maintaining oral health has been addressed by Kumar and Mason, where the interaction of the microbiome and the host receives the attention (Kumar and Mason, 2015). Continuing along the same lines on the interaction of microbes with the immune system, Zaura and collaborators propose an interesting hypothesis about how a normal oral microbiome is acquired (Zaura et al., 2014). Their view is that fetal tolerance toward the mother's microbiota during pregnancy is a major factor selecting for the acquisition of the oral microbiome.

There is no doubt that the next generation sequencing (NGS) technologies have revolutionized the field of microbiology. However, the price to pay for the high throughput output is low taxonomical accuracy and sequencing bias. Schulze-Schweifing and colleagues compare the different approaches for microbiome characterization: culture, traditional cloning and sequencing as well as high throughput sequencing (Schulze-Schweifing et al., 2014), while Lazarevic and colleagues demonstrate the difficulties in work with low DNA yield samples (Lazarevic et al., 2014).

The overall purpose of the studies on ecological interactions of oral microbial communities is to be able to apply that knowledge to understand and prevent oral diseases. In this direction, Rosier and colleagues present a comprehensive and helpful review on the historical hypotheses that have attempted to explain the development of oral diseases (Rosier et al., 2014). With this topic we have summarized the current insights and identified the goals for future research in oral microbial ecology. We believe the field will benefit enormously from these ecological approaches, which certainly show that oral microbial communities cannot be understood by the isolated study of their individual microorganisms and that they are much more complex than the addition of its microbial and host components.

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CELLULAR AND INFECTION MICROBIOLOGY

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Functional expression of dental plaque microbiota

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Dental caries remains a significant public health problem and is considered pandemic worldwide. The prediction of dental caries based on profiling of microbial species involved in disease and equally important, the identification of species conferring dental health has proven more difficult than anticipated due to high interpersonal and geographical variability of dental plaque microbiota. We have used RNA-Seq to perform global gene expression analysis of dental plaque microbiota derived from 19 twin pairs that were either concordant (caries-active or caries-free) or discordant for dental caries. The transcription profiling allowed us to define a functional core microbiota consisting of nearly 60 species. Similarities in gene expression patterns allowed a preliminary assessment of the relative contribution of human genetics, environmental factors and caries phenotype on the microbiota's transcriptome. Correlation analysis of transcription allowed the identification of numerous functional networks, suggesting that inter-personal environmental variables may co-select for groups of genera and species. Analysis of functional role categories allowed the identification of dominant functions expressed by dental plaque biofilm communities, that highlight the biochemical priorities of dental plaque microbes to metabolize diverse sugars and cope with the acid and oxidative stress resulting from sugar fermentation. The wealth of data generated by deep sequencing of expressed transcripts enables a greatly expanded perspective concerning the functional expression of dental plaque microbiota.

Keywords: caries, oral microbiota, dental plaque, biofilm, transcriptome

INTRODUCTION

Members of the oral microbial community play key roles in maintaining oral health and as putative agents responsible for the onset and progression of oral diseases. Previous studies have estimated that greater than 700 species of microorganisms inhabit the oral cavity (Moore and Moore, 1994; Darveau et al., 1997; Kolenbrander, 2000; Hutter et al., 2003). The application of high throughput, culture-independent metagenomics methodologies represents an approach that is well aligned with the high species diversity of oral microbiota. Our previous efforts to define the population structure of dental plaque microbiota revealed an impressive radiation of species derived from a substantially smaller set of genera (Peterson et al., 2011; Walter and Ley, 2011). The *Streptococcus* are dominant in dental plaque microbiota but include a variety of additional genera such as: *Veillonella, Campylobacter, Neisseria, Gemella, Granulicatella Capnocytophaga*, and *Fusobacterium*. A comparison of the saliva community composition of human subjects from China (Luo et al., 2012; Ling et al., 2013), the USA (Cephas et al., 2011) and the African continent (Nasidze et al., 2011) display a high level of variability. In each case the dominant genera identified are unique. The saliva microbiota of Chinese children featured a high proportion of Streptococcus (∼40%) and Prevotella (∼25%) and was complemented by 17 lower abundance genera (Ling et al., 2013). Despite the commonality of a plant-based diet, the saliva microbiota of human subjects from Sierra Leone (SL), the Democratic Republic of Congo (DRC), and the Batwa pygmies (BP) of Uganda display clear distinctions in community structure. *Streptococcus* spp. represented ∼20% of the total in all groups. The SL saliva microbiota is dominated by *Enterobacter* spp. (∼60%), whereas the subjects from the DRC displayed a high proportion of *Serratia* spp (∼25%) and a relatively high abundance of *Rothia* spp. The other observed genera include taxa that are not significantly represented in saliva microbiota previously reported. These studies and others indicate that the microbiota may adopt a relatively large number of configurations in both health and disease (Cephas et al., 2011; Nasidze et al., 2011; Luo et al., 2012; Ling et al., 2013). The phylogenetic representation of related species in bacterial communities confer functional redundancy since their genomes encode a relatively high frequency of homologous protein functions. Such redundancy ensures that the loss of individual species within the community is functionally well tolerated and represents a likely basis of the high interpersonal variation observed in oral microbiota.

A variety of factors such as: genetic, immunological, behavioral, environmental, and mechanisms of vertical inheritance all play a role in defining the oral microbial community composition. Among these factors those pertaining to environment and

particularly diet may be the most influential. In this manner, any case-control study attempting to relate microbial composition to features of the oral cavity in a state of health or disease is severely hampered by the fact that unrelated individuals participating in these studies do not share the same environment. The advantages of utilizing a twin study model, are numerous and importantly allow control over host genetics and relevant environmental factors, e.g., diet, vertical inheritance and lifestyle that serve to increase study power.

The healthy adult oral microbiota represents a highly tuned ensemble of species, selected for survival in a highly competitive and challenging environment that features frequent flux in dietary nutrients (Van der Hoeven and Camp, 1991), O_2 concentration (Diaz et al., 2002), temperature (Fedi and Killoy, 1992), pH (Svensater et al., 1997), and energy metabolism (Palmer et al., 2006; Jakubovics et al., 2008). The dental plaque biofilm contains phylogenetically diverse acidogenic (acid-producers) species many that are also aciduric (acid-tolerant). Dental plaque biofilm-mediated sugar metabolism leads to the production of organic acids that reduce the pH of the biofilm microenvironment and represent key factor in the demineralization of the tooth surface (van Houte, 1994). The availability of dietary carbohydrates is key to biofilm initiation and development (Paes Leme et al., 2006). The production of acid may differentially inhibit resident microbial populations. Microbial metabolism of nitrogenous substrates has been attributed to the production of small arginine peptides that may elevate pH (Burne and Marquis, 2000). Likewise urease activity may also serve to elevate pH of the dental biofilm (Kleinberg, 2002). The dominance of the *Streptococci* and other members of the Firmicutes, dictate the overall fermentative activities in dental plaque.

The individual members of the dental plaque community are likely to belong to numerous and diverse functional networks. These networks may largely reflect cooperative activities of species to maintain environmental homeostasis. For example, the *Veillonella* exploit the metabolic activities of the dominant fermentative microbes. The *Veillonella* are asaccharolytic and derive energy from the metabolism of SCFAs (van der Hoeven et al., 1975; Noorda et al., 1988) producing shorter chain length acids with higher dissociation constants, thereby increasing the pH of the biofilm microenvironment. These acid sinks are critical to the growth and activity of the fermentative species. Interestingly, some acidogenic *Streptococcus* and *Granulicatella* encode the L-lactate dehydrogenase gene suggesting that they too may contribute to acid remediation of dental biofilms (McLean et al., 2012; Edlund et al., 2013).

In order to overcome the challenges associated with determining the species and functional activities of oral microbiota that maintain oral health or drive disease we must improve our understanding of how complex communities function and interact with one another. We have sampled the dental plaque of a large cohort of twin pairs in a longitudinal analysis spanning 3 years. Here we report on the transcriptional activity of the dental plaque microbiota of a twin cohort to improve our understanding of fundamental biochemical features of biofilm communities and the inter-relationships that exist between species in a feast or famine microenvironment.

MATERIALS AND METHODS

DENTAL CARIES PHENOTYPE DETERMINATION

Dental caries examinations were performed on 38 subjects [19 twin pairs, 6 monozygotic (mz), 13 dizygotic (dz)]. The twin pairs were either concordant for dental health, C-F ($n = 4$ pairs), concordant for dental caries C-A ($n = 6$ pairs), or discordant for dental caries ($n = 9$ pairs). 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 optimal (<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 (Kaste et al., 1996) to include white spot lesions and cavitated lesions; (2) *Digital imaging fiber-optic trans-illumination* (DIFOTI) recorded images of dental lesions (incipient and frank lesions) to complement the caries clinical examination (Schneiderman et al., 1997); (3) *Quantitative light fluorescence* (QLF) profiled images of dental lesions similar to the DIFOTI procedure that are not readily captured by visual examinations and complemented the caries clinical examination. C-A subjects had a range of 1–17 decayed tooth surfaces whereas C-F subjects presented with a decay component = 0. Caries-inactive (C-I) subjects presented with surfaces that had restorations provided in previous visits.

DENTAL PLAQUE BIOFILM SAMPLING

Subjects were instructed to refrain from brushing or eating prior to sampling. Therefore, the subjects had not consumed a meal in at least 12 h prior to sample collection. Dental plaque samples were obtained using a sterile toothbrush passed slowly across all tooth surfaces. 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 (Corby et al., 2005). Dental plaque was dislodged from the toothbrush by agitation for 1 min into tubes containing 8 mL of sterile reduced transport fluid (RTF) (Syed and Loesche, 1972) held at 4°C prior to storage at −80°C.

BACTERIAL mRNA ISOLATION FROM DENTAL PLAQUE

Dental plaque samples were thawed and resuspended in RNAprotect reagent (Qiagen Inc) and stored at −80°C. RNA isolation was performed following the procedure recommended by

the manufacturer for the mirVana RNA isolation kit (Ambion). The purified RNA was evaluated subjectively using the Agilent Bioanalyzer and quantitated using a UV spectrophotometer. We used hybridization-based subtraction methods to remove human and bacterial rRNA sequences from samples as described in detail http://www.hmpdacc.org/RSEQ/.

RNA-Seq DATA ANALYSIS

We assembled a reference genome database comprised of 206 oral species (134 unique species groups) for read mapping. The RNA-Seq data was processed through a pipeline that performs a series of quality control steps. First, raw reads were examined using the FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) tool. Quality scores were calculated based on Illumina 1.5 encoding. Trimming of low quality base calls were conducted using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic) by removing terminally located low quality bases (Phred scores <30) and cutting of reads when average quality dropped below 30. Finally, reads of 60 bases or less were removed from further analysis. The remaining reads were then evaluated for the presence of bacterial or human rDNA sequences using SortMeRNA (http://bioinfo.lifl.fr/RNA/sortmerna/) by filtering based on the default databases that include 16S, 23S, 18S, 5.8S, 5S, and 28S rRNAs. Sequences with similarity to these sequences are removed from further analysis. The remaining reads were then aligned to reference genomes using STAR aligner (https://code.google.com/p/rna-star/). In cases where sequence reads map to more than one location in a reference genome, or to more than one reference genome, the best alignment was selected. Sequence reads mapping sporadically and at a very low frequency to reference genomes (104 genomes) were dropped from further analysis.

GENE EXPRESSION ANALYSIS

Raw read counts from sequence alignment were assessed using htseq-count tool within the HTSeq python suite (http://www-huber.embl.de/users/anders/HTSeq/). For further analysis, the raw read counts were read into R/Bioconductor version 3/2.13 and were scaled using DESeq scaling factors (Anders and Huber, 2010) following $\log_2$ transformation (a constant +1 was added prior $\log_2$ transformation). Reads greater than 40 bases were used for mapping and 2 mismatches were allowed for mapping reads to reference genomes.

FUNCTIONAL ANALYSIS

rRNA filtered reads were uploaded to the MG-RAST analysis platform for functional analysis (Meyer et al., 2008). Functional data from MG-RAST analysis was retrieved using matR package (https://github.com/MG-RAST/matR) and further analyzed using R/Bioconductor.

RESULTS AND DISCUSSION

The majority of metagenomic analyses conducted thus far on the dental plaque microbiota have surveyed and compared the phylogenetic composition of communities associated with dental health and disease in the supra- and sub-gingival domains of the oral cavity. While informative, these studies do not provide insights into the functional features of these communities. Dental biofilms are comprised of metabolically active, metabolically inactive and dead cells. In order to evaluate the metabolically active cells of supragingival dental plaque biofilms we have conducted a survey of the RNA expression to gain insights of those functions that are important for survival and fitness in the highly competitive dental plaque biofilm. The species and relative contribution of transcripts to the transcriptome is largely consistent with our previous phylogenetic profiling results (Peterson et al., 2013) with respect to the genera and species present and their overall proportions within the community.

HUMAN COHORT AND RNA-Seq ANALYSIS

We performed RNA-Seq analysis of RNA isolated from dental plaque biofilms derived from 19 twin pairs. These subjects were given dental examinations that allowed each twin pair to be classified as belonging to one of three phenotypic classes (C-F, C-A, or C-I). RNA from each sample was subjected to RNA-Seq using the Illumina GSA platform to generate 100 base reads. An average of ∼32.4 million reads/sample (range = 23–40 million) were generated. We created an oral cavity reference genome database consisting of a total of 206 reference genome sequences, representing 134 unique oral species. These sequences and the associated SOPs developed for microbial mRNA enrichment is available through http://www.hmpdacc.org/RSEQ/. Despite attempts to remove human and bacterial rDNA sequences, high levels of these sequences remained and were removed *in silico*. **Table 1** summarizes the human cohort and bacterial sequences used for mapping. An average of ∼55% of reads generated were readily mapped to HMP reference genomes emphasizing the overall relevance of selected genomes and utility of this community resource.

GENETICS, ENVIRONMENT, AND CARIES PHENOTYPE AS DETERMINANTS OF TRANSCRIPTIONAL RELATEDNESS

Based on the relative abundance and origin of the profiled transcripts, we generated a dendrogram of the samples to assess whether twin pairs clustered more tightly than unrelated individuals and whether caries phenotype altered those relationships (**Figure 1**). Fourteen of the 19 twin pairs were most similar to each other with respect to their gene expression patterns, suggesting that either genetic and/or environmental factors are significant determinants of dental plaque microbiota gene expression patterns. To evaluate the influence of host genetics on transcriptional profiles, we compared the linkage of MZ and DZ twin pairs. Among the six MZ twin pairs, four (66%), displayed linkage, compared to 10 of the 13 DZ twin pairs (76%). A comparison of the linkage relationships among discordant MZ and DZ twin pairs revealed that 100% of all discordant MZ ($n = 3$) and DZ ($n = 6$) twin pairs displayed linkage. These results suggest that genetic and/or environmental factors are dominant to caries status as determinants of gene expression patterns. This conclusion is supported by the finding that only 50% of the concordant twin pairs displayed linkage. The sample size evaluated here does not allow definitive conclusions with respect to the relative influence of genetic determinants compared to environmental factors. To achieve statistical support for these conclusions will require analysis of a larger number of twin pairs in longitudinal studies.

Table 1 | Human subjects and RNA-Seq statistics.

Subject	Caries status	Twin type	Filtered reads	Reads mapped	%
2011	CA (1DS)	DZ	10404431	4690029	45.00
2012	CA (1DS)	DZ	5048821	2354837	46.64
2051	CF	MZ	15163860	6133805	40.45
2052	CF	MZ	8491819	3268038	38.49
2061	CA (2 DS)	DZ	5800355	2923616	50.41
2062	CF	DZ	4998861	2226509	44.54
2125	CA (17 DS)	MZ	5073718	2840183	55.98
2126	CA (6 DS, 3 FS)	MZ	10255337	5437838	53.03
2169	CF	MZ	5972974	3424681	57.34
2170	CA (1 DS)	MZ	7949869	4376808	55.05
2191	CF	MZ	6787165	3702907	54.56
2192	CF	MZ	5722083	2966892	51.85
2225	CF	MZ	7297864	3168223	43.42
2226	CI (1 FS)	MZ	9301393	2330247	25.06
2233	CF	DZ	4126376	1797372	43.56
2234	CF	DZ	5318936	2206502	41.48
2241	CA (1 DS)	DZ	3397928	1915754	56.38
2242	CA (1 DS)	DZ	4145884	2601872	62.76
2269	CA (2 DS)	DZ	3734615	2510588	67.23
2270	CF	DZ	3417500	2042613	59.77
2283	CI (2 FS)	DZ	5031738	3139713	62.40
2284	CA (1 DS)	DZ	4358907	2199217	50.45
2309	CF	DZ	9571270	3922570	40.98
2310	CF	DZ	7731245	2351980	30.42
2354	CA (1 DS)	DZ	4077295	2275040	55.80
2355	CA (1 DS)	DZ	1073838	464318	43.24
2930	CF	DZ	3873870	2279747	58.85
2931	CA (3 DS)	DZ	4071273	2225255	54.65
2954	CA (15 DS, 2 FS)	DZ	7338017	3247379	44.26
2955	CA (9 DS, 2 FS)	DZ	10133068	4293062	42.37
2991	CA (1 DS)	DZ	5331302	3270152	61.34
2992	CF	DZ	3267126	1265071	38.72
3214	CF	MZ	4008044	1758551	43.87
3215	CA (1 DS)	MZ	6357380	3360013	52.85
3306	CA (1 DS)	DZ	3773687	2163623	57.34
3307	CF	DZ	3748232	2198571	58.65
4131	CA (1 DS)	DZ	3656778	2075715	56.77
4132	CF	DZ	6734529	4223948	62.73

DS, decayed surface; FS, filled surface.

The apparent lack of association between caries status and gene expression linkage in twin pairs must be interpreted with caution since linkage is dictated by global features of the transcriptome. It is expected that gene expression patterns that distinguish C-F and C-A subjects may involve the altered expression of a small fraction of the transcriptome and therefore would not be revealed by this analysis. In addition, some subjects went from a state of health to disease within a follow-up visit. It is possible therefore, that these subjects possessed a C-A signature at baseline, despite being clinically C-F. Detailed analysis of expression patterns to identify those genes that may clearly distinguish C-F and C-A subjects are ongoing and not reported here.

GENE EXPRESSION OF GENERA PRESENT IN DENTAL PLAQUE MICROBIOTA

In contrast to phylogenetic profiling of microbiota, RNA-Seq data permits the analysis of metabolically active members of the supragingival dental plaque biofilm. We detected transcripts mapping to 27 genera that spanned six orders of magnitude in abundance (**Figure 2**). Consistent with their numerical dominance in dental plaque, transcripts expressed by *Streptococcus* spp. were the most abundant (53% of total), nearly five times more than those expressed by the next most prevalent genera *Veillonella* spp. (11%) and *Capnocytophaga* spp. (11%). Transcripts from these genera together with *Gemella* spp. (5%) and *Neisseria* spp. (3%) comprised 83% of all mapped transcripts. Within individuals, additional genera contributed significantly to the dental plaque transcriptome (>1%) including: *Aggregatibacter* spp. (6 subjects), *Fusobacterium* (3 subjects), *Haemophilus* spp. (8 subjects), *Lachnoanaerobaculum* spp. (8 subjects), *Lachnospiraceae* spp (4 subjects), *Leptotrichia* (6 subjects), and *Parvimonas* spp. (1 subject). These findings underscore the interpersonal variation in the genera contributing to the dental plaque biofilm transcriptome. It will be of interest to correlate the 16S rDNA profiles of these subjects to determine the extent that transcript abundance is related to relative species abundance.

GENE EXPRESSION OF SPECIES PRESENT IN DENTAL PLAQUE MICROBIOTA

Several studies to date have surveyed the dental plaque biofilm and saliva microbiota using culture independent 16S rDNA sequencing (Lazarevic et al., 2010; Jiang et al., 2013; Yang et al., 2014). Attempts to define a core microbiome are challenging due to high interpersonal and geographic variability in microbiota composition and a strong shift toward the use of short read sequencing technologies that generally allow only genus level enumeration (Griffen et al., 2011; Li et al., 2013). The RNA-Seq data mapped to the reference genomes of 79 unique species. A significant number of reads mapped to 58 unique species in all subjects suggesting that they may represent a substantial fraction of a core dental plaque biofilm microbiota. It will be of interest to determine whether this core microbiome definition extends to additional geographies beyond the cohort examined here. Displayed as an aggregate, the transcripts expressed by individual species are relatively continuous over a broad abundance range (Figure S1). Transcripts derived from just 9 species including: *S. sanguinis* (16%), *S. mitis* (10%), *V. parvula* (9%), *Capnocytaphaga* sp. (9%), *S. oralis* (8%), *Streptococcus* sp. (7%), *G. haemolysans* (5%), *S. gordonii* (4%), and *Neisseria* sp. (3%) represented 71% of the dental plaque microbiota's transcriptome. An additional six species produced transcripts at 2% abundance. *S. mutans* was among these moderate abundance species. An additional 16 species produced transcripts at 1% of the total transcriptome. Together these 31 species account for 99% of the mapped transcripts observed in this cohort.

The frequency of observed transcripts expressed by individual species also spanned six logs in magnitude (**Figure 3**). The abundance of transcripts generated by individual species is variable across subjects, varying by approximately two logs or less. The variation in transcript abundance across human subjects is

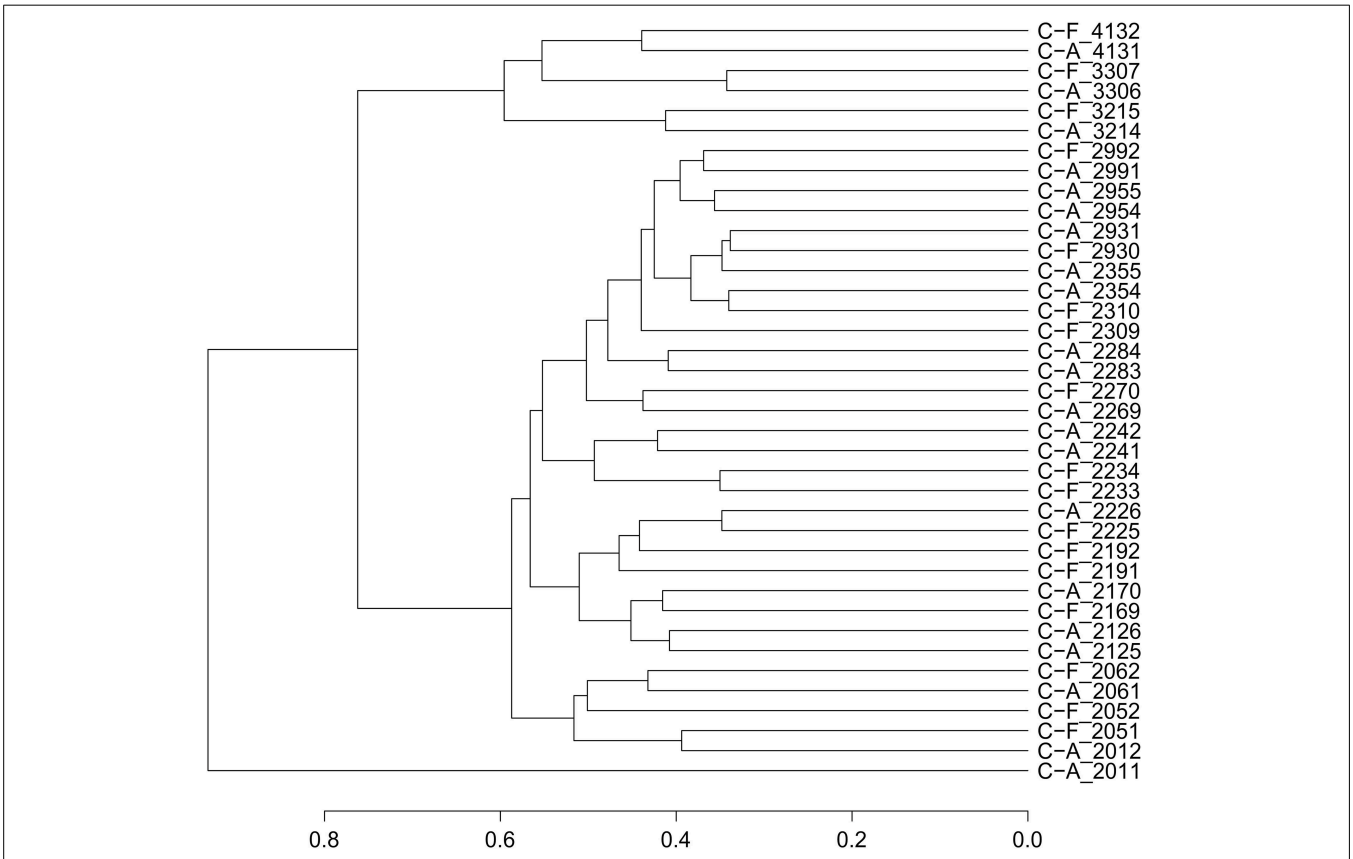

FIGURE 1 | Dendrogram of twin pairs based on RNA expression patterns.

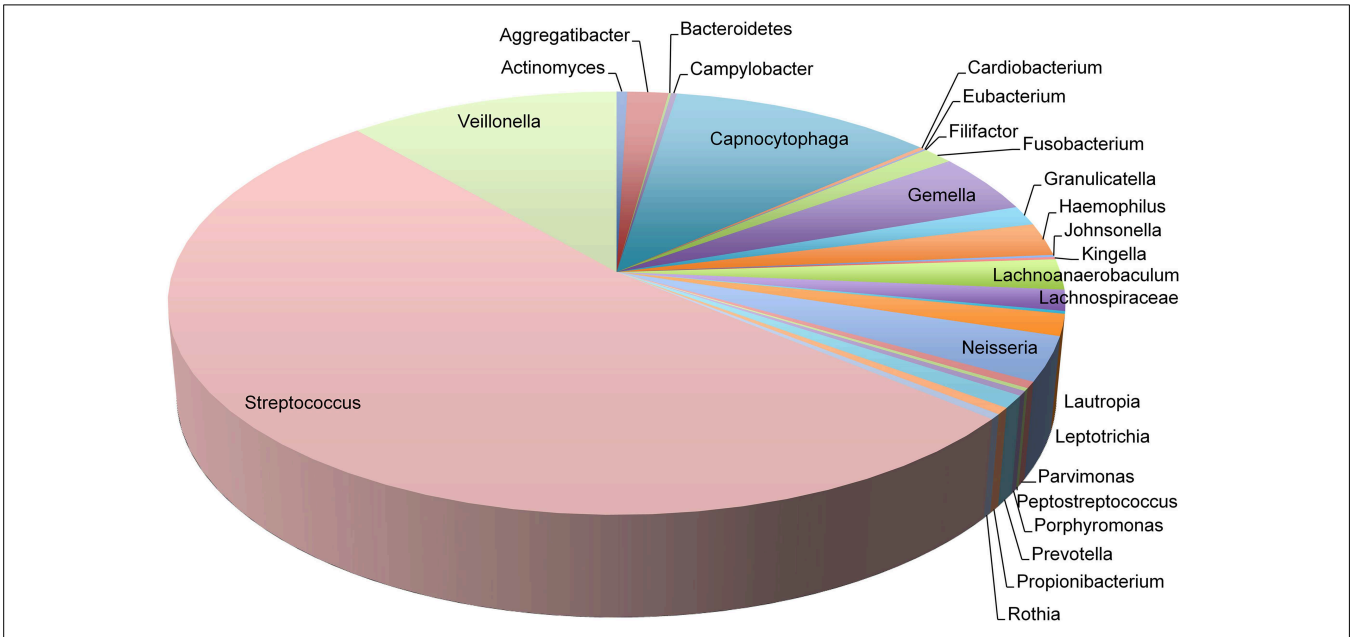

FIGURE 2 | Transcript abundance of representative genera in dental plaque biofilms. Pie chart representing the proportions of transcripts mapping to 27 genera observed in 19 twin pairs.

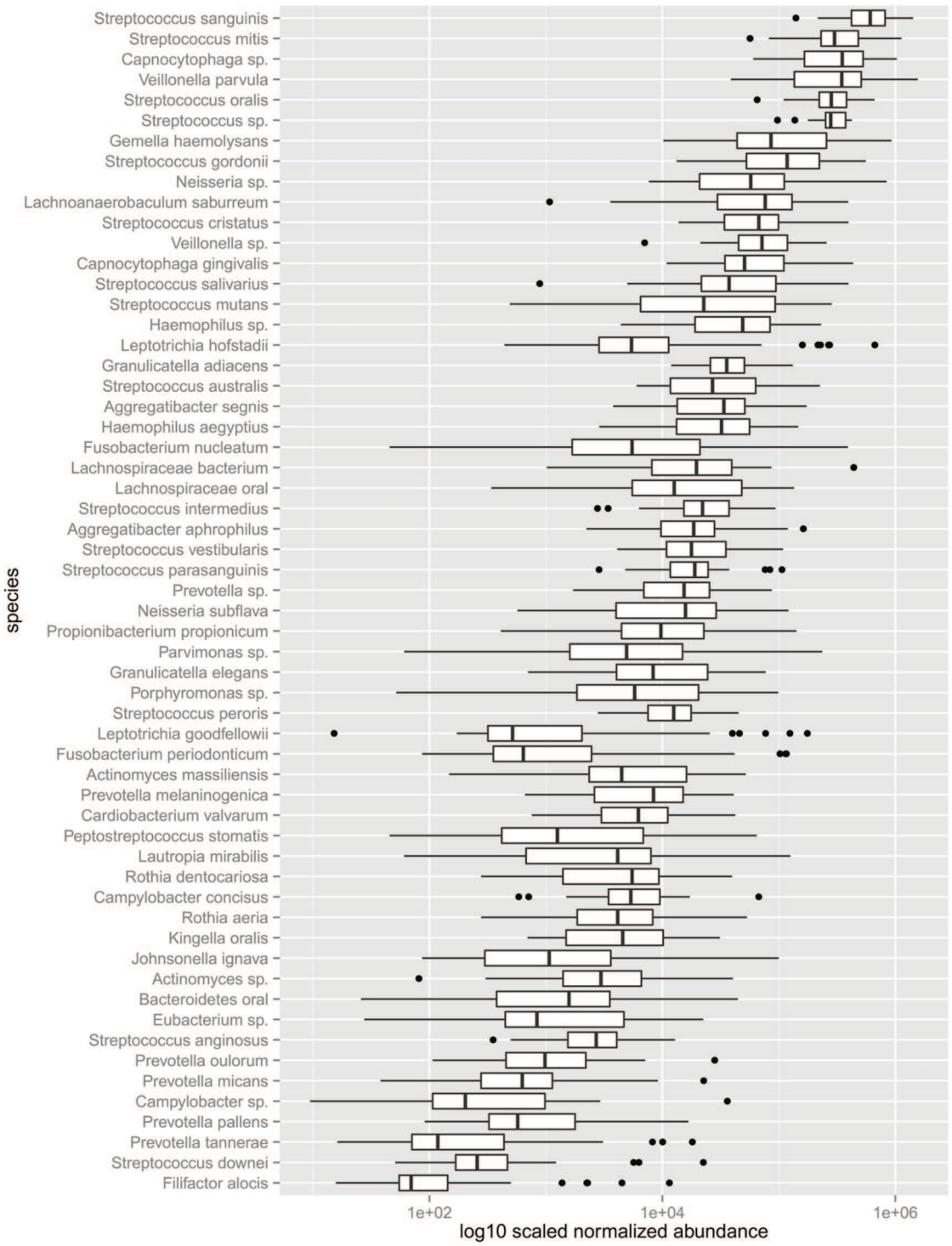

FIGURE 3 | Abundance of the dental plaque core microbiota. Box and whisker plot. Outliers shown as dots represent values 1.5 times great or less than the upper and lower quartile, respectively.

consistent with numerous reports describing the high interpersonal variation in phylogenetic representation of supragingival dental plaque (Bik et al., 2010; Gross et al., 2010). *Streptococcus* spp., with the exception of *S. mutans* displayed rather limited variation in transcript abundance across subjects, whereas other species display markedly increased variability. The transcripts expressed by *Fusobacterium nucleatum* were detected over a range of 4 logs. Approximately 40% of the species displayed extreme

transcript abundance variation (>2 SD from the mean). It is notable that many of the outliers tend to be over-represented with respect to the mean as illustrated by those expressed by *Leptotrichia hofstadii*, suggesting that in some instances the abundance of these species may be highly over-represented in subjects. The transcript abundance generated by species, of moderate and low abundance display the largest variation. These results provide a set of parameters that define fluctuations in phyla as it relates to health and disease states. It is evident from these results that species-specific transcriptional variability may range from biologically significant to inconsequential.

FUNCTIONAL NETWORKS BASED ON GENE EXPRESSION CORRELATIONS

The relative abundance of species within the dental plaque biofilm community is dictated by numerous and mostly undefined signals present in the microenvironment. These signals are both host and microbe-generated. The cooperative and antagonistic relationships amongst resident species in dental plaque biofilms suggest that the abundance of individual species and their transcripts is not independent of the activities of other species (Tong et al., 2008; Tamura et al., 2009). This speculation is strongly supported by the data. We used Spearman correlation to address whether the transcriptional activities of individual taxa display relationships (**Figure 4**). Correlations based on transcript abundance across subjects indicate that the majority of correlations amongst genera are positive. Multiple *Streptococcus* spp. display weak positive correlations with one another. This relationship may reflect the large overlap in sugar utilization potential encoded by these genomes that provide broad similarities in environmental conditions that co-select for increased growth and/or metabolic activity. This trend is evident for a number of other genera wherein member species display correlated transcriptional activity. Exceptions included: *Gemella* spp. (*G. elegans* and *G. adjacens*) and *Rothia* spp. (*R. aeria* and *R. dentocariosa*) that do not appear significantly correlated. *Actinomyces* spp. and *Lautropia mirabilis* display transcriptional activity that is largely anti-correlated with the majority of the dental plaque community, suggesting that the signals favoring their metabolic activity may be distinct compared to the majority of the dental plaque microbiota.

FIGURE 4 | Spearman Correlation matrix of the dental plaque core microbiota. Blue indicates positive correlation and yellow indicates negative correlation.

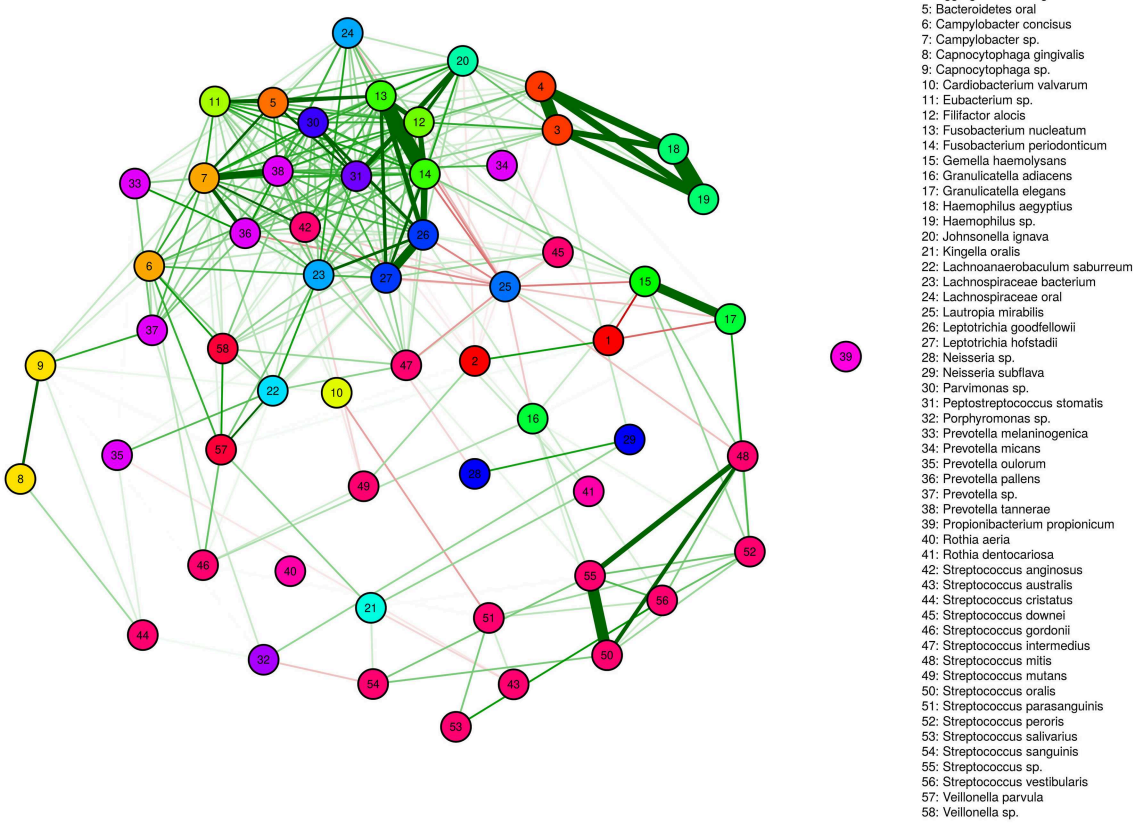

FIGURE 5 | Functional Networks. Positively correlated (green) and anti-correlated (red) transcripts define networks. The thickness of lines indicates the strength of the correlation.

We identified several genera/species displaying both correlated and anti-correlated transcriptional activity. These relationships define functional networks representing a spectrum of simple and complex community relationships (**Figure 5**). A deeply integrated network involving, predominantly positive interactions are evident. It is interesting that this high-density network is highly diverse in its membership and includes more than half of the observed genera. The high-density cluster of interactions is relatively devoid of *Streptococcus* spp. Somewhat surprising, the *Streptococcus* display a range of mostly weak positively correlated networks that are relatively independent of the expression patterns of the majority of the microbial community. High interpersonal and geographic variation of dental plaque microbiota has hampered our ability to identify the microbial signatures associated with dental health and disease. The network relationships observed suggest that the fluctuations of single species are in many instances likely to be accompanied by shifts in other species in the network. These functional networks represent a potentially simplifying framework and may represent a more effective way to compare features of microbiota associated with health and disease. It is possible that genera unique to particular geographies may belong to the functional networks observed and described below.

One positively correlated network includes the genera *Bacteroides*, *Eubacterium*, *Filifactor*, and *Fusobacterium* (complex I). The abundance of transcripts generated by these genera span approximately two logs and vary across subjects in a coordinated manner (**Figure 6**). With some exceptions, the *Fusobacterium* are the most abundant genera within this network, whereas the remaining genera are more variable in relative abundance. The species membership of this network includes: *F. nucleatum, Fusobacterium periodonticum, B. oral* an uncharacterized *Eubacterium* spp., and *Filifactor alocis.* The mixed dominance relationships of this network across subjects may suggest that the signals that regulate the growth behavior are complex.

Another positively associated network includes the genera *Peptostreptococcus* spp., *Bacteroides* spp., *Campylobacter* spp., *Johnsonella* spp., and *Parvimonas* spp. (complex II) The relative abundance of this network is more tightly linked, compared to complex I. The variation in abundance across subjects is slightly more than 1 log (Figure S2). However, the dominance relationships of complex II are more variable. The species comprising this network are *Peptostreptococcus stomatis, Bacteroidetes oral, Campylobacter concisus,* an uncharacterized *Campylobacter* spp., *Johnsonella ignava,* an uncharacterized *Parvimonas* spp., *Lachnospiraceae bacterium*, and *Lachnospiraceae*

oral. The observed fluctuations are relevant in that certain configurations drive the abundance of *Parvimonas* and other genera into an abundance range that may be of biochemical consequence to the microenvironment.

A third network including: *Haemophilus* spp., *Lachnoanaerobaculum* spp., and *Aggregatibacteria* spp. (complex III) display both positive and negative correlations (**Figure 7**). The abundance of transcripts expressed by *Haemophilus* spp. and

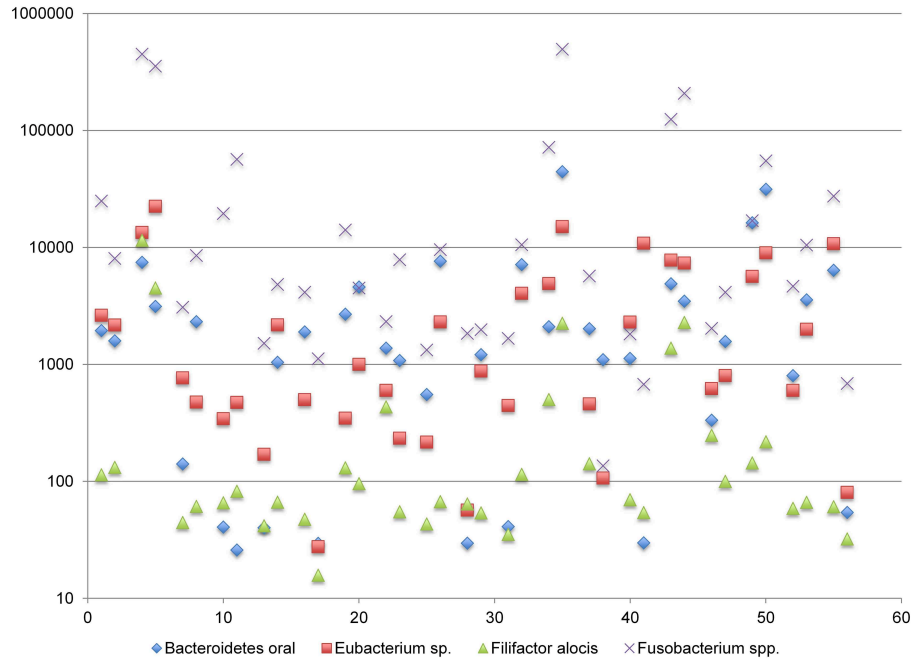

FIGURE 6 | Functional Network in dental plaque microbiota, complex I. The y-axis displays read counts on a log scale. The x-axis displays subjects in the order shown in **Table 1**.

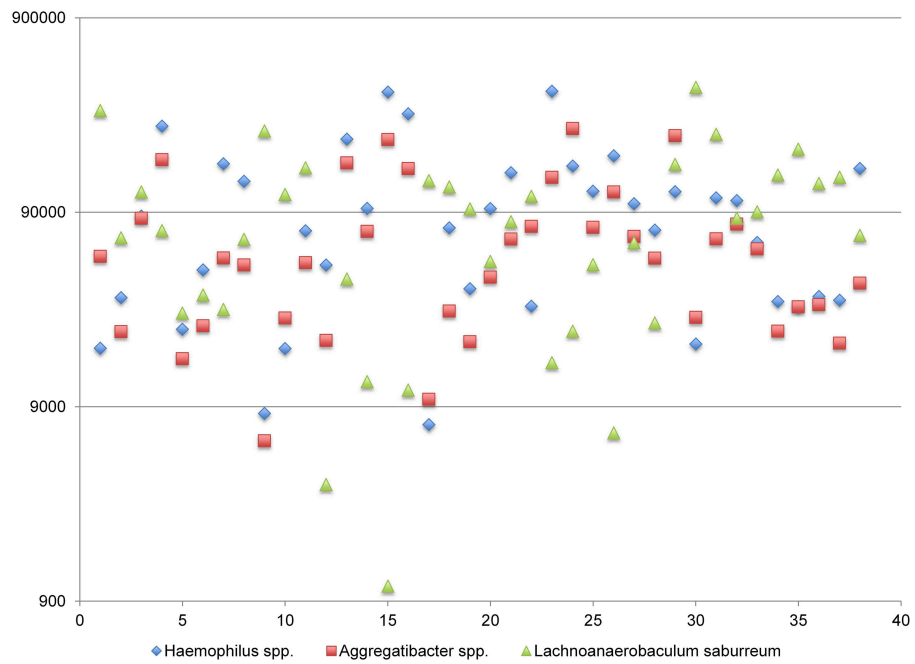

FIGURE 7 | Functional Network in dental plaque microbiota, complex III. The y-axis displays read counts on a log scale. The x-axis displays subjects in the order shown in **Table 1**.

Aggregatibacteria spp. are tightly linked, generally differing by less than 5-fold. By contrast, the abundance of *Lachnoanaerobaculum* spp. is anti-correlated with respect to these genera. When *Haemophilus* spp. and *Aggregatibacteria* spp. transcript levels are high, *Lachnoanaerobaculum* spp. transcript levels are low and *vice-versa*. Inspection of **Figure 5** shows that *Aggregatibacteria* spp. are positively correlated with the high density functional network, whereas the *Haemophilus* spp. is only indirectly linked to the large network, based on its strong positive interactions with *Aggregatibacteria* spp. The behavior of this complex may be the result of specific niche associated signals that favor the outgrowth of one group and reciprocally inhibit the other. It is of potential interest that members of this complex "aggregate" at a point where transcripts generated by each member genera are in the range of 0.5% of the total. It is unclear whether this aggregation point has biological significance or represents a point of complex equilibrium, the balance of which can be disturbed in predictable ways. The species involved in this network are *H. aegyptus* and an uncharacterized *Haemophilus* spp. and two Aggregatibacter spp., *A. segnis* and *A. aphrophilus*.

The genera: *Leptotrichia* spp., *Lautropia* spp., and *Lachnospiraceae* spp. define another network involving positive and negative correlations (Figure S3). The transcript abundance generated by *Leptotrichia* spp. and *Lachnospiraceae* spp. is positively correlated and anti-correlated with respect to *Lautropia* spp. (complex IV). An analysis of these correlations at the species level revealed the network members to include: *L. hofstadii, L. bacterium, L. oral, Leptotrichia goodfellowii*, and *L. mirabilis*. Referring to **Figure 5**, we see that *L. mirabilis* is conspicuous in its nearly exclusively anti-correlated relationships. The majority of the anti-correlated relationships involve many of the genera making up the high-density network. The *Lachnospiraceae* are generally dominant in this network, although relatively frequent co-dominance with *Leptotrichia* are observed. One subject, showed *Lautropia* dominance and was associated with uncharacteristically low abundance of transcripts expressed by *Lachnospiraceae* and *Leptotrichia.*

A fifth functional network consisting of the genera *Streptococcus* spp., *Parvimonas* spp., *Eubacterium* spp. and *Campylobacter* spp. (complex V) was noted (Figure S4). The transcripts produced by these genera are positively correlated but anti-correlated with *Streptococcus* spp. The species involved in this network include: *Campylobacter concisus,* and an uncharacterized *Campylobacter* spp., *Parvimonas* spp. *Eubacterium* spp. It was difficult to identify any species within the *Streptococcus* that exhibited uniform anti-correlated transcription suggesting that the growth inhibiting influence of the *Streptococcus* within this network likely involves the combined activities of two or more species. It may be speculated that the overall balance between the *Streptococcus* and the remainder of the complex is based on sugar availability, since the fermentative *Streptococcus* may thrive under conditions that differ from the remainder of the complex that are primarily asaccharolytlic (*Campylobacter* spp., *Parvimonas* spp. *Eubacterium* spp.). Despite various reports of antagonistic relationships among *Streptococcus* spp., we do not observe such anti-correlated relationships at the level of transcription. The transcripts produced by the two most abundant species, *S. sanguinis* and *S. mitis* are positively correlated, however it is of potential interest that in instances where *S. mitis* is numerically dominant to *S. sanguinis,* the level of the *S. sanguinis* transcripts are reduced, suggesting that *S. mitis* may directly inhibit *S. sanguinis* when it is the most abundant species present.

An additional anti-correlated network (complex VI) including the numerically dominant genera *Gemella* spp. are strongly anti-correlated with those produced by *Actinomyces* spp. (Figure S5). From this data it is not clear, whether the conditions promoting high transcriptional activity of the *Gemella* spp. is inhibitory to *Actinomyces* spp., or if conversely the conditions selecting for elevated transcriptional activity of *Actinomyces* is inhibitory to *Gemella* spp. When analyzed at the species level, we identified *A. massiliensis* and *G. haemolysans* as the members of this network.

FUNCTIONAL ANALYSIS OF DENTAL PLAQUE BIOFILM GENE EXPRESSION

Using the SEED subsystems role categories within the MG-RAST metagenomic analysis tool we see that despite the relatively large interpersonal variation in species-specific transcription, the representation of functional role categories is more homogeneous across subjects (**Figure 8**). This result is consistent with previous reports based on the analysis of functional annotation of metagenomic DNA sequences (Human Microbiome Project, 2012). The breadth of functions expressed at >1% of the total is substantial and descriptions of each are beyond the scope of this manuscript. A detailed analysis of these functions is ongoing. The most abundant and variable functional role category involves transcripts encoding functions pertaining to protein translation (range = 13–28%). The most abundant transcripts expressed by the dental plaque microbiota encode ribosomal subunit biogenesis (8.9% of total), and transcripts derived from the translation elongation factors, EF-Tu and EF-G (4% of total). The next most transcriptionally abundant functional category relates to carbohydrate utilization (∼10% of total). The functions contributing to this role category include those involved in glycolysis/gluconeogenesis and the Entner-Doudoroff pathway that convert glucose to pyruvate. Transcripts encoding RNA polymerase subunits were also prevalent.

Transcripts encoding functions related to monosaccharide and disaccharide metabolism represent a significant portion of the dental biofilm transcriptome (∼15% of total). Transcripts encoding enzymes for the metabolism of allose, galacuronate/glucuronate, gluconate, ribose, sorbitol/sorbose, tagatose/galctitol, fucose, rhamnose, and xylose were observed. In most subjects the transcripts encoding enzymes for the metabolism of tagatose (a stereoisomer of fructose) and galactitol (generated by the metabolism of lactose and subsequent conversion to galactose) were the most highly represented, although in three subjects the dominant transcripts in this category encoded enzymes involved in sorbitol (the sugar alcohol form of glucose) and sorbose metabolism. In general, transcripts encoding disaccharide metabolism were more prevalent than those encoding monosaccharides by a factor of ∼2. The most abundant transcripts associated with di-saccharide metabolism, encoded functions involved in lactose and galactose metabolism, although in a

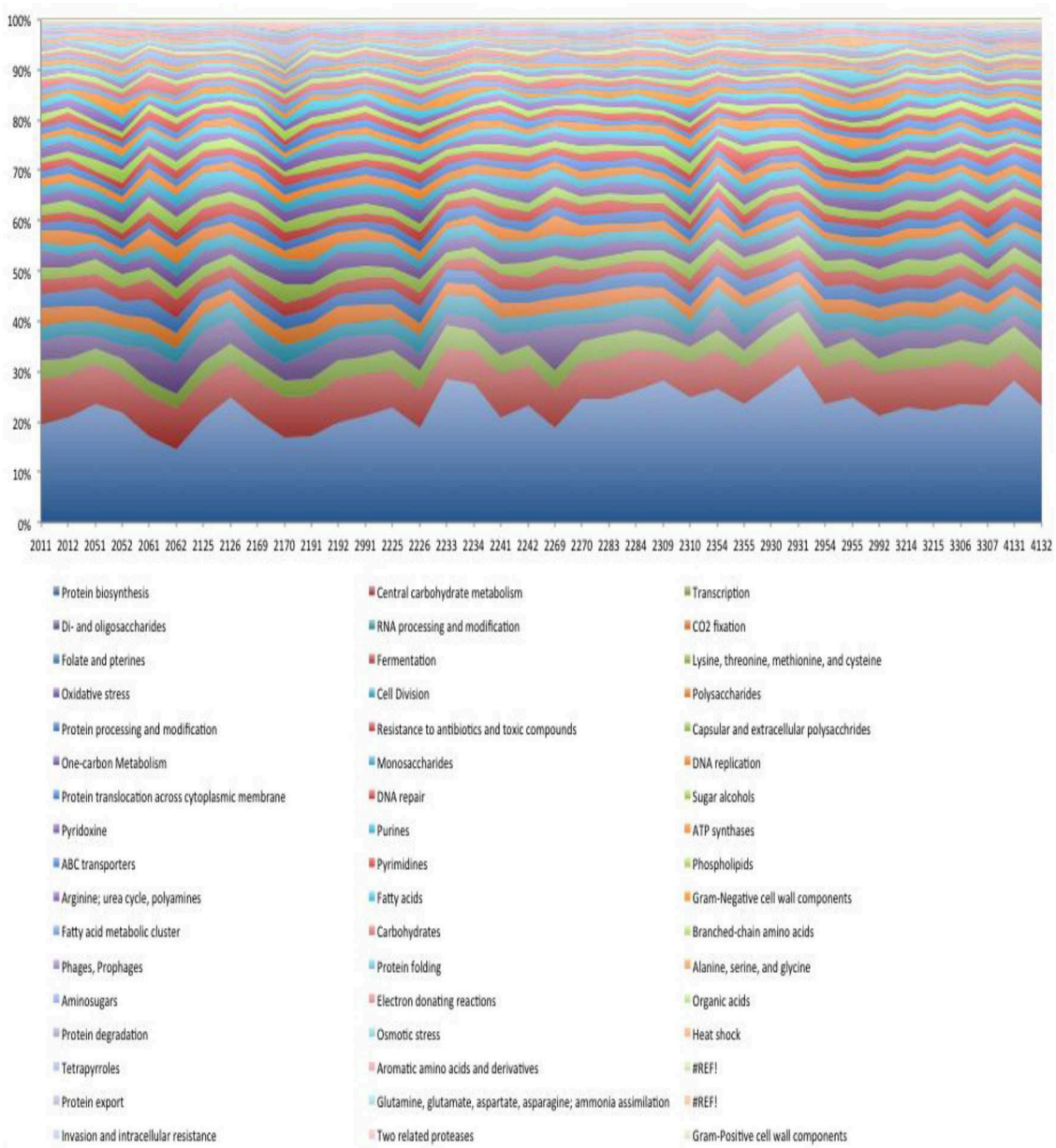

FIGURE 8 | Functional role categories expressed in dental plaque biofilm.

few subjects, transcripts encoding trehalose metabolism functions were the most abundant in this category.

We observed a relatively large number of transcripts encoding functions related to the Calvin Benson cycle, a CO_2 fixation pathway used in plants. The vast majority of reads (74%) related to this pathway corresponded to GAPDH, fructose-bisphosphate aldolase (9%), phosphoglycerate kinase (5%), triose phosphate isomerase (2%) likely reflecting the glycolytic pathway. Other transcripts encoding transketolase, ribose-5-phosphate isomerase, ribulose phosphate 3-epimerase, participate in the pentose phosphate pathway. Therefore, the reads assigned to the Calvin Benson cycle in dental plaque microbiota samples represent enzymes with overlapping functions in glycolysis and the pentose phosphate pathway. The transcripts encoding for sugar alcohol utilization were predominantly associated with ethanolamine utilization (∼50% of total) and mannitol utilization (∼25% of total).

OXIDATIVE STRESS

The dental plaque microbiota produces a substantial number of transcripts encoding stress response adaptations including detoxification, heat shock, osmotic and oxidative stress. The majority of stress response transcripts (50–75% of total) were associated with oxidative stress suggesting that oxidative stress may

be the dominant stressor of dental biofilm microbial communities (∼2% of all transcripts). Two inter-related systems are dominant within this group, transcripts encoding superoxide dismutase, that mediate the conversion of superoxide to molecular O_2 and H_2O_2. These transcripts represented 22% of those related to oxidative stress and 0.4% of the total transcriptome. It is interesting to note that this reaction is H^+ consuming and given its relative abundance may play a role in acid remediation in dental plaque. An impressive number of transcripts are produced by dental plaque biofilm encoding functions that serve to metabolize (detoxify) superoxides and peroxides. Peroxiredoxins (9% of oxidative stress transcripts, 0.2% of total transcriptome) alter their redox state in order to convert H_2O_2 to H_2O. This enzyme activity represents a potential biomarker of squamous cell carcinomas (Huang et al., 2011; Jancsik et al., 2013). Transcripts encoding catalase (1% of oxidative stress genes) performs the same conversions in the biofilm community using an Fe redox process. Interestingly, another prominent set of transcripts encoding ferroxidase (4% of oxidative stress genes), an enzyme that reduces Fe^{2+} to Fe^{3+} while consuming H^+. Ferritin-like proteins also have ferroxidase activity and were expressed at similar levels as ferroxidase. The relevance of this pathway is based on the damaging effects of ferrous Fe^{2+} in the presence of H_2O_2 on Fe-S cluster containing proteins.

RESISTANCE TO ANTIBIOTICS AND TOXIC COMPOUNDS

The transcripts encoding resistance to antibiotics and toxic compounds was of interest. The majority of transcripts encode functions pertaining to metal tolerance and regulation. Large and diverse systems devoted to the maintenance of metal homeostasis underscores the relative importance of these systems. More than 16% of transcripts within this group encode mercury (II) reductase (EC 1.16.1.1). This enzyme mediates the generation of NADPH accompanied by the reduction of Hg to Hg^{2+}. Copper homeostasis is maintained by P-type ATPases that use cellular energy (ATP) to pump Cu^{2+} ions out of the cell. These systems are greatly expanded compared to those functions related to other metals and toxins including cadmium, cobalt and arsenic. Transcripts involved in antibiotic resistance were difficult to interpret since many transcripts assigned to this group pertain to proteins that when mutated confer resistance to antimicrobial drugs. However, transcripts encoding the acriflavin resistance complex (AcrA and AcrB), that confers protection to cells from hydrophobic inhibitors including many common antibiotics in use today were prevalent. Transcripts corresponding to this multi-drug efflux system were 4% of the total in this group. Additionally, 1.6% of transcripts within this group encode a putative macrolide-specific efflux system. Transcripts encoding for proteins involved in acid stress and bacteriocins represented only minor components of the dental plaque biofilm's transcriptome across all subjects, including those with high caries activity.

SUMMARY

The results presented here have provided a number of unique insights with regard to the biochemical priorities and the environmental and/or genetic influence on these patterns of the dental plaque biofilm microbiota. Gene expression patterns amongst some genera are coordinated. Previous studies have attempted to recognize associations between features of the microbiota (individual genera or species) and dental health/disease. These studies have been confounded by an inability to control genetic and environmental factors, high interpersonal and geographical variation of dental biofilm communities. The recognition of functional networks operational in dental plaque communities may be of importance since it reduces the number of independent variables that may define dental health and caries activity. A longitudinal study of these networks as human subjects transition from C-F to a C-A phenotype will provide a direct test of the biological significance of these networks. The dental biofilm microbiota devotes a significant amount of its transcriptional potential to the expression of proteins that substantially remediate superoxides and peroxides and H^+ produced by fermentative bacterial species. Maintenance of metal homeostasis, particularly of Fe^{2+} that are damaging to Fe-S cluster containing proteins in the presence of H_2O_2, also uses biochemical processes that consume H^+. These important stress response pathways may represent a previously overlooked system used by dental biofilm microbiota to cope with a low pH microenvironment.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00108/abstract

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Transcriptomic analysis of three *Veillonella* spp. present in carious dentine and in the saliva of caries-free individuals

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Veillonella spp. are predominant bacteria found in all oral biofilms. In this study, a metatranscriptomic approach was used to investigate the gene expression levels of three oral *Veillonella* spp. (*V. parvula, V. dispar* and *V. atypica*) in whole stimulated saliva from caries-free volunteers and in carious lesions ($n = 11$ for each group). In the lesions the greatest proportion of reads were assigned to *V. parvula* and genes with the highest level of expression in carious samples were those coding for membrane transport systems. All three *Veillonella* spp. increased expression of genes involved in the catabolism of lactate and succinate, notably the alpha- and beta-subunits of L(+)-tartrate dehydratase (EC 4.2.1.32). There was also significantly increased expression of histidine biosynthesis pathway in *V. parvula*, suggesting higher intra-cellular levels of histidine that could provide intra-cellular buffering capacity and, therefore, assist survival in the acidic environment. Various other systems such as potassium uptake systems were also up regulated that may aid in the survival and proliferation of *V. parvula* in carious lesions.

Keywords: *Veillonella*, caries, stress proteins, pH regulation, RNA-sequencing

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Introduction

Veillonella are obligate anaerobic Gram-negative small cocci isolated from the oral cavity and intestinal tract of humans and animals that gain energy from the utilization of short-chain organic acids, particularly lactate and succinate (Delwiche et al., 1985). The human *Veillonella* are *Veillonella parvula*, *V. atypica*, *V. dispar*, *V. montpellierensis*, *V. denticariosi,* and *V. rogosae* (Mays et al., 1982; Rogosa, 1984; Jumas-Bilak et al., 2004; Byun et al., 2007; Arif et al., 2008). The predominant *Veillonella* species on the tongue were *V. rogosae*, *V. atypical,* and *V. dispar* (Beighton et al., 2008; Mashima et al., 2011). *V. parvula* has often been detected as the predominant *Veillonella* species isolated from active occlusal carious lesions (Arif et al., 2008; Beighton et al., 2008). Based on these studies, each *Veillonella* species seems to occupy different intra-oral habitats with limited degree of overlap between species. With the pH of carious lesions reported to be below 5 (Hojo et al., 1994), the bacteria's ability to colonize and proliferate in such an environment necessitates them to exhibit a phenotype characterized by acid resistance. The objective of this study was to determine and compare the transcriptome of three of the predominant human oral *Veillonella* (*V. parvula*, *V. dispar,* and *V. atypica*) present in caries lesions and in the saliva of caries-free individuals.

Many bacterial genome sequence data are now publicly available, making it possible to exploit the opportunities offered by next generation sequencing (NGS) approaches to determine the *in vivo* expression of specific bacterial genes of individual species present in mixed-population biofilms. The short reads obtained from NGS can be aligned to bacterial genomes, enabling transcriptomic analysis of species without the need for species-specific protocols, as is necessary with the microarray approach. The functional potential of the oral microbiome has been investigated using metagenomic approaches in which genomic DNA is extracted, sequenced and the resulting sequences annotated by comparison to extant complete and partial genome sequences (Belda-Ferre et al., 2012; Luo et al., 2012). To investigate gene expression, the metatranscriptome of an individual species within a natural biofilm may be determined using RNA sequencing (RNA-seq). The application of RNA-seq to the study of bacterial transcriptomes has been reviewed by Pinto et al. (2011) and McLean (2014). Several studies have also recently described the use of RNA-Seq as a tool to investigate the oral microbiome in health and disease (Duran-Pinedo et al., 2014; Jorth et al., 2014) as well as interrogate specific metabolic pathways in oral bacterial species *in vitro* (Zeng et al., 2013).

In this study, we adopted a metatranscriptomic approach to investigate the level of genes expressed by the three *Veillonella* in both active carious lesions and saliva of caries-free subjects, in order to observe metabolic activities occurring in their natural environment, which may give an insight into their intra-oral distribution.

Materials and Methods

Samples Collection and RNA Isolation

Ethical approval was obtained for the collection of carious lesions ($n = 11$) and saliva ($n = 11$) samples. All subjects ($n = 22$) gave informed consent prior to collection of the clinical material. Extracted teeth with large occlusal soft, active carious lesions were obtained from patients attending dental clinics at Guy's Hospital dental surgery. The teeth were immediately placed in 5 ml RNAprotect® Bacteria Reagent (Qiagen) and transferred to the laboratory. The superficial biofilm was carefully removed and discarded. The infected soft dentine was collected using sterile excavators, and placed in 1 ml RNAprotect reagent, disaggregated, centrifuged (4°C at 10,000 × g) and the pellets stored at −80°C. Whole mouth wax-stimulated saliva samples, collected for 5 min, were obtained from caries-free volunteers who refrained from eating for at least 2 h prior to sampling. Immediately after collection, RNAprotect reagent was added to the saliva (1:1 v/v), the samples were centrifuged and the pellets stored at −80°C until further processing. Total RNA was extracted using the UltraClean® Microbial RNA isolation kit (MOBIO Laboratories, Inc.), including a DNase treatment step using the RNase-Free DNase Set (Qiagen) prior RNA elution.

cDNA Synthesis and Library Preparation for High-Throughput Sequencing

A minimum of 100 ng of total RNA was extracted from each clinical sample. The total RNA was processed using reagents provided in the Illumina® TruSeq™ RNA Sample Preparation Kit. Briefly, the RNA extracts were further purified, and fragmented. First and second strands cDNA were synthesized with Superscript II Reverse Transcriptase (Invitrogen). End repair was performed on the nucleic acid fragments, 3' ends were adenylated and adapter indexes ligated. The processed cDNA were amplified and further purified, prior library validation with the Agilent DNA 1000 Bioanalyzer (Agilent Technologies) and dsDNA BR Qubit assays (Invitrogen). The resulting libraries were processed for cluster generation using the TruSeq paired end cluster kit v.2, Illumina Inc., and an equimolar amount of each library was run in a separate flowcell lane. Paired end sequencing was then carried out using a Genome Analyzer IIx Illumina platform to produce 76 bp reads.

Data Handling and Gene Expression Analyses

A FASTQ file was obtained for each of the 22 cDNA libraries. Initial checks of the sequencing read base qualities were done via the local server provided by the Genomics facilities at Guy's Hospital Biomedical Research Centre, the data were then imported into the CLC Genomics Workbench software (CLC Bio, Qiagen). Within the CLC environment, adapter sequences were removed and for each sample file a short read mapping was performed simultaneously against 144 annotated oral bacterial genomes which were previously imported from various databases (the DNA Data Bank of Japan, NCBI, the Broad Institute and HOMD databases) (**Supplementary File 5**). The read mapping was carried out using the RNA-Seq analysis package default settings (mismatch cost: 2, insertion cost: 3, deletion cost: 3, length fraction: 0.8, and similarity fraction: 0.8; with the maximum number of hits for a read set to 1) within the CLC software, which employs the CLC Assembly Cell (CLC3) read mapper (http://www.clcbio.com/products/clc-assembly-cell/).

In this study, we are concerned with reads that mapped to 3 *Veillonella* strains: *V. parvula* DSM2008, *V. dispar* ATCC 17748, and *V. atypica* ACS 0049 V Sch6 only (**Table 1**). In order to facilitate comparison between these *Veillonella* strains, a RAST annotation (Rapid Annotation using Subsystem Technology) (Aziz et al., 2008) was carried out on their genomes and used with the other oral strains in the read mapping. All of the 22 sequence data files were processed for read mapping against the 144 oral genomes. Results were exported as excel files containing raw read counts determined for each of the genes from the 144 oral strains (total of 351,456 genes) (**Supplementary File 1**). In order to compare expression levels between the 22 biological samples, the raw count data were gathered into a single excel spreadsheet for normalization (**Supplementary File 1**). The read counts were scaled by determining the effective library size of each sample, using the estimateSizeFactors and counts accessor functions within the Bioconductor R package DESeq (Anders and Huber, 2010), which provided an output table displaying normalized expression values for each gene and for each of the 22 samples. Data corresponding to the 3 *Veillonella* strains were manually extracted from the spreadsheet and used separately for further analysis to infer on their gene expression levels in caries lesions and caries-free saliva samples. Median values were calculated for both caries and saliva sample groups ($n = 11$ each) (**Supplementary File 2**), which we called relative median expression (RME) values. The RME values of identical genes found in the 3 *Veillonella*

TABLE 1 | Characteristics of the 3 *Veillonella* strains selected in this study, with their relative proportions in caries and saliva metatranscriptomes (CDS refers to coding genes).

Feature code	*Veillonella* species	strain	Number of CDS (from RAST annotation)	Relative proportion (%) of transcripts in caries ($n = 11$) ±SD	Relative proportion (%) of transcripts in saliva ($n = 11$) ±SD
HMPREF	*V. atypica*	ACS-049-V-Sch6	1840	0.91 ± 0.43	4.09 ± 3.47
VEIDISOL	*V. dispar*	ATCC 17748	1954	2.18 ± 1.13	7.08 ± 5.07
Vpar	*V. parvula*	DSM 2008	1904	16.62 ± 11.17	4.76 ± 7.21

strains were summed and ranked from highest to lowest values, to observe the most highly expressed *Veillonella* transcripts in caries and caries-free saliva samples (**Supplementary File 2**). The gene identities were obtained from the RAST annotations and was supplemented by BLAST searching within Uniprot (http://www.uniprot.org/), InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and PATRIC (http://patricbrc.org/) when necessary.

The raw read count data from all 144 oral strains were also used to carry out differential gene expression analysis between both sample groups using the statistical software R package DESeq2 (Love et al., 2014) based on the negative binomial model. Differential expression analysis results for the 3 *Veillonella* strains were manually extracted from the total R result outputs into excel spreadsheets, and the largest negative and positive Log2 Fold Change values, with adjusted *p*-values (padj) $< 10^{-3}$ were considered as significant.

The **Supplementary File 1** contains the raw count input information used for the DESeq and DESeq2 analyses.

Sequence Data Accession Numbers

RNA-Seq sequencing data are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive; biosamples accession numbers for this study are SRS741215 and SRS752041.

Results and Discussion

Analysis of Read Count and Ecological Considerations

Here we have determined gene expression levels by mapping reads to bacterial species which form part of the oral microbial populations. The total number of mapped reads ranged between 25,593,022 and 88,238,546 for the caries-free saliva samples and between 20,088,245 and 32,910,299 for the caries samples (**Supplementary File 1**). In the carious lesions, 16.62 ± 11.17 per cent, 2.18 ± 1.13 per cent and 0.91 ± 0.43 percent of the mapped reads were assigned to *V. parvula, V. dispar,* and *V. atypica*, respectively, compared with 4.76 ± 7.21, 7.08 ± 5.07, and 4.09 ± 3.47 in the saliva samples (all $p < 0.05$) (**Table 1**). The pattern of the distribution of reads mirrored the reported distribution of these three species based on cultivable bacterial studies (Arif et al., 2008; Beighton et al., 2008). Belda-Ferre et al. (2012) also reported *V. parvula* to be the most predominant species in biofilm infecting dentine, with 166 contigs (>500 bp) assigned to *V. parvula* from their metagenomic data.

The major environmental factors affecting the *Veillonella* strains in the carious lesions and in saliva are suspected to be the low pH and availability of organic acids (lactate and succinate) required for the generation of ATP. The acidic environment within carious lesions is unlikely to be homogenous despite lactic acid being the major organic acid present (Palmer et al., 2006), resulting in areas that might be more alkaline (i.e., pH > 6). Nevertheless, it should be expected that the concentration of organic acids in saliva is less than that of carious lesions, since subjects had refrained from eating for 2 h prior sample collection, hence organic acids and dietary components should have cleared from the mouth. Moreover, we should emphasize that the microbiota present in wax-stimulated saliva is likely to derive from the intra-oral mucosal surfaces and from the supra-gingival plaque, providing an average composition of intra-oral surfaces, but mostly of the tongue surface (Simon-Soro et al., 2013). These ecological aspects have been taken into account and explain the differences in metabolic activities occurring within the *Veillonella* species in both sample groups.

Gene Expression Analysis

The combined expression level of the 3 *Veillonella* species was determined for each condition. The relative mean expression (RME) values for each identical gene product were added and the top 30 most highly expressed gene products in saliva were ranked. The corresponding values for the caries samples are also displayed together in **Figure 1**.

Overall, the 3 *Veillonella* species present in the caries and saliva samples display a similar profile of transcripts. *V. parvula* expressed more genes in the caries samples, whereas *V. dispar* expressed more genes in the saliva samples (**Table 1**, **Supplementary File 2**).

The most abundant transcripts were related to the production of cell surface proteins (RME = 13175), outer membrane synthesis (S-layer proteins, RME = 6592), translation elongation factors (G and Tu, RME = 11860 and 7731 respectively), transport systems (RME = 5481), ribosomal subunit proteins (protein biosynthesis, RME = 3028), and carbohydrate metabolism (particularly the glyoxylate and dicarboxylate metabolism, EC 4.2.1.32, EC 6.4.1.2, EC 1.1.1.37; RME = 2147, 2126, and 1461 respectively). These results are consistent with those described by Peterson et al. (2014) in plaque biofilm. Similarly Benítez-Páez et al. (2014) found evidence of overrepresentation of translation functions, together with high expressions of elongation factors Tu and G, emphasizing their importance and involvement in oral biofilm formation especially in early biofilms.

We also report high levels of transcripts encoding membrane transport proteins (cadmium-exporting ATPase, RME = 3861; autotransporter adhesin, RME = 5481; ABC tranporters,

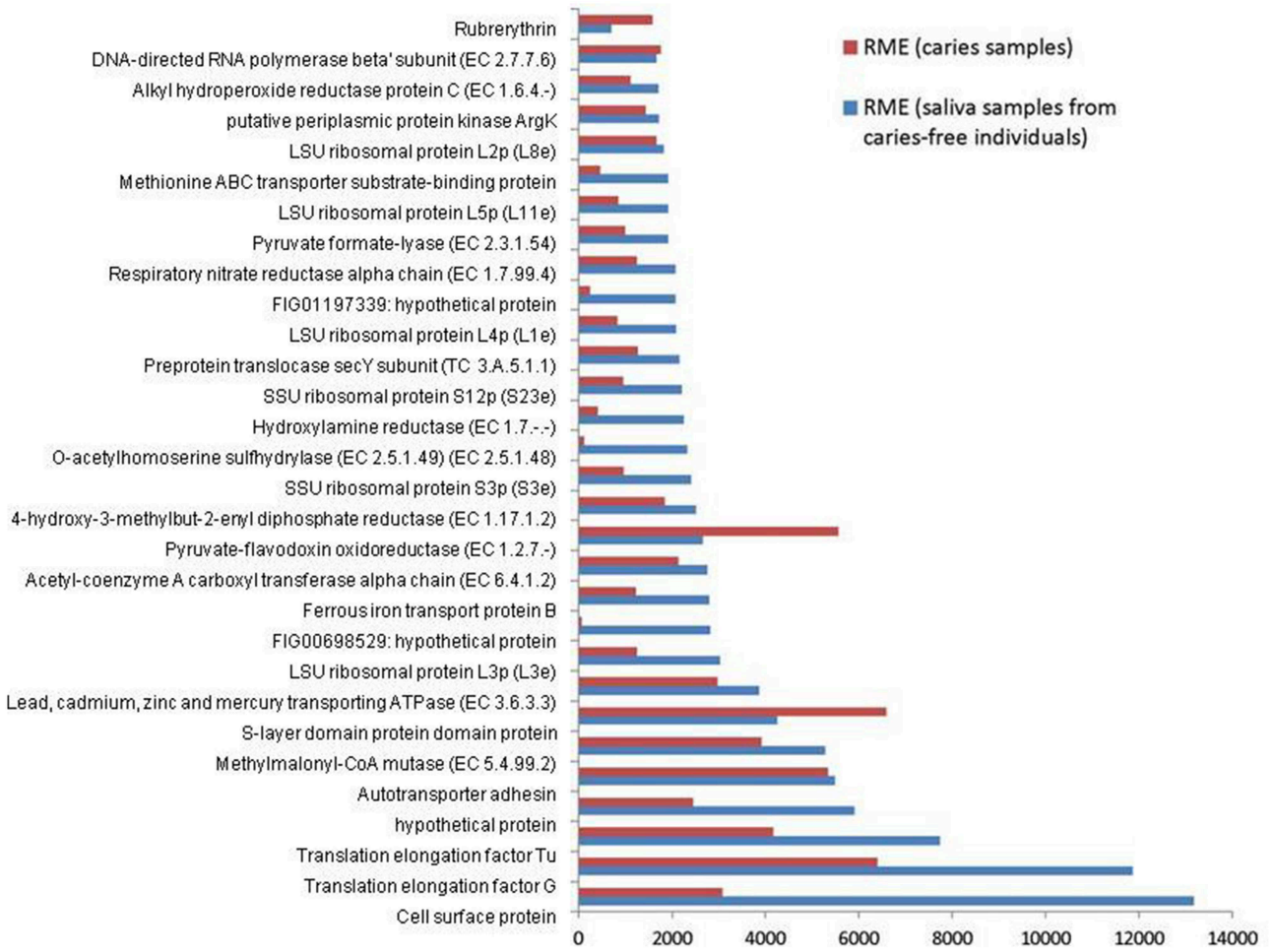

FIGURE 1 | Relative median expression (RME) levels in 3 *Veillonella* strains (*V. parvula* DSM2008, *V. dispar* ATCC 17748 and *V. atypica* ACS 0049 V Sch6). RME were calculated from the median values of normalized read counts in the caries ($n = 11$) and saliva ($n = 11$) samples. The 30 highest RME values were sorted in ascending order for the genes in saliva samples and are displayed with the RME values of corresponding genes in caries samples.

RME = 1998), as well as transcripts involved in oxidative stress protection (rubrerythrin, RME = 1577; and alkyl hydroperoxide reductase protein C, EC 1.6.4.-, RME = 1700), in both caries and saliva groups (**Supplementary File 2**). The overall similarity in transcription profiles in both sample groups suggest that the selected *Veillonella* species are actively expressing genes that are involved in cellular maintenance and survival within diverse environments.

Differential Expression Analysis

Differential gene expression between the caries and saliva groups was investigated using the R package DESeq2 (Love et al., 2014).

Sample to sample distances were calculated within the DESeq2 package. The principal components analysis and the heatmap of Euclidian distance between samples were based on the metatranscriptomic data mapped to 144 oral strains, and show caries and saliva samples to form distinct clusters. The PCA plot displays larger differences between saliva samples than between caries samples (**Figure 2**), suggesting that metabolic functions in the caries lesions are more conserved that in the caries-free samples. Likewise, the heatmap indicates the overall similarity between samples of the same group, with the exception of saliva sample number 9 (H9 in **Figure 3**), which seems to cluster with the caries samples, indicating that it shares similar functions found in caries.

Jorth et al. (2014) found more similar functional features in microbiota associated with disease compared to health-associated microbiota, even though great variations in the oral microbial composition were observed between and within patients. Other papers have described inter-patients variations in terms of bacterial profiles, and these seem to reduce in diversity when changing from healthy to a disease status (Munson et al., 2004; Preza et al., 2008). Our data suggest that in the caries lesions, metabolic functions in the 3 *Veillonella* species are more similar, than in caries-free saliva samples.

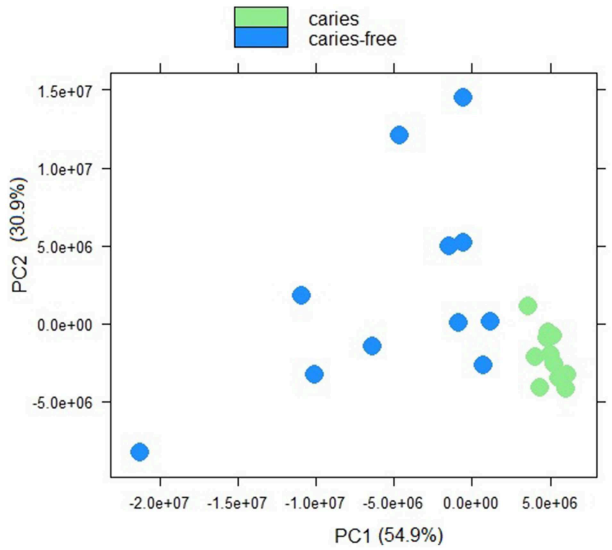

FIGURE 2 | Principal component analysis plot displaying sample-to-sample distances for caries and saliva samples. The PCA plot is based on the differential expression analysis of 144 oral bacterial strains, carried out using the R package DEseq2 (Love et al., 2014).

In order to identify the main functional differences between the caries lesions and saliva samples, output data from the DESeq2 analysis were sorted according to the log2 fold change values (**Supplementary File 3**). Since the transcriptomic data ($n = 22$) were analyzed with the caries-free vs. caries condition (used as the default DESeq2 condition setting), negative log2 fold change values, with corresponding Benjamini-Hochberg (BH) adjusted *p*-values (padj) $< 10^{-3}$ considered as significant (Benjamini and Hochberg, 1995), indicate genes with the strongest down-regulation in saliva (or strongest up-regulation in caries). Conversely, the largest log2 fold change values, with corresponding significant BH padj $< 10^{-3}$, indicate genes which are the most differentially expressed in saliva. Only the top 15 genes in both conditions are displayed in **Table 2**, and ranked according to the log2FoldChange values. A heatmap was also constructed within the DESeq2 package, and displays the top 30 differentially expressed genes across all 22 samples for the 3 *Veillonella* species (**Supplementary File 4**).

Genes that were differentially expressed in caries lesions (padj $< 10^{-3}$) were those expressed by *V. parvula*, and were mainly involved in pyruvate metabolism, transferases and membrane transport systems (including the biosynthesis of efflux pump components, ABC transporter and sulfur carrier proteins) (**Table 2**), inferring a role of these functions in disease. Similar findings were reported by Benítez-Páez et al. (2014) who found that ABC transporters were significantly up-regulated in mature biofilms, with cell motility function associated with bacterial chemotaxis, whereas Duran-Pinedo et al. (2014) reported significant levels of ABC transporters in periodontitis samples that seem associated with high levels of expression of virulence factors. Other specific pathways associated with disease have also been reported. In the case of periodontitis, a significant enrichment in butyrate production was detected (Jorth et al., 2014), iron acquisition and membrane synthesis have also been described as important metabolic activities defining disease (Duran-Pinedo et al., 2014).

However, in our data all 3 *Veillonella* species (especially *V. parvula*) expressed genes involved in glyoxylate and dicarboxylate metabolism, and alanine aspartate and glutamate metabolism, in particular genes encoding the alpha- and beta-subunits of L(+)-tartrate dehydratase (EC 4.2.1.32). These are involved in the production of ATP through catabolism of lactate and succinate. Overall, the data suggest that all species responded to growth in the carious lesions by increasing the expression of many genes associated with the utilization of lactate and succinate with the consequent generation of ATP via the sodium ion-translocating methylmalonyl-CoA decarboxylase (Buckel, 2001). We also found significant up-regulation of genes encoding aspartate aminotransferases (Vpar_1105, Vpar_0075, HMPREF9321_0571, HMPREF9321_1684) in both caries and saliva samples (**Supplementary File 3**); these enzymes catalyze the reaction L-aspartate + 2-oxoglutarate into oxaloacetate + L-glutamate and may be an alternative method of entering intermediates into the lactate metabolic pathway, for producing ATP.

Genes involved in histidine metabolism were also up-regulated in caries by *V. parvula*, but not in the other 2 species (**Supplementary File 3**). Of particular importance is the up-regulation of ATP phosphoribosyltransferase (EC 2.4.2.17) which has a central role in histidine biosynthesis. Similar up-regulation was observed in *Corynebacterium glutamicum* and *Salmonella typhimurium*, as well as in *Lactobacillus casei*, in response to acid adaptation (20 min at pH 4.5) (Foster, 1995; Brockmann-Gretza and Kalinowski, 2006; Broadbent et al., 2010). It was suggested that the up-regulation of the histidine operon resulted in increased intra-cellular levels of His which may contribute to intracellular buffering capacity as the pK_a value of the imidazole groups of histidine and histidine-containing peptides is near 6.0 and these have been shown to contribute to intracellular buffering in vertebrate cells (Abe, 2000).

Additionally, a potassium uptake system in *V. parvula* (Vpar_1334 and Vpar_1335; KtrA and KtrB) was also significantly up-regulated in caries, but not by the other 2 species. K^+ uptake in prokaryotes is essential for maintenance of cytoplasmic pH (Csonka and Epstein, 1996; Stumpe et al., 1996), this system may also assist in the survival of *V. parvula* in the acidic environment of the carious dentine. In *V. dispar* and *V. dispar,* these genes were significantly up-regulated in saliva, which may explain their lower ability to control their intracellular pH in the caries lesions. Clearly, *V. parvula* exhibits several distinct systems for intracellular pH control which do not appear to function as well in either *V. atypica* or *V. dispar,* and this may explain the ability of *V. parvula* to be better fitted to growth and proliferation in the acidic environment of carious lesions compared to the other two species.

Most of the differentially expressed genes in the saliva samples are those expressed by *V. atypica and V. dispar,* and encode for

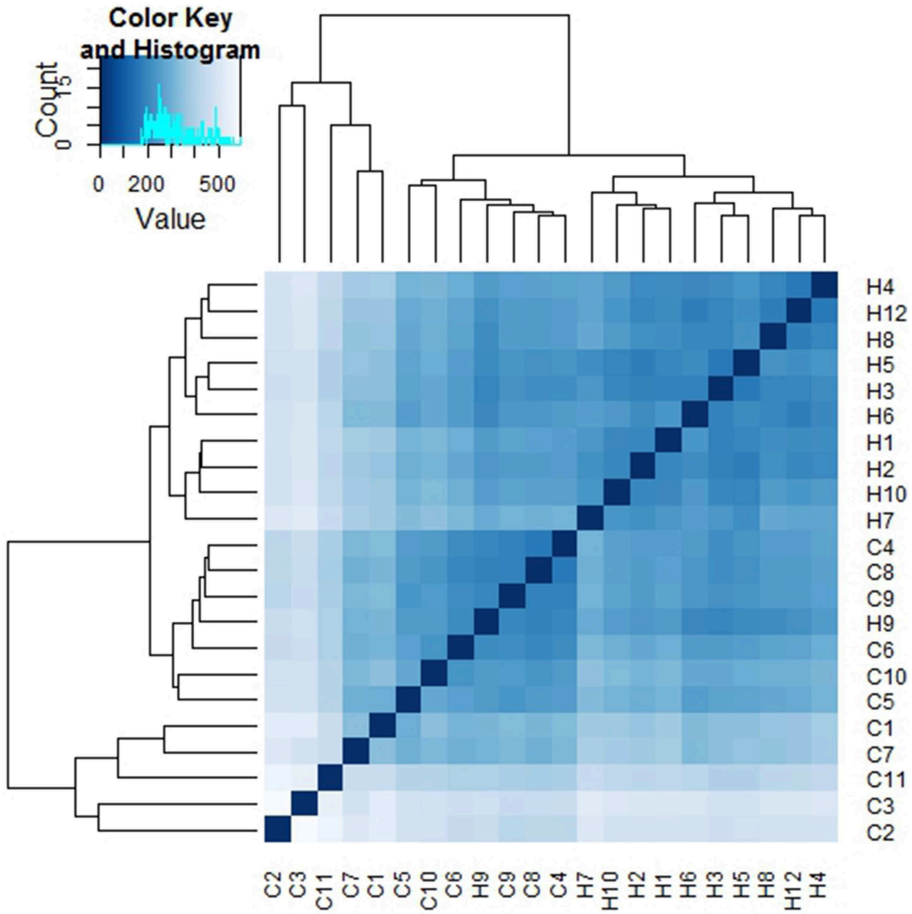

FIGURE 3 | Heatmap of Euclidian distances between samples ($n = 22$). The heatmap was constructed using the R package DESeq2 (Love et al., 2014), and is based on the differential expression analysis of 144 oral bacterial strains.

the oligopeptide, sulfonate transporter systems, and cysteine and methionine metabolism (EC 2.1.1.10). Others include genes involved in purine metabolism (EC 3.6.1.11), ferrichrome and other transport systems, molybdenum cofactor biosynthesis, as well as oxidoreductases which involve the use of NAD+ or NADP+ as acceptor in the chemical reaction leading to the formation of siroheme from uroporphyrinogen III (EC 1.3.1.76) (**Table 2**).

General stress response genes have also been identified in the carious lesions and saliva samples in all 3 *Veillonella* species (**Supplementary File 3**). Several genes encoding heat shock and chaperonin proteins were found up-regulated in caries (Vpar_1034, Vpar_1035, Vpar_0881), and others up-regulated in saliva (VEIDISOL_01212, HMPREF9321_0106, VEIDISOL_01142). Stress proteins such as chaperonin heat shock protein 33 (HMPREF9321_0536) and putative peroxide-responsive repressor PerR (HMPREF9321_0995) were up-regulated by *V. atypica* in the saliva samples (**Supplementary File 3**). In *V. parvula* and *V. dispar,* several genes associated with extracellular S-layer formation, were significantly up-regulated, which is a well characterized stress-associated response (Xiao et al., 2012).

Conclusion

Recent reports of metagenomic and metatranscriptomic analyses of oral samples are providing extensive information on the microbial populations and functions characterizing health and disease. These studies have also confirmed previous culturable observations regarding the intra-oral distribution of particular species but also found novel taxa which have not previously been identified amongst cultured bacteria and phyla for which only limited cultivated isolates are extant.

Here we have applied a metatranscriptomic approach to study 3 predominant oral *Veillonella* spp. in their natural habitat and have shown that their gene expression profiles are overall similar in both caries lesions and saliva (caries-free) samples. However, through differential expression analysis, *V. parvula* seems to exhibit a distinct method of intra-cellular pH control not evident in the other two species investigated, which might explain the preponderance of *V. parvula* in carious lesions and the reduced ability of *V. atypica* and *V. dispar* to proliferate in this acid environment. Other important functions related to membrane transport systems are reported to be over-expressed in the caries lesions inferring a role in disease.

TABLE 2 | Up-regulated genes in the caries samples (top of table) and in the saliva samples (bottom of table).

Feature ID	Gene product	base mean	log2Fold change	lfcSE	padj
Vpar_1291	L(+)-tartrate dehydratase beta subunit (EC 4.2.1.32)	357.26	–8.90	0.72	2E-31
Vpar_1292	L(+)-tartrate dehydratase alpha subunit (EC 4.2.1.32)	646.86	–7.83	0.74	2E-22
VEIDISOL_00680	L(+)-tartrate dehydratase beta subunit (EC 4.2.1.32)	84.70	–6.60	0.81	1E-13
VEIDISOL_00681	Possible membrane transport protein	5.72	–6.28	1.09	2E-07
Vpar_0720	hypothetical protein	2.99	–6.17	1.32	4E-05
Vpar_1308	Ornithine carbamoyltransferase (EC 2.1.3.3)	41.52	–5.84	1.03	3E-07
Vpar_0455	Sulfur carrier protein ThiS	6.55	–5.73	1.18	2E-05
Vpar_1307	N-acetyl-gamma-glutamyl-phosphate reductase (EC 1.2.1.38)	36.32	–5.51	1.02	1E-06
Vpar_1306	Acetylglutamate kinase (EC 2.7.2.8)	28.84	–5.28	1.11	3E-05
Vpar_0164	FIG01197475: hypothetical protein	20.37	–5.28	1.04	7E-06
Vpar_1004	Alpha-aspartyl dipeptidase Peptidase E (EC 3.4.13.21)	18.93	–5.26	1.12	3E-05
Vpar_0330	FIG01197189: hypothetical protein	159.84	–5.00	0.80	2E-08
VEIDISOL_00679	L(+)-tartrate dehydratase alpha subunit (EC 4.2.1.32)	63.25	–4.96	0.94	3E-06
Vpar_1022	Putative ATP:guanido phosphotransferase (EC 2.7.3.-)	114.18	–4.74	0.92	5E-06
Vpar_1367	RND efflux system, outer membrane lipoprotein, NodT family	40.60	–4.63	0.86	1E-06
HMPREF9321_0616	Siroheme synthase / Precorrin-2 oxidase (EC 1.3.1.76)	6.02	6.78	1.66	3.8E-04
VEIDISOL_01296	Cold shock protein CspC	5.35	6.80	1.63	2.8E-04
HMPREF9321_1331	Ferrichrome transport ATP-binding protein FhuC (TC 3.A.1.14.3)	14.49	6.86	1.21	3.4E-07
HMPREF9321_0811	FIG002958: hypothetical protein	5.70	6.90	1.62	2.0E-04
HMPREF9321_0294	Small-conductance mechanosensitive channel	5.82	6.94	1.62	1.7E-04
HMPREF9321_1565	Exopolyphosphatase (EC 3.6.1.11)	5.67	6.94	1.61	1.6E-04
HMPREF9321_0879	Homocysteine S-methyltransferase (EC 2.1.1.10)	18.40	7.04	1.21	1.5E-07
HMPREF9321_1453	Molybdenum cofactor biosynthesis protein MoaB	6.44	7.10	1.60	9.3E-05
VEIDISOL_01157	Sodium-dependent transporter	7.78	7.28	1.59	5.8E-05
HMPREF9321_0702	FIG01197118: hypothetical protein	8.58	7.33	1.60	5.4E-05
HMPREF9321_0668	NAD(P)HX epimerase/NAD(P)HX dehydratase	8.15	7.34	1.59	4.4E-05
HMPREF9321_0246	Mobile element protein	11.60	7.74	1.56	1.1E-05
HMPREF9321_1665	binding-protein-dependent transport systems inner membrane component	13.47	7.88	1.56	7.5E-06
VEIDISOL_00207	Alkanesulfonates/ Sulfonate ABC transporter, ATP-binding protein	14.99	7.96	1.57	6.2E-06
HMPREF9321_1134	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (TC 3.A.1.5.1)	17.60	8.24	1.53	1.6E-06

Genes expressed with the strongest down-regulation in the saliva samples (or up-regulation in the caries lesions) and genes with the strongest up-regulation in saliva samples were determined using the R package DESeq2 (Love et al., 2014). The list of genes is ranked according to the Log2FoldChange values from the negative lowest values (strongest down-regulation in saliva) to the positive highest values (strongest up-regulation). The baseMean corresponds to the average of the normalized count values (divided by size factors), the log2FoldChange corresponds to the effect size estimate indicating the change in gene expression between both sample groups; lfcSE corresponds to the standard error of the log2FoldChange estimate, and padj corresponds to the Benjamini & Hochberg adjusted p-values.

We have shown here that RNA-Seq is a powerful technique that can be used to observe the transcriptome of selected species or strain, provided their genome sequence data are available. The obvious drawbacks from such technique relate to the limited number of reference genomes available for reads mapping, and also to the fact that non-core genome sequences are not captured using the current methodology. Further analyses including larger samples and samples from similar biofilms such as plaque instead of saliva would be beneficial to add to our understanding of the oral microbial functions during initiation and development of disease.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2015.00025/abstract

Supplementary File 1 | Read count data for the 144 oral strains (including *V. parvula* DSM2008, *V. dispar* ATCC 17748 and *V. atypica* ACS 0049 V Sch6), used as input file for analyses using DESeq and DESeq2 in R.

Supplementary File 2 | Relative median expression (RME) values based on the median and 75th percentile (Q3) values of normalized read counts in the caries ($n = 11$) and saliva samples ($n = 11$), for the 3 *Veillonella* species.

Supplementary File 3 | Data output from the differential expression analysis for the 3 *Veillonella* species, obtained from DESeq2 analysis.

Supplementary File 4 | Heatmap constructed using the R package DESeq2, displaying the highest most variable genes across all samples ($n = 22$), the analysis was based on the 3 *Veillonella* strains only.

Supplementary File 5 | List of reference strains used in the CLC Genomics Workbench (CLC Bio, Qiagen) for RNA-Seq analysis.

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The impact of horizontal gene transfer on the adaptive ability of the human oral microbiome

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The oral microbiome is composed of a multitude of different species of bacteria, each capable of occupying one or more of the many different niches found within the human oral cavity. This community exhibits many types of complex interactions which enable it to colonize and rapidly respond to changes in the environment in which they live. One of these interactions is the transfer, or acquisition, of DNA within this environment, either from co-resident bacterial species or from exogenous sources. Horizontal gene transfer in the oral cavity gives some of the resident bacteria the opportunity to sample a truly enormous metagenome affording them considerable adaptive potential which may be key to survival in such a varying environment. In this review the underlying mechanisms of HGT are discussed in relation to the oral microbiome with numerous examples described where the direct acquisition of exogenous DNA has contributed to the fitness of the bacterial host within the human oral cavity.

Keywords: horizontal gene transfer, mobile genetic elements, conjugation, transformation, hydrogen peroxide, extracellular DNA, oral cavity, biofilm

INTRODUCTION

The human oral microbiome is an incredible example of a species rich collection of micro-organisms living together primarily as a multispecies biofilm. The constant challenges the biofilm inhabitants have to cope with include interactions of co-operation and antagonism whilst the individual cells have to adjust to an ever changing onslaught of environmental perturbations. Availability of carbohydrate sources, temperature changes and the interaction with transient, non-oral species of bacteria are just a few examples of the challenges the individual members of the multispecies oral biofilm have to adjust to.

There are many recent reviews concerning the actual numbers, and species composition, of bacteria within the human oral cavity and the reader is directed to these (e.g., Curtis et al., 2011; Wade, 2013), and accompanying articles in this special issue for the background knowledge on the composition of this community.

The dynamic environment of the oral cavity pressures cells of the oral biofilm to not only adjust at the metabolic level but also evolve their genomic content and potential. The species richness and diversity of the oral cavity creates complex bacterial interactions including the exchange of genetic material via horizontal gene transfer (HGT). A recent study on the genome evolution of the genus *Streptococcus*, which is the most abundant genus in the oral cavity (Rosan and Lamont, 2000; Diaz et al., 2006), demonstrated HGT as important mechanism for the acquisition of new genetic traits and significantly contributed to the genomic expansion and streamlining of *Streptococcus* (Richards et al., 2014). Indeed Smillie *et al* have recently shown that the driver for HGT between bacteria is primarily due to the ecology with most gene transfers (determined where genes are >99% identical at the nucleotide level in different bacteria) occurring in bacteria from ecologically similar environments (Smillie et al., 2011) and is less dependent on geography or phylogeny of the microbial community. This makes strategic sense for the organisms which inhabit these different ecological niches as the available, accessory metagenome will be enriched for genes which allow adaption to local stresses and maximization of opportunities within a particular environment. Each environment will have an individual, and often unique, set of parameters which must be tolerated and exploited by the microbial inhabitants in order to successfully colonize that environment over time.

THE VARIABLE ORAL ENVIRONMENT

The oral cavity is by no means a static environment; rather it is an environment where diverse ecological pressures exist. As a portal to the distal part of the digestive tract the oral cavity is open to the environment and also has a variety of foods (substrates) pass through it. There is therefore a great deal of variability encountered in terms of physical, chemical and physicochemical characteristics.

Bacteria will have to cope with multiple defense mechanisms within the oral cavity including, but not limited to the production of host antimicrobial compounds such as lactoperoxidase and lysozyme, bacterially derived antimicrobials and bacteriocins, production of immunoglobulins A, G, and M, mucus layers on mucosal surfaces and the constant shedding of epithelial cells. There are also relatively strong mechanical forces which result during chewing, talking and the movement of the tongue. Forces up to 150 Newtons (N) are generated whilst chewing foods such as meat whilst the maximal biting forces have been estimated to be between 500 and 700 N (Wilson, 2005). In addition to the

mechanical forces there are also hydrodynamic shear forces that occur due to the flow of saliva and, to a lesser extent gingival crevicular fluid.

Chemically the mouth is a very diverse environment which can be subject to extremely rapid change when food and liquids are consumed. Whilst the main source of nutrients for oral bacteria is the saliva there is a diverse range of carbohydrates and other sources of energy which can be temporarily elevated following feeding and the ability to utilize these substrates rapidly provides an advantage for the microbes. Additionally with so many different species inhabiting the oral cavity, inevitable syntrophic relationships have evolved such as species of *Veillonella* utilizing the lactate produced by cariogenic streptococci (Chalmers et al., 2008).

Differences in mechanical force, nutritional variation and availability, temperature, pH levels, oxidative stress and redox potential, presence of both host and bacterially derived antibacterial enzymes all provide challenges to the microbial inhabitants which inevitably select for evolved advantageous traits. When such traits are encoded by mobile DNA this environment will also therefore select for the transfer of such genes to other inhabitants.

HORIZONTAL GENE TRANSFER IN THE ORAL CAVITY

Conjugation, transduction and transformation are the three main mechanisms of HGT. Conjugation is the direct transfer between live donor and recipient cells in a DNase insensitive manner and is the mechanisms of transfer used by conjugative plasmids and conjugative transposons. Transduction is the transfer of host genomic DNA by bacteriophage which package the host DNA into the bacteriophage head structures and transformation is the uptake of exogenous, extracellular DNA often released from dead bacteria cells in the environment. The mechanics of these three mechanisms have been reviewed in detail previously for specific pathogens (e.g., Lindsay, 2014). Recently however another process involved in HGT has been reported which deserves mention here as it could be directly relevant to the biofilm way of life.

Membrane vesicles are released from the cell surface by many Gram-negative, and some Gram-positive, bacteria and can contain proteins, polysaccharides and importantly for microbial adaptation, DNA (Yaron et al., 2000). This DNA can be utilized by other competent bacteria as a substrate for transformation. Virulence genes, plasmid located antibiotic resistance genes and *gfp* (encoding green fluorescent protein) have been shown to be exported from *Escherichia coli* in vesicles and furthermore have been shown to successfully transform *Salmonella* (Yaron et al., 2000; Mashburn-Warren and Whiteley, 2006). It has been revealed that a small proportion of the membrane vesicles from the psychrotropic bacterium *Shewanella* have a double membrane and therefore contain cytoplasmic contents, providing a much needed explanation of how DNA can be incorporated into the vesicles without being either transported to the periplasm of the cell or to the external environment and subsequently integrated into a vesicle composed only of the outer membrane (Pérez-Cruz et al., 2013). These DNA containing membrane vesicles have also recently been shown for *Acinetobacter baylyi* (Fulsundar et al., 2014). More recently, the oral biofilm relevant *Streptococcus mutans* has been shown to release extracellular DNA (eDNA) via membrane vesicles into the developing biofilm and provides therefore an important source for genetic material via this novel mechanism (Liao et al., 2014).

HGT has been demonstrated to occur between a wide range of bacteria which inhabit the human oral cavity. Using *in vitro* models both conjugation and transformation have been shown to occur between different species of bacteria (Roberts et al., 1999, 2001; Ready et al., 2006; Hannan et al., 2010) and acquisition of doxycycline resistance encoding transposons has also been demonstrated within a patient receiving doxycycline therapy for the treatment of periodontitis (Warburton et al., 2007). Importantly, gene transfer from transient bacteria unable to colonize oral biofilms themselves has been previously demonstrated to occur from a *Bacillus subtilis* donor of the conjugative transposon Tn*5397* to an oral *Streptococcus* sp. in a mixed species oral biofilm growing in a constant depth film fermentor (Roberts et al., 1999). Additionally bacterial DNA from transient species has been detected in metagenomic libraries made from DNA isolated from the pooled saliva of 20 healthy individuals (Seville et al., 2009); in this case the likely source of the cloned DNA was from a transient *Phytoplasma* sp., which usually resides within plant tissues. This work demonstrates that the plasticity of the oral metagenome may be influenced by the members of transient bacteria which interact with the oral community and will depend themselves on the diet and habits of the individual human host.

Whilst there have been no reported observations of transduction in the oral cavity, or relevant *in vitro* models, there is now good evidence that bacteriophages are abundant in the oral cavity. Studies on saliva have isolated bacteriophages able to lysogenize specific bacterial pathogens such as *Enterococcus faecalis* (Bachrach et al., 2003; Stevens et al., 2009) and *Aggregatibacter actinomycetemcomitans* (Sandmeier et al., 1995; Willi et al., 1997). More recently direct observation and metagenomic analysis of viral plaque and saliva fractions have revealed that bacteriophages are extremely common (Al-Jarbou, 2012; Pride et al., 2012). It has been determined that viral particles are present at approximately 10^8 particles per milliliter of saliva and that the vast majority of these particles are bacteriophages that may be a reservoir of genes involved in pathogenicity (Pride et al., 2012).

Recent reviews have summarized examples of HGT within and between species which inhabit the human oral cavity (Roberts and Mullany, 2006, 2010; Olsen et al., 2013) however some individual examples pertaining to life in the oral cavity will be discussed below.

ADHERENCE AND BIOFILM FORMATION

Many, if not most bacteria which inhabit the oral cavity grow as a biofilm on the non-shedding surfaces of the teeth. Therefore the ability to adhere to the oral surfaces and to grow within a biofilm will give these bacteria a significant advantage in this environment due to the advantages of the biofilm mode of growth, many of which are discussed above. One recent example demonstrating the extent to which HGT can allow an organism to survive, and cause disease in the oral cavity is the recently published genome and transcriptome of *Streptococcus parasanguinis* FW213 (Geng et al., 2012). The genome of this bacterium contains at least five acquired genomic islands (GIs). The first two GIs, Fwisland_1 and

Fwisland_2 contain genes which are likely to be involved in the production of the lantibiotic salivaricin B and a lactococcin 972 type bacteriocin respectively. The production of bacteriocins and other bacterial inhibitors, particularly by the oral streptococci, which usually are active against similar bacteria to the producing strain, gives them a clear advantage during growth in a relatively nutrient limited environment such as the oral cavity (see below). The third GI, Fwisland_3 encodes long fimbriae that are involved in enhanced biofilm formation. Fwisland_4 encodes genes whose predicted products are involved with the biogenesis and export of extracellular polysaccharides whose roles in biofilm formation, adherence and resistance to host immune systems, such as phagocytosis are well known. Finally Fwisland_5 encodes genes which are believed to be involved in the modulation of biofilm formation. All of the predicted function of the genes products from the 5 GIs appear to be involved in adaptation to the oral cavity (Geng et al., 2012). A similar contribution of HGT to the genomes of other oral species have also been demonstrated, e.g., *Porphyromonas gingivalis* (Tribble et al., 2007, 2012; Kerr et al., 2014) and the mitis group streptococci (Zähner et al., 2011). The ability of bacteria to grow as a biofilm within the oral cavity can also protect them from exogenous antibacterial compounds such as disinfectants and antibiotics. Another way HGT can contribute to the survival under antibiotic pressure is to allow the bacteria to acquire specific genes encoding antibiotic resistance proteins which will be discussed in the next section.

ACQUIRED ANTIBIOTIC RESISTANCE AND MOBILE GENETIC ELEMENTS

One of the most prominent phenotypic advantages a bacterial cell can exhibit is resistance to antimicrobial compounds which are present in great abundance in the oral environment. Acquired antibiotic resistance genes have been found in many species of bacteria which inhabit the oral cavity (Ciric et al., 2012) and additionally recent studies have focussed on metagenomes from the oral cavity (saliva) and have shown a myriad of different resistance genes being present (Seville et al., 2009). Often these resistance genes have been found to be localized on putative and sometimes proven mobile genetic elements (MGEs) (e.g. Ciric et al., 2011, 2014). The conjugative transposon Tn*916* is a prime example of a MGE which is found to be responsible for the transfer of a multitude of different resistances in the oral microflora.

Tn*916* normally confers tetracycline and minocycline resistance by encoding for the Tet(M) protein, a ribosomal protection protein which reversibly binds to the 23S rRNA subunit of the ribosome and prevents tetracycline binding therefore preventing protein synthesis, or removing a bound tetracycline molecule before binding itself (Connell et al., 2003). Tn*916* is the paradigm of a large family of MGEs, many of which encode additional resistance genes e.g. elements such as Tn*2009* contain *erm*(B) conferring macrolide, lincosamide and streptogramin resistance, Tn*6009* encodes resistance to both inorganic and organic mercury by the action of MerA and MerB respectively and Tn*1545* and Tn*6003* both encode resistance to kanamycin via the product of *aphA*-3 (reviewed in Roberts and Mullany, 2011). Interestingly the first antiseptic resistance gene has recently been found associated with a Tn*916* like element designated Tn*6078*. This antiseptic resistance gene; *qrg*, is itself flanked by two copies of a commonly found insertion sequence IS*1216*. This composite transposon has inserted into a gene encoding a protein Orf15 which is believed to be essential in the conjugation of the host Tn*916*-like element. Experimentation failed to demonstrate transfer by conjugation of this element, presumably due to the insertion in *orf15*, however it was demonstrated that it could successfully transform a competent oral streptococci (Ciric et al., 2011) to CTAB resistance. This study is one of a number to highlight the redundancy in the mechanisms of HGT of resistance genes in this environment and it shows that both conjugation and transformation are important in terms of antimicrobial resistance transfer (Hannan et al., 2010). The regulation of competence and uptake of naked DNA in the oral environment is pervasive and is perhaps the driving force for adaptation in some species of oral bacteria such as the streptococci. Most of the previously mentioned MGEs with their different resistances have recently been found in a survey of oral streptococci from 20 healthy adult UK volunteers who provided saliva (Ciric et al., 2012) demonstrating how common these elements, and resistances, are in the general population. Similarly two recent surveys of plasmids from endodontic derived enterococci identified a large number of different replicons containing a larger number of different resistance genes (Song et al., 2013; Wardal et al., 2013).

METABOLIC ADAPTABILITY

An interesting example of the influence of HGT on the metabolic capability is provided by the lactobacilli. Mammalian associated lactobacilli are common in the human gastrointestinal tract and in the oral cavity. A recent report has described the catabolic versatility of the different species found in this environment and shown that the ability to use dietary carbohydrates is commonly associated with the acquisition of a particular plasmid encoding the relevant metabolic pathways (O'Donnell et al., 2013). One interesting and well characterized strain is *Lactobacillus salivarius* UCC118 which was originally isolated from the terminal ileum of a patient undergoing reconstructive surgery on their urinary tract (Claesson et al., 2006). This strain contains a 242 kb megaplasmid designated pMP118 and two cryptic plasmids (Li et al., 2007; O'Donnell et al., 2013). The pMP118 megaplasmid contains, among others, genes predicted to encode proteins involved in pentose and polyol utilization and genes involved in glycolysis making this particular plasmid extremely beneficial to the carrying strains in carbohydrate rich environments such as the oral cavity and GI tract.

HORIZONTAL GENE TRANSFER AND EXTRACELLULAR DNA

The molecular mechanisms of HGT have been investigated in great detail (Frost et al., 2005; Thomas and Nielsen, 2005). While the source of DNA for HGT through mechanisms like conjugation and transduction is obvious, the generation of DNA in the oral biofilm for transformation of competent bacteria is not well understood. In general, DNA for transformation has to be extracellular DNA accessible for competent bacteria. A regulatory relationship between competence development and the generation of eDNA has been shown for pneumococci *in vitro*

(Steinmoen et al., 2002; Moscoso and Claverys, 2004), where the release of chromosomal DNA is part of a lytic process controlled by the competence system, termed fratricide (Claverys et al., 2007). The regulatory coordination of DNA release and competence development ensures that the population wide decision to direct energy toward competence development is rewarded by providing the substrate for uptake at the same time. Taking in consideration that an initial clonal population would diverge due to mutation, the extracellular DNA generated during competence development would have enough diversity (mutations) that could favor establishment of new phenotypic traits under the right selective pressure (Luria and Delbrück, 1943). The homologous extracellular DNA released by a competent population of pneumococcus would facilitate easy integration via homologous recombination. The homologous extracellular DNA, however, might pose an evolutionary disadvantage by not providing complex diversity, e.g., genes for new or alternative metabolic pathways or antimicrobial resistance genes as discussed above. Therefore, diversity is most likely achieved by incorporating heterologous DNA from species that are ecologically similar and not of clonal origin (Smillie et al., 2011). This diversity is actually provided in human associated bacterial communities, such as the oral biofilm.

EXTRACELLULAR DNA IN THE ORAL CAVITY

A prominent genus of the oral biofilm is *Streptococcus* and competence development is wide spread among this genus (Rosan and Lamont, 2000; Martin et al., 2006; Havarstein, 2010). The biofilm environment of oral streptococci creates a special situation due to the high cell density and species richness (Kreth et al., 2009). Several seminal findings show that oral streptococci are able to co-aggregate with other bacterial species to built up the mature oral biofilm community, which is one of the most diverse human associated communities identified so far (Valm et al., 2011 and references in Kolenbrander et al., 2006). This diversity does not only create cooperation, but also fierce competition (Kreth et al., 2011). Although, the release of eDNA is most-likely mechanistically explained by bacterial lysis, the molecular details and motivation might be diverse.

EXPERIMENTALLY CONFIRMED MECHANISMS FOR eDNA RELEASE

BACTERIOCIN DEPENDENT eDNA RELEASE

That bacterial competition causes the release of DNA has been shown (Kreth et al., 2005a; Johnsborg et al., 2008). Initial investigations between the clinically relevant antagonism of *S. mutans* and oral commensal *S. gordonii* have revealed an interesting mechanism of eDNA release. The molecular basis for this antagonism is dependent on bacteriocin production (Kreth et al., 2005a). Bacteriocins are antimicrobial peptides inhibitory toward competing bacterial species. *S. mutans* produces a wide array of bacteriocins and several are regulated by the genetic competence system (Merritt and Qi, 2012). The regulation is due to a specific binding site for the ComE transcriptional regulator found in the promoter sequence of several bacteriocins (van der Ploeg, 2005; Kreth et al., 2006). Once ComE becomes phosphorylated during the activation of the competence cascade, it can bind to the respective bacteriocin gene promoters and activate transcription, while also activating the genes responsible for DNA uptake and homologous recombination. *S. mutans* therefore coordinates its bacteriocin production with competence development (Kreth et al., 2005a), reminiscent of what has been shown for *S. pneumoniae*. The significant difference is that the production of these competence-regulated bacteriocins causes the release of DNA from surrounding competitors, not from itself. Dual species, *in vitro* culture experiments with *S. mutans* and *S. gordonii* have confirmed that induction of the competence system with *S. mutans* specific competence stimulating peptide can result in the release and transfer of transforming DNA from *S. gordonii* to *S. mutans* (Kreth et al., 2005a). Interestingly, the oral bacterial species most susceptible to the bacteriocins under the control of the competence system (namely mutacin IV, V, VI, and Smb) are closely related to *S. mutans* (Merritt and Qi, 2012). This seems to be an ideal mechanism to ensure the availability of DNA with some evolutionary distance but close enough for a high chance of chromosomal integration via homologous recombination. In a recent review about mutacins of *S. mutans*, Merritt and Qi explain the potential ecological role of the coordinated competence and mutacin regulation. *S. mutans* is not an early colonizer and therefore has to face stiff competition from species like *S. sanguinis* and *S. gordonii*, which are abundant species during early biofilm development. By producing mutacins controlled by the competence system, *S. mutans* can eliminate the competition potentially freeing occupied space for its own colonization. The released DNA from the closely related species can easily be taken up and has a high potential to provide new genotypic traits. Conversely, other non-related early colonizers like *Actinomyces* are not targeted by competence-regulated mutacins (Merritt and Qi, 2012). It is tempting to speculate that this is due to the low chance for integration of DNA from distant species into the streptococcal chromosome. In addition, uptake of extracellular DNA released by the action of competence-regulated mutacins might be mainly for homologous recombination and not as a food source or for the generation of DNA building blocks. Competence development in streptococci might in general induce bacteriocins or lytic enzymes to cause lysis of evolutionary related species, as discussed in the next section, to serve in the acquisition of new genetic traits or for DNA repair.

MUREIN HYDROLASE DEPENDENT eDNA RELEASE

The importance of competence and HGT in its ecological context becomes evident when we revisit the aforementioned pneumococcal fratricide. Initially investigated only in pure single species cultures, fratricide seemed to be an event directed toward its own kind (Claverys et al., 2007). However, in a recent study Johnsborg *et al* demonstrated that fratricide has a broader ecological impact (Johnsborg et al., 2008). *S. pneumoniae* resides in a niche, the oral cavity and nasopharynx that is also inhabited by closely related streptococci like *S. mitis* and *S. oralis* (Dewhirst et al., 2010). Therefore it is not surprising that pneumococcal competence regulated fratricide and the release of DNA affects nearby species. The center of the pneumococcal fratricide is the murein hydrolase CbpD (Wei and Havarstein, 2012). In a concerted action of the autolytic enzymes LytA, LytC and CbpD target cells are

lysed to release DNA (Eldholm et al., 2009). While *lytA* and *lytC* are constitutively expressed with an increase of *lytA* expression during competence development, *cbpD* is only expressed in competent cells (Johnsborg et al., 2008). Co-cultivation of *S. pneumoniae* with the closely related *S. mitis* or *S. oralis* demonstrated that CbpD is required for cross species lysis and deletion of CbpD abolished the ability. Further investigation demonstrated that competence induced cell lysis significantly increases HGT between species possessing the CbpD lysis mechanism (Johnsborg et al., 2008; Eldholm et al., 2010). What is the ecological implication? Competence is regulated by a small peptide, termed CSP (competence stimulating peptide), which is secreted and accumulates in the extracellular environment to trigger competence development in a quorum sensing dependent way (Johnsborg and Havarstein, 2009). The CSP sequence is strain and species specific (Johnsborg et al., 2007). In the nasopharynx and oral cavity a diverse number of different CSP pherogroups exist. Once one pherogroup starts to develop competence it also produces the ComM protein rendering the cells immune to the muralytic attack of CbpD. Other pherotypes not developing competence at the same pace, for example due to a lower initial density can now be attacked and the extracellular DNA released during the attack can be taken up by the competent community (Johnsborg et al., 2008; Eldholm et al., 2010). Interestingly, in a follow up study it was shown that the muralytic activity of CbpD at the target cells is dependent on its functional binding to choline-decorated teichoic acids. This limits cross-species attack to streptococcal species with choline-decorated teichoic acids such as *S. mitis* and *S. oralis* (Eldholm et al., 2010). Incidentally, *S. mitis* and *S. pneumoniae* both belong to the mitis group of streptococci and homologous recombination has been discussed as a major driving force for their evolution reflected by a mosaic structure in several gene sequences (Kilian et al., 2008).

SELF-ACTING INTRACELLULAR BACTERIOCIN DEPENDENT eDNA RELEASE

While the action of CbpD can be directed toward itself and other species dependent on the competence state of the respective species, *S. mutans* has a dedicated bacteriocin for intracellular action against itself (Perry et al., 2009). The production of this bacteriocin, termed CipB or mutacin V, is also under the control of the competence system and is stress induced. CipB activity against itself is due to intracellular accumulation in the producer, therefore strictly autolytic. In general, stress situations like low antibiotic concentrations, high cell density and oxygen can induce the competence pathway (Claverys et al., 2006). It is believed that competence and lysis would allow the exchange of fitness-enhancing DNA under those stress conditions (Perry et al., 2009). Since it was shown that the CipB induced lysis only occurs in a sub fraction of cells a valid question is how beneficial the extracellular DNA can be? It is well established that antibiotics can cause DNA damage (Cheng et al., 2013; Dwyer et al., 2014). However, this requires active metabolism and DNA replication. If DNA is released into the environment, the mutagenic potential of certain antibiotics is not working. Hence, cells with damaged DNA could take up the released DNA from its own kind for repair. Obviously this works also in the other direction; if DNA mutation happened due to the presence of antibiotic stress, cells could benefit from the mutated and released DNA, if selection pressure establishes the incorporated DNA as fitness enhancing.

The complexity of bacteriocin production and regulation is best understood in *S. mutans* (Merritt and Qi, 2012). However, the cross-species attack in addition to the internal action of CipB raises the question about the coordination of bacteriocin production. Several mutations in other global regulatory proteins abolish bacteriocin production indicating that the network controlling bacteriocin production is intricate (Chong et al., 2008; Okinaga et al., 2010; Xie et al., 2010). The fluctuating environment of *S. mutans* makes it certain that there are yet to be identified regulatory mechanisms fine tuning bacteriocins production, competence and transformation dependent on environmental signals. In addition, it is not entirely clear when the bacteriocins are produced in the ecological context *in vivo*. Surprisingly, there is a great diversity of bacteriocins produced by different *S. mutans* strains, but all investigated strains produce bacteriocins indicating the importance of this mechanism to ensure the availability of extracellular DNA (Merritt and Qi, 2012).

H_2O_2 DEPENDENT eDNA RELEASE

A very different mechanism of extracellular DNA generation is used by commensal streptococci *S. gordonii* and *S. sanguinis* (Kreth et al., 2008). Initial investigations on the dual species antagonism with *S. mutans* concentrated on bacteriocin production (Kreth et al., 2005a,b). But the commensals are not the mere victims of this antagonism. *S. sanguinis* and *S. gordonii* can inhibit *S. mutans* in a very efficient way using hydrogen peroxide (H_2O_2), and compared to the commensals itself, *S. mutans* is highly H_2O_2 susceptible (Kreth et al., 2008). Interestingly, the production of H_2O_2 is intimately correlated to the release of DNA. The enzyme responsible for the production of H_2O_2 in *S. sanguinis* and *S. gordonii* was identified as pyruvate oxidase or SpxB. SpxB is an oxido-reductase that catalyzes the conversion of pyruvate, inorganic phosphate (Pi) and molecular oxygen (O_2) to H_2O_2, carbon dioxide (CO_2) and the high-energy phosphoryl group donor acetyl phosphate in an aerobic environment. Knock-out studies with putative pyruvate oxidase orthologs in *S. sanguinis* and *S. gordonii* confirmed SpxB as main H_2O_2 producer (Kreth et al., 2008). However, the production of growth inhibiting amounts of H_2O_2 is not exclusive to the pyruvate oxidase. Mutational studies with *Streptococcus oligofermentans* showed that at least two other enzymes in addition to the pyruvate oxidase are capable of producing growth-inhibiting amounts of H_2O_2. The lactate oxidase LctO catalyzes the formation of pyruvate and H_2O_2 from L-lactate and oxygen and an L-amino acid oxidase generates H_2O_2 from amino acids and peptones. Antagonism assays of *S. oligofermentans* and *S. mutans* grown in dual species biofilms demonstrated that LctO dependent H_2O_2 production is sufficient to antagonize *S. mutans* in an SpxB mutant. The role of the L-amino acid oxidase in interspecies competition is not clear since its inhibiting activity is only visible in a *lctO/spxB* double knock-out mutant (Tong et al., 2007, 2008; Liu et al., 2012). In general, SpxB is a very conserved enzyme encoded by several oral streptococci, including the aforementioned streptococci as well as *S. oralis*, *S. mitis* as abundant members of the oral biofilm

(Zhu and Kreth, 2012; Zhu et al., 2014). Expression of *spxB* in the oral biofilm has recently been confirmed pointing to its active biological function during biofilm development (Zhu et al., 2014). The release of chromosomal DNA into the environment by *S. gordonii* and *S. sanguinis* is closely associated with the production of H_2O_2 and an SpxB deletion affected the release significantly (Kreth et al., 2008). In agreement with a diminished production of H_2O_2 under anaerobic conditions, a significant reduced concentration of extracellular DNA was detected under oxygen limited growth conditions (Itzek et al., 2011). Interestingly, addition of H_2O_2 to anaerobically grown cells does induce DNA release. The H_2O_2 induced release process is not entirely understood. An obvious time delay was observed between the addition of H_2O_2 and the first detectable amounts of extracellular DNA, suggesting that H_2O_2 does not induce immediate lysis of the bacterial cells. No release was observed when cells were treated with chloramphenicol, a known protein biosynthesis inhibitor and traditionally used to show that a process requires newly synthesized proteins. A possible signal for the cell to release DNA could be H_2O_2 induced DNA damage. Treatment with DNA damaging agents like UV-light and mitomycin C also triggered the release of DNA under anaerobic conditions (Itzek et al., 2011). Further mechanistic studies showed that the release process seems to be a lytic process (Xu and Kreth, 2013), but different from the complete cell lysis observed for the DNA release in pneumococci and enterococci since extracellular DNA release can be induced by H_2O_2 without any obvious bacterial cell lysis (Itzek et al., 2011). A connection of H_2O_2 dependent lysis and extracellular DNA release with competence development is not surprising considering the already described examples for other streptococci. *S. gordonii* expresses the murein hydrolase, LytF. In fact, *lytF* is only expressed during competence because its expression is under the control of the competence stimulating peptide CSP. This observation is reminiscent of the expression of CbpD in *S. pneumoniae* and LytF has been proposed as functional analog of CbpD. DNA transfer experiments relying on LytF dependent cell lysis and subsequent DNA uptake by *S. gordonii* showed that most cells are protected from the muralytic activity of LytF (Berg et al., 2012). This is in agreement with the observed lysis resistant population in S. gordonii after H_2O_2 addition (Itzek et al., 2011). A close association, however, of H_2O_2 induced release of DNA and competence development is evident since cells grown under H_2O_2 producing conditions are also induced for competence development (Itzek et al., 2011). Interestingly, competence development in *S. pneumoniae* can be initiated by mitomycin C induced DNA damage, which also leads to the release of DNA similar to what was reported for *S. gordonii* (Prudhomme et al., 2006).

Combining the experimental findings in *S. pneumoniae* and *S. gordonii* a clearer picture emerges about the association and ecological advantage of H_2O_2 induced DNA release with the adaptation of oral streptococci to stress. *S. gordonii* and probably other H_2O_2 producing oral streptococci release DNA into the environment as a consequence of DNA damage. This pool of released DNA likely contains mutations in various genes because of the DNA damage. If such mutated DNA is taken up and integrated into the chromosome, the transformation event would lead to a bacterium able to grow and out compete bacteria without the respective mutation under selective conditions or as a template for the repair of stress-induced DNA damage.

LIMITATIONS OF HORIZONTAL GENE TRANSFER

The successful transfer of a new genetic trait is dependent on several events. In the case of eDNA, the genetic material needs to persist in the environment long enough to be taken up by a competent bacterium. The eDNA integrity and persistence is compromised by host and bacterial derived extracellular nucleases (Kishi et al., 2001; Palmer et al., 2012). Once taken up by competent bacteria, eDNA is subject to cellular defense mechanism which have evolved to prevent the invasion and incorporation of potentially harmful DNA. Restriction modification systems are a well-characterized and common bacterial defense system that methylates genomic DNA at specific target sequences. These sequences are also recognized and cleaved by cognate restriction enzymes when they are not methylated, which will likely be the case on newly acquired, foreign DNA that is taken up by the bacterium, thus preventing integration into the chromosome (Bickle and Kruger, 1993). Another important defense mechanism is the CRISPR-Cas system (clustered regularly interspaced short palindromic repeats), which is considered a bacterial immune system. It is an adaptive and inheritable system which recognizes and destroys foreign DNA therefore preventing infection by bacteriophages, transposons and plasmids. It is an RNA based protection mechanism, which stores parts of the DNA of previously encountered bacteriophage, transposons and plasmids in the CRISPR chromosomal locus. The cell is therefore able to prevent potential harmful DNA of integrating into the chromosome by RNA interference using the stored information of the CRISPR (Gasiunas et al., 2014). In addition, an important limitation is the selective pressure for the respective new genetic trait. If there is no advantage gained by the host bacterium to keep the acquired DNA there will be no selective pressure to maintain it.

CONCLUSIONS

The increasing amount of evidence for HGT in the human oral cavity shows that these processes are important in the adaptability of the oral community. The nature of some of the evolutionary strategies involving HGT is much more complex than simple acquisition of DNA released from dead cells or acquisition of a plasmid or transposon from a donor member of the oral community. More evidence for this is found in oral metagenomes; it has recently been found that the incidence of CRISPRs and the numbers of MGEs associated with oral cavity derived metagenomes is far more than in the GI tract of man (Zhang et al., 2013). The pervasiveness of HGT and the incredibly large number of MGEs found in oral bacteria is arguably a result of the oral environment or at the very least is influenced and selected by the conditions found within it. In other words, if the oral bacterial consortium requires a specific gene, for example conferring antibiotic resistance, it is most-likely already present and only needs to be acquired by the various mechanisms of HGT discussed.

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AI-2 of *Aggregatibacter actinomycetemcomitans* inhibits *Candida albicans* biofilm formation

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Aggregatibacter actinomycetemcomitans, a Gram-negative bacterium, and *Candida albicans*, a polymorphic fungus, are both commensals of the oral cavity but both are opportunistic pathogens that can cause oral diseases. *A. actinomycetemcomitans* produces a quorum-sensing molecule called autoinducer-2 (AI-2), synthesized by LuxS, that plays an important role in expression of virulence factors, in intra- but also in interspecies communication. The aim of this study was to investigate the role of AI-2 based signaling in the interactions between *C. albicans* and *A. actinomycetemcomitans*. *A. actinomycetemcomitans* adhered to *C. albicans* and inhibited biofilm formation by means of a molecule that was secreted during growth. *C. albicans* biofilm formation increased significantly when co-cultured with *A. actinomycetemcomitans luxS*, lacking AI-2 production. Addition of wild-type-derived spent medium or synthetic AI-2 to spent medium of the *luxS* strain, restored inhibition of *C. albicans* biofilm formation to wild-type levels. Addition of synthetic AI-2 significantly inhibited hypha formation of *C. albicans* possibly explaining the inhibition of biofilm formation. AI-2 of *A. actinomycetemcomitans* is synthesized by LuxS, accumulates during growth and inhibits *C. albicans* hypha- and biofilm formation. Identifying the molecular mechanisms underlying the interaction between bacteria and fungi may provide important insight into the balance within complex oral microbial communities.

Keywords: oral microbiology, interspecies interaction, quorum sensing

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a non-motile, Gram-negative coccobacillus, which can be found as a commensal in the oral cavity. In addition, it is also the principal cause of aggressive periodontal disease (Saarela et al., 1999). *A. actinomycetemcomitans* uses chemical signals to sense cell density and alter expression of virulence factors (Novak et al., 2010). This microbial communication is known as quorum sensing (QS) and the only identified cell-cell signaling molecule in *A. actinomycetemcomitans* this far is autoinducer 2 (AI-2). AI-2, which has been proposed to be a general interspecies, concentration dependent signal, is synthesized by LuxS as a precursor, 4,5,-dihydroxy-2,3-pentanedione (DPD), followed by secretion into the medium where it spontaneously undergoes cyclization into AI-2 which accumulates. Interestingly, DPD can thus be converted into several structures that can be recognized by different species (Miller et al., 2004). For instance, *Vibrio harveyi* produces an unusual furanosyl borate diester ((3aS,6S,6aR)-2,2,6,6a-tetrahydroxy-3a-methyltetrahydrofuro[3,2-d][1,3,2] dio xaborol-2-uide), while *Salmonella* Typhimurium recognizes (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran.

In addition, the polymorphic fungus *Candida albicans* is one of the most commonly isolated fungi from the oral cavity (Cannon and Chaffin, 1999). In healthy individuals, *C. albicans* grows as a commensal, mostly in the unicellular yeast morphology, but in immune-compromised individuals this species is capable of producing multicellular filamentous forms, a pathogenic morphology (Odds, 1988). The morphological transition from the yeast-to-hyphal mode of growth is influenced by many factors, including pH, nutrient availability, temperature and the presence of QS molecules, that stimulate or repress filamentation as a function of cell density (Hornby et al., 2001; Hazan et al., 2002). Recently, the interaction of bacteria with *C. albicans* through QS molecules has received increasing attention (Shirtliff et al., 2009). In the oral cavity two Gram-positive bacteria have been shown to affect *C. albicans* biofilm formation through QS molecules. *Streptococcus mutans* was shown to inhibit hyphal formation through the QS molecule competence-stimulating peptide (CSP) (Jarosz et al., 2009) and the fatty acid signaling molecule trans-2-decenoic acid (Vilchez et al., 2010). In addition, *S. gordonii* was shown to secrete AI-2, which was shown to repress *C. albicans* QS by inhibiting the action of farnesol (Bamford et al., 2009) upon

contact. In the healthy oral cavity a multitude of bacterial species co-exist with *C. albicans*, both Gram-negative and Gram-positive (Zaura et al., 2009). In contrast to the limited information on the effect of Gram-positive oral bacteria on *C. albicans*, no information is available on the effect of QS molecules of Gram-negative oral bacteria. Therefore, in the present study, it was our aim to investigate the effect of AI-2 produced by the Gram-negative oral bacterium *A. actinomycetemcomitans* on *C. albicans*.

MATERIALS AND METHODS

MICROBIAL STRAINS AND GROWTH CONDITIONS

A. actinomycetemcomitans spp. were routinely cultured for 18 h at 37°C under microaerobic conditions (10% CO_2) on trypticase soy agar or broth containing 0.6% yeast extract (TSB-YE/Difco). When appropriate, standardized cell suspensions were prepared with a density of 2.1×10^8 CFU mL^{-1} as determined with serial dilution plating and determination of the optical density measured at 655 nm (OD_{655}). *Escherichia coli* was routinely grown in Luria-Bertani broth at 37°C with constant aeration. For solid medium, 15 g agar per liter was added to the liquid medium. When required, ampicillin (100 μg mL^{-1}) or kanamycin (30 μg mL^{-1}) was added to the medium. *C. albicans* strain ATCC 10231 or SC5314 were taken from stock cultures frozen in 15% glycerol at −80°C and sub-cultured twice onto yeast peptone agar plates with 2% glucose (YPD) or when indicated in yeast nitrogen base medium pH 7, supplemented with 50 mM glucose (YNB).

ADHESION OF *A. ACTINOMYCETEMCOMITANS* TO *C. ALBICANS*

Adhesion of *A. actinomycetemcomitans* Y4 to *C. albicans* strain SC5314 was studied using a Bioflux 1000Z setup. Briefly, *C. albicans* was seeded into a 48-well microfluidics plate (Fluxion Biosciences) at an initial optical density measured at 600 nm of 0.2 ($OD_{600} = 0.2$). After 30 min adhesion to the bottom plate at 37°C, flow with prewarmed YNB was started at 0.5 dyne cm^{-2} for 4 h. A bacterial suspension at an initial $OD_{600} = 0.2$ in phosphate buffered saline (PBS; 10 mM potassium phosphate, 0.15 M sodium chloride, pH 7) containing 0.2 μL mL^{-1} Syto9 and 0.2 μL mL^{-1} propidium iodide (*Bac*light, Invitrogen) was flowed through the microfluidics channels at 0.5 dyne cm^{-2} and images were captured every 30 s using the appropriate filter settings. Images were translated to AVI-movies using ImageJ (1.46r).

CONSTRUCTION OF AN *A. ACTINOMYCETEMCOMITANS luxS* MUTANT

A. actinomycetemcomitans (serotype b) was isolated in our periodontal clinic (Universitas Indonesia, Jakarta) from periodontitis patients, with their consent. The isolated bacteria were identified by means of Gram staining, characteristic star-positive colonies on agar plates and tight adherence to surfaces when grown in broth (Slots et al., 1982). Positive clones were confirmed and serotyped using PCR (Suzuki et al., 2001). A single isolate, *A. actinomycetemcomitans* UI-09, was selected as wild-type strain for all other experiments. In addition, the commonly used *A. actinomycetemcomitans* Y4 was used as a reference in certain experiments and showed similar results compared to *A. actinomycetemcomitans* UI-09.

All primers for plasmid construction were designed using *A. actinomycetemcomitans* database (http://www.oralgen.org, ID for *luxS* in *A. actinomycetemcomitans* HK 1651 is AA00516). In order to create a *luxS* defective mutant, a suicide vector was constructed using the neighbor-joining technique as previously described for *Campylobacter jejuni* (Bachtiar et al., 2007). Firstly, a 229-bp DNA fragment containing part of the upstream sequence adjacent to *luxS* was PCR-amplified using the primer *Eco*RI-L1 (ACGAATTCAATCCACCGCACTT, forward) and primer *Bam*HI-L2 (TCGGATCCAAGTTTTCTTGTTAGG, reverse). The PCR product was cloned into pBluescript in the forward direction via the *Eco*RI and *Bam*HI sites, and confirmed by restriction analysis. The construct (pBl-L1) was subsequently introduced into *E. coli* JM 107. Positive clones were selected on LB agar supplemented with ampicillin, X-Gal and IPTG. Secondly, a 409-bp DNA fragment containing the downstream flanking region of *luxS* was amplified using primers *Bam*H1-L3 (CATGGATCCGAAGAAGCACATCAA, forward) and *Xba*I-L4 (ATCTAGAGCAAGTTGCTCGTAA, reverse). The amplified fragment was inserted into pBl-L1 between *Bam*HI and *Xba*I sites. The resulted intermediate plasmid (pBl-L2) was cut at the unique *Bam*HI site and a 1.4-kbp fragment containing a kanamycin cassette (obtained from vector pMW2 digested with *Bam*HI) was ligated into the plasmid to obtain the suicide plasmid, $pBLkm^r$.

NATURAL TRANSFORMATION

A biphasic system for *A. actinomycetemcomitans* transformation was performed as described previously (Wang et al., 2002). Transformation was done by incubating a suspension of 10^8 CFU mL^{-1} of *A. actinomycetemcomitans* at 37°C under microaerobic conditions for 3 h. Subsequently, 10 μg of the suicide vector was added and cells were incubated for 3 h at 37°C. Cells were then harvested and plated on medium supplemented with kanamycin and incubated at 37°C, under microaerobic conditions for 2 days to select for transformants. Homologous recombination and disruption of *luxS* was confirmed using PCR analysis with primers flanking the target site (*Eco*RI-L1 and *Xba*I-L4).

SPENT MEDIUM PREPARATION

Spent medium of *A. actinomycetemcomitans* cultures was prepared as described previously (Jarosz et al., 2009). Protein concentration in the spent medium was measured using the Bradford method. Spent medium was diluted in PBS to yield 10 and 100 μg mL^{-1} concentrations and used immediately or stored for short periods of time at −20°C. The pH of the spent medium was adjusted to pH 7.

SYNTHESIS OF (S)-4,5,-DIHYDROXY-2,3-PENTANEDIONE (DPD)

DPD was synthesized following a procedure published by Ganin et al. (2009). Lyophilized DPD was dissolved in DMSO at 33 mM stock concentrations and stored at −20°C until required. Synthetic DPD concentrations used in the described experiments are assumed to be physiologically relevant as they are in line with concentrations of AI-2 reported to be produced in saliva-fed natural oral biofilms (Rickard et al., 2008).

BIOFILM FORMATION OF *C. ALBICANS* AND *A. ACTINOMYCETEMCOMITANS*

Quantification of *A. actinomycetemcomitans* biofilms was achieved by staining with Crystal Violet (CV).

A. actinomycetemcomitans strains (5 μL) were used to inoculated wells of 96-well (flat-bottom) cell culture plates (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) containing 95 μL TSB-YE in each well. After, 18 h of incubation under microaerobic condition, the culture medium containing planktonic cells was removed and the wells were carefully washed with 200 μL of distilled water. Adherent bacteria were stained with 50 μL of 0.1% CV for 15 min at room temperature. After rinsing twice with 200 μL of distilled water, CV bound to the biofilm was extracted with 200 μL of 99% ethanol for 20 min and quantified by measuring the absorbance at 655 nm with a microplate reader (Model 3550, Bio-Rad Laboratories, Hercules, CA, USA).

Biofilm formation of *C. albicans* was induced in YNB as previously reported (Krom et al., 2007). Mixed species biofilms of *C. albicans* (2×10^6 CFU mL^{-1}) and *A. actinomycetemcomitans* (2.1×10^7 CFU mL^{-1}) were grown in medium containing 70% YNB and 30% TSB-YE (vol/vol). Where indicated, the 30% fresh TSB-YE fraction was replaced by spent medium as described previously (Jarosz et al., 2009). In addition, when indicated, sterile spent medium from the *A. actinomycetemcomitans* was added to YNB at 1, 10, and 100 μg mL^{-1} protein concentration. After 24 and 48 h of growth, biofilm formation on the well of microtiter plates were washed once with PBS and metabolic activity of the biofilms was quantified using [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) as described previously (Krom et al., 2007). Since *C. albicans* rapidly metabolized MTT, in contrast to *A. actinomycetemcomitans* (**Supplementary Figure S1**) this assay could be used to determine *C. albicans* biofilm formation even in co-cultures with *A. actinomycetemcomitans*. All assays were carried out on at least two separate occasions with at least duplicate determinations on each occasion. Microtiter wells containing only YNB broth but no cells were used as negative controls. To mimic the conditions in the oral cavity initial experiments were also performed in the presence of pooled sterilized human saliva, however no differences were observed relative to experiments without saliva. Therefore, all other experiments were performed without saliva.

INHIBITION OF HYPHA FORMATION

Hypha formation was assayed as described previously (Jarosz et al., 2009) using *C. albicans* strain SC5314. The morphology of cells was analyzed using a 20x objective on an inverted microscope (Olympus, Tokyo, Japan).

STATISTICAL ANALYSES

Differences between means were analyzed for statistical significance using two-tailed Student's *t*-tests. Differences were considered significant when $p \leq 0.05$ level. For multiple comparisons, a One-Way ANOVA followed by a TUKEY HSD test was performed (http://vassarstats.net/anova1u.html).

RESULTS

ADHESION OF *A. ACTINOMYCETEMCOMITANS* TO *C. ALBICANS*

Adhesion to surfaces is a critical initial step in microbial biofilm formation. Using a microfluidics setup, adhesion under flow conditions were studied. Single *A. actinomycetemcomitans* Y4 cells adhere to hyphae and yeast cells of *C. albicans* SC5314, as illustrated by the increased green fluorescent spots associated with *C. albicans* (**Figure 1**). In addition, adhesion of *A. actinomycetemcomitans* to the glass surface could be observed.

EFFECTS OF *luxS* DELETION ON BIOFILM FORMATION OF *A. ACTINOMYCETEMCOMITANS*

A. actinomycetemcomitans luxS formed significantly less biofilm compared to the wild type strain (**Figure 2**) but growth rate in planktonic cultures was not affected, in line with previous reports (Novak et al., 2010). Spent medium of a 4 h-old culture of the wild type strain was able to rescue biofilm formation of the *luxS* strain at 100 μg protein mL^{-1} (**Figure 2**, left panel). Medium of 6 h-old cultures also rescued the phenotype to a similar, but not medium of 8 and 24 h-old cultures. Addition of synthetic DPD rescued this phenotype specifically at 100 nM DPD, but not at lower or higher concentrations (**Figure 2**, right panel).

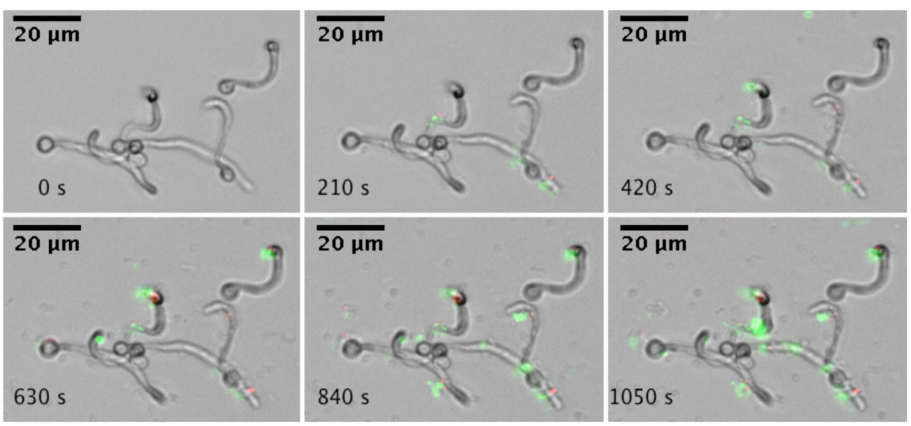

FIGURE 1 | Real-time microscopic analysis of adhesion of *A. actinomycetemcomitans* to *C. albicans* SC5314. Hyphae of *C. albicans* SC5314 were allowed to form on the bottom of a microfluidics plate. Bacteria were stained with Syto9 and PI and allowed to adhere to *C. albicans* SC5314 while flowing at 0.5 dyne cm^{-2}. Images were captured every 30 s for a total of 10 min (montage shows images every 210 s). Images were edited for brightness and contrast.

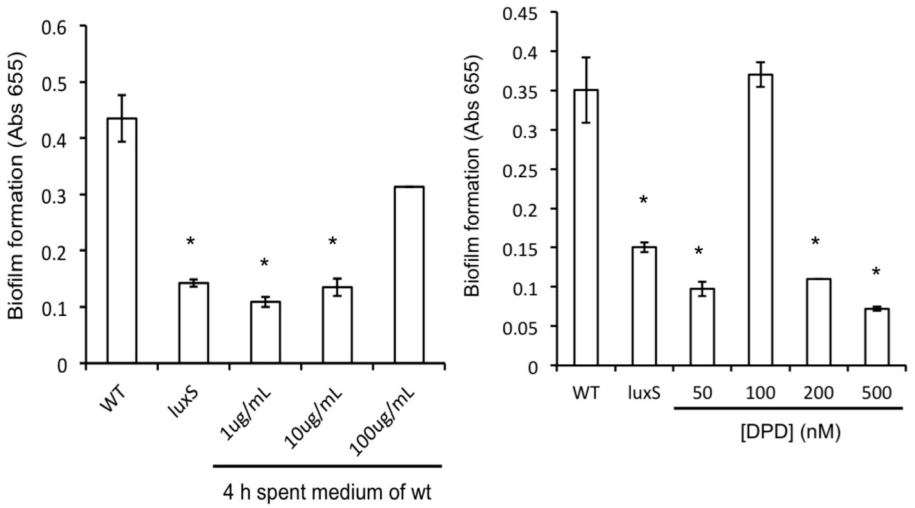

FIGURE 2 | AI-2 plays a role in biofilm formation of *A. actinomycetemcomitans*. Biofilm formation of *A. actinomycetemcomitans luxS* is restored to near wild-type levels by the addition of sterile spent medium **(left panel)** as well as by the addition of 100 nM synthetic DPD **(right panel)**. *Indicates significantly different from control.

ROLE OF AI-2 ON MIXED SPECIES BIOFILMS OF *A. ACTINOMYCETEMCOMITANS* AND *C. ALBICANS*

Compared to mono-species biofilm formation of *C. albicans*, mixed species biofilms of *C. albicans* with *A. actinomycetemcomitans* resulted in a reduction of more than 50% in metabolic activity after 24 h of culturing (**Figure 3A**). A similar decrease in biofilm formation was observed after 48 h of culturing (not shown). Mixed species biofilms of *C. albicans* with *A. actinomycetemcomitans luxS* had no significant effect compared to mono-species biofilms.

EFFECT OF SECRETED FACTORS ON *C. ALBICANS* BIOFILM FORMATION

To further determine whether the inhibition effect was modulated by secreted compounds of the bacteria, the spent medium of the wild type *A. actinomycetemcomitans* was used as a source of secreted molecules to complement *A. actinomycetemcomitans luxS*. When spent medium from 4 and 6 h-old wild-type cultures was added, the *A. actinomycetemcomitans luxS* mutant was able to inhibit *C. albicans* biofilm formation, but this inhibition was not observed for spent medium derived from 8 and 24-h old cultures (**Figure 3B**). Conversely, when spent medium of *A. actinomycetemcomitans luxS* was added to mixed species biofilms of *C. albicans* and *A. actinomycetemcomitans luxS*, no significant inhibitions of biofilm formation was observed (**Supplementary Figure S2**).

EFFECT OF SYNTHETIC DPD ON *C. ALBICANS* BIOFILM GROWTH AND HYPHA FORMATION

When synthetic DPD was added to spent medium of *A. actinomycetemcomitans luxS* during *C. albicans* biofilm growth, a concentration dependent inhibition of *C. albicans* biofilm formation was observed with the maximum inhibition reached at 100 nM DPD (**Figure 4**, right panel). Hypha formation is a key process in biofilm formation. Synthetic DPD was added to *C. albicans*' under hypha inducing conditions. DPD inhibited hypha formation by 30 and 70% at 100 nM and 1 μM, respectively (**Figure 4**, left panel).

A. ACTINOMYCETEMCOMITANS SPENT MEDIUM DISRUPTS ESTABLISHED *C. ALBICANS* BIOFILM

To test the ability of *A. actinomycetemcomitans* to disrupt *C. albicans* biofilms, we challenged established biofilms (24 h old) of *C. albicans* with spent medium from *A. actinomycetemcomitans* WT and *luxS* cultures of increasing age. A culture age dependent decrease in viability was observed when *C. albicans* biofilms were exposed to spent medium of cultures of the WT strain, but not to spent medium derived from the same aged cultures of the *luxS* strain (**Figure 5**).

DISCUSSION

A. actinomycetemcomitans is related to severe periodontitis. In addition, several studies have reported on the isolation of a large number *Candida* spp., from periodontal pockets of patients with periodontitis (Jarvensivu et al., 2004; Urzua et al., 2008). Recently, co-isolation of *Candida* spp. and *A. actinomycetemcomitans* was correlated with the occurrence of severe periodontitis (Brusca et al., 2010). However, due to limited information it is currently unclear if *C. albicans* participates in the etiology of any kind of periodontitis. Biofilms are the most common mode of growth of *Candida spp.*, as observed *in vivo*. Therefore, interspecies interactions occur preferentially in mixed species biofilms such as those found in the periodontal pocket. A better fundamental understanding of interspecies interaction in the oral cavity is therefore of great relevance. Here we show for the first time that a commonly isolated periodontal pathogen *A. actinomycetemcomitans* adheres to *C. albicans* under flow condition (**Figure 1**). Physical interactions, such as adhesion are initial and probably critical stages in microbial biofilm formation. Following adhesion growth

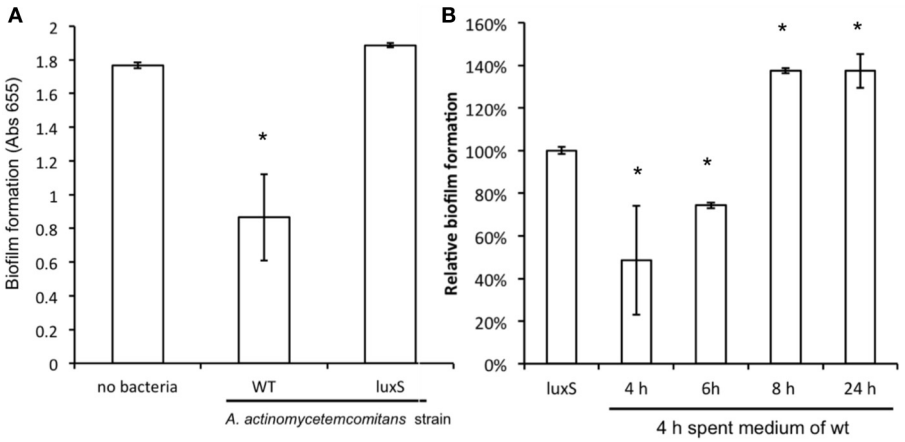

FIGURE 3 | The effect of co-culture with *A. actinomycetemcomitans* on *C. albicans* ATCC 10231 biofilm formation. (A) *C. albicans* biofilms were grown without any bacteria or with *A. actinomycetemcomitans* wild-type or *luxS* mutant. Biofilm formation, quantified using the MTT assay, was measured after 24 h of growth. Data represent the mean and standard deviations of six biofilms grown on two separate occasions. **(B)** The effect of spent medium on co-cultures between *C. albicans* and *A. actinomycetemcomitans luxS*. Spent medium of *A. actinomycetemcomitans* wild-type cultures grown for different times was added to the mixed species culture of *C. albicans* and *A. actinomycetemcomitans luxS*. Biofilm formation was quantified after 24 h of growth using the MTT assay. The relative biofilm formation compared to *C. albicans* + *A. actinomycetemcomitans luxS* without any spent medium was calculated. Data represent the mean and standard deviations of six biofilms grown on two separate occasions. *Indicates significantly different from control.

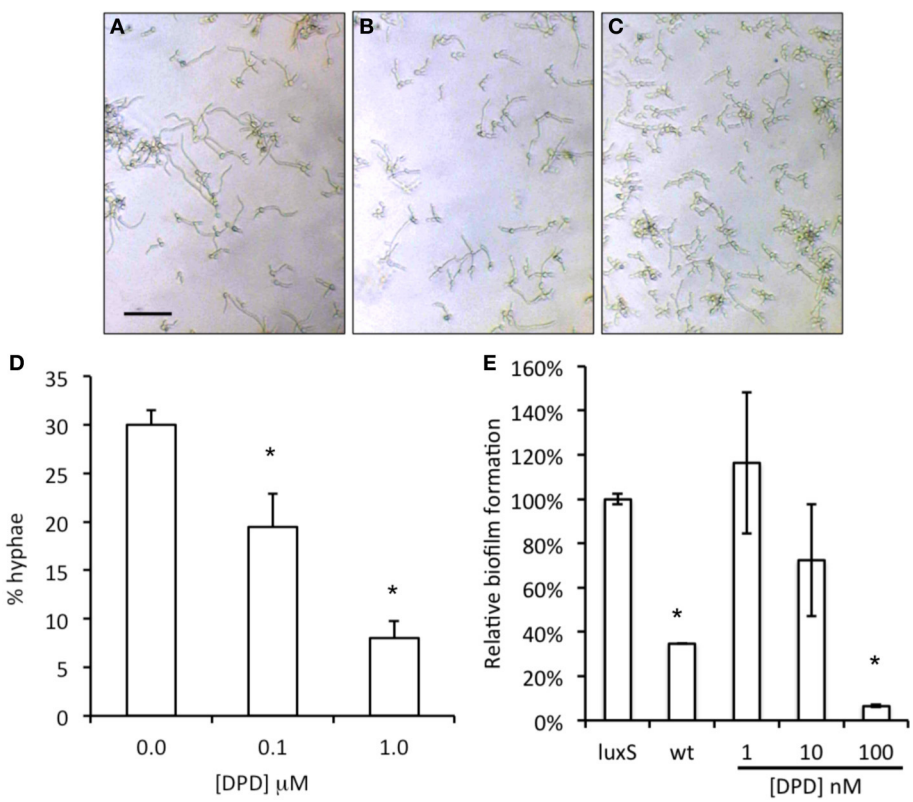

FIGURE 4 | Effect of synthetic DPD on *C. albicans* SC5134. Hypha-formation was induced by switching fresh cultures to 37°C for 3–4 h. Representative microscopic images show a decreased hypha formation (**A** = control, **B** = 0.1 μM DPD, **C** = 1.0 μM DPD; bar represent 40 μm for all images). Hyphae and yeast morphologies were counted and plotted as % of all cells **(D)**. The results represent the mean of two independent experiments, each consisting of at least 100 cells per sample. Synthetic DPD added to spent medium of *A. actinomycetemcomitans luxS* inhibits *C. albicans* SC5314 biofilm formation **(E)**. *Indicates significantly different from control.

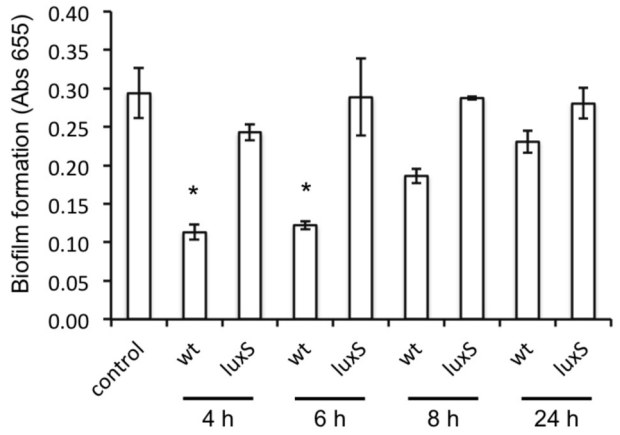

FIGURE 5 | Effect of spent medium of *A. actinomycetemcomitans* wt and *luxS* on preformed *C. albicans* ATCC 10231 biofilms. Biofilms of *C. albicans* were grown for 24 h after which they were exposed to spent medium of *A. actinomycetemcomitans* cultures of increasing age for an additional 24 h. Total amount of biofilm was determined using the MTT assay and results depict the averages of a total of 4 wells in 2 independent experiments. *Indicates significantly different from control as determined using a One-Way ANOVA followed by a TUKEY HSD test.

and accumulation results in interspecies chemical interactions, amongst others mediated by quorum sensing (Jarosz and Krom, 2011).

AI-2 activity has been discovered in spent culture supernatants of many oral bacteria (Fong et al., 2001; Blehert et al., 2003; Wen and Burne, 2004). However, the function of AI-2 as a general bacterial signaling molecule is an issue that is yet to be resolved. Several studies have suggested that AI-2 is involved in biofilm formation (Chung et al., 2001; Yoshida et al., 2005). On the other hand, it has also been suggested that the main function of the LuxS enzyme is in the regulation of metabolic processes (Winzer et al., 2002). Inactivation of *luxS* in *A. actinomycetemcomitans* does not affect growth rates but does result in phenotypic alterations, including biofilm formation and reduced colonization in various experimental infection models (Hardie and Heurlier, 2008). Our data demonstrated that biofilm formation of the *A. actinomycetemcomitans luxS* strain was decreased as compared with its parent strain. Complementation of the *luxS* deletion by both spent medium of wild-type *A. actinomycetemcomitans* as well as by addition of synthetic DPD would indicate that the *luxS* mutant phenotype is due to absence of the quorum sensing molecule and not related to any metabolic defect. It should however be noted that it is not straightforward to uncouple signaling and metabolic functions of LuxS (Redanz et al., 2012). The remarkable concentration dependent complementation of the *luxS* mutant by DPD—reaching a maximum at 100 nM, followed by a sharp decrease at higher concentrations - is interesting and currently we are unable to explain this specific behavior. It is however important to notice that such a behavior has been observed in another study. Rickard and coworkers described a similar sharp concentration optimum for synthetic AI-2 in a mixed-species biofilm model consisting of *Actinomyces naeslundii* and *Streptococcus oralis* be it at approximately 100-fold lower concentration (Rickard et al., 2006). Spent medium of 8 h cultures and older no longer inhibited *C. albicans* biofilm formation (see **Figure 5**). This could be related to decreased AI-2 presence at later stages of growth which is in line with the growth-phase specific production of AI-2, higher in the first 6 h of growth compared to later stages, observed in several oral species (Fong et al., 2001; Blehert et al., 2003; Wen and Burne, 2004).

Bamford et al. showed that *S. gordonii* increases *C. albicans* biofilm formation in a LuxS dependent fashion, however, no effect of synthetic DPD on *C. albicans* hypha formation was observed (Bamford et al., 2009). In contrast, we observed that the exogenous addition of AI-2/DPD to the growth medium restored the *luxS* phenotype in a dose-dependent manner and that addition of synthetic DPD inhibited hypha formation of *C. albicans*. This effect was only seen in medium consisting of 70% YNB/30% TSB, and not in YNB alone. In contrast to Bamford we did not use saliva in the germ-tube assay. Both minimal medium and saliva are strong inducers of hypha formation and this might explain the observed different response of *C. albicans* to synthetic DPD. Additionally, use of different *C. albicans* strains in the present study compared to Bamford and coworkers could account for the observed differences in response. Alternatively, the different response under different hypha inducing conditions could indicate a difference in the chemical structure of AI-2 produced by *S. gordonii* or *A. actinomycetemcomitans*, and intriguingly that *C. albicans* is able to recognize this chemical difference. Detailed chemical characterization of the AI-2 for both species should be performed to further support this statement.

Communication within oral microorganisms involves several classes of signal molecules; autoinducing peptides synthesized by Gram-positive bacteria, such as competence-stimulating peptide of *S. mutans* (Jarosz et al., 2009), fatty acid signaling molecules such as farnesol and trans-2-decenoic acid (SDSF) (Vilchez et al., 2010) and AI-2 as a proposed universal signal produced by both Gram-positive and Gram-negative bacteria (Federle and Bassler, 2003). The different responses of *C. albicans* to AI-2, depending on its origin, is intriguing and could illustrate that the already complicated interspecies chemical communication might be even more complicated, but a more extensive study is needed to provide solid evidence for such a tempting, but speculative conclusion.

Oral biofilms are very complex multi-species communities in which we assume that interspecies interaction plays a role in establishing and maintaining a balance. A recent study on bacterial and yeast colonization in a group of mucositis patients showed a complete absence of *A. actinomycetemcomitans* and a more than average presence of *C. albicans* (Laheij et al., 2012). As hypha formation is a pivotal step in *C. albicans* biofilm formation (Krueger et al., 2004) and adhesion, the LuxS mediated interaction with *A. actinomycetemcomitans* would decrease fungal biofilm formation. Additionally, the function of LuxS is related to the regulation of bacteria virulence factors, including cytolethal distending toxin (CDT), as reported in *Campylobacter jejuni* (Jeon et al., 2005). For *A. actinomycetemcomitans* it has been shown that CDT (CDTB) is toxic to the yeast *Saccharomyces cereviseae* (Matangkasombut et al., 2010). There are therefore potentially multiple systems, one based on

a secreted QS molecule and a second on a toxin, involved in the observed negative interactions between *A. actinomycetemcomitans* and *C. albicans*. A preliminary study indicated that spent medium of wild-type *A. actinomycetemcomitans* has no toxic effects on *C. albicans* (**Supplementary Figure S3**). Similar dual mechanisms of inhibition have been observed between *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Li and Krom, unpublished data). It is tempting to hypothesize that the QS molecule is sensed as a defense mechanism to protect against the upcoming battle with a toxin-producing competitor (Jarosz et al., 2011). Additional studies are required to further elucidate the molecular and biochemical mechanisms involved in the interspecies interaction between *A. actinomycetemcomitans* and *C. albicans*.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00094/abstract

Supplementary Figure S1 | Comparison of MTT and XTT reduction by *C. albicans* SC5314 and *A. actinomycetemcomitans*. Metabolic activity of *C. albicans* yields a purple or orange color upon reduction of MTT and XTT respectively. Incubation of *A. actinomycetemcomitans* does not result in a significant color change.

Supplementary Figure S2 | Complementation of the *luxS*-effect using spent medium of wild-type *A. actinomycetemcomitans*. Addition of 4 and 6 h-old wt-spent medium to mixed species biofilms of *C. albicans* ATCC 10231 and *A. actinomycetemcomitans* results in inhibition of biofilm formation. Addition of spent medium of *A. actinomycetemcomitans luxS* did not affect biofilm formation.

Supplementary Figure S3 | Effect of spent medium of wild-type *A. actinomycetemcomitans* on growth kinetics of *C. albicans* SC5134. An overnight culture of *C. albicans* was diluted 1:20 or 1:50 in medium consisting of 70% YNB + 30% filter sterilized 4 h-old spent medium of wild-type *A. actinomycetemcomitans*. As a control, the same inoculum was grown in 70% YNB + 30% fresh TSB + YE. No significant differences were observed illustrating the absence of any toxicity related agent in the spent medium of wild-type *A. actinomycetemcomitans*.

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Fungal-bacterial interactions and their relevance to oral health: linking the clinic and the bench

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High throughput sequencing has accelerated knowledge on the oral microbiome. While the bacterial component of oral communities has been extensively characterized, the role of the fungal microbiota in the oral cavity is largely unknown. Interactions among fungi and bacteria are likely to influence oral health as exemplified by the synergistic relationship between *Candida albicans* and oral streptococci. In this perspective, we discuss the current state of the field of fungal-bacterial interactions in the context of the oral cavity. We highlight the need to conduct longitudinal clinical studies to simultaneously characterize the bacterial and fungal components of the human oral microbiome in health and during disease progression. Such studies need to be coupled with investigations using disease-relevant models to mechanistically test the associations observed in humans and eventually identify fungal-bacterial interactions that could serve as preventive or therapeutic targets for oral diseases.

Keywords: oral health, microbiome, mycobiome, fungi, bacteria, interactions

INTRODUCTION

Around 600 bacterial species and a still undetermined number of fungal species inhabit the oral cavity of humans (Dewhirst et al., 2010; Ghannoum et al., 2010; Dupuy et al., 2014). Oral microbial cells arrange in organized biofilm structures on non-shedding surfaces such as teeth. Organized aggregates are also formed on mucosal surfaces and even in the salivary fluid phase, via specific cell to cell adhesion events (Dongari-Bagtzoglou et al., 2009; Kolenbrander et al., 2010). Such physical proximity facilitates metabolic interactions among microbial cells (Egland et al., 2004; Jakubovics et al., 2008; Kim et al., 2008), while a defined spatial structure has been shown to increase stability of microbial communities, allowing creation of chemical gradients (Kim et al., 2008). Microorganisms in these diverse assemblages interact through various types of metabolic exchanges. For example, bacterial consortia cooperate to release nutrients from macromolecules available in oral fluids (Bradshaw et al., 1994). Cross-feeding events have been identified in which metabolic end-products of one species are used as carbon sources by another community member (Diaz et al., 2002; Marsh and Martin, 2009). Also, through various signaling events, bacterial cells coexisting in a community alter the phenotype of their neighbors (Kuboniwa et al., 2009; Frias-Lopez and Duran-Pinedo, 2012). Due to their specific nature, microbial interactions are likely to influence community assembly and may also determine resistance and resilience of communities to perturbations. Since most oral diseases are associated with perturbations of community balance, understanding the interactions among species that maintain community stability or allow microbial shifts to occur is an essential part of the development of strategies to preserve and restore oral health. Interactions among oral microbial cells, however, have almost been exclusively studied in bacteria, while little is known regarding fungi-bacteria relationships.

Limited interest on fungal-bacterial interactions and their role in health and disease is a consequence of incomplete knowledge on the fungal microbiota. Most studies on oral fungi have focused on *Candida* species, which are highly amenable to cultivation. *Candida* species establish in the oral cavity as commensals but may become virulent, causing mucosal lesions, under certain conditions (Lalla et al., 2013). The role oral bacteria play in candidiasis has only recently received attention (Diaz et al., 2012b; Xu et al., 2013). Interest in fungi other than *Candida* and their role in oral health and disease is also emerging as recent molecular characterizations of the oral mycobiome have highlighted the great diversity of fungi present in the oral cavity (Ghannoum et al., 2010; Dupuy et al., 2014). In this perspective we will summarize current understanding of fungal-bacterial ecology in relation to oral health, arguing that while the advent of "omics" approaches will facilitate the identification of potential fungal-bacterial relationships associated with health and disease, parallel mechanistic studies using *in vitro* and *in vivo* models are needed. Thus, a link between clinical studies in humans conducted via a systems biology perspective and laboratory experimental approaches using models to study candidate fungal-bacterial relationships will provide the key to understand the roles of fungal-bacterial interactions in oral health maintenance (**Figure 1**).

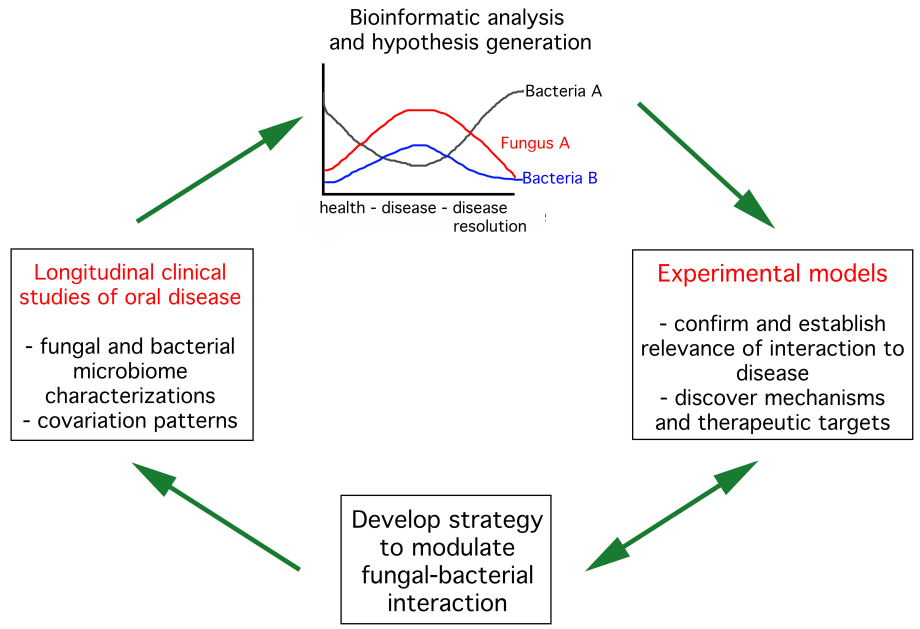

FIGURE 1 | Strategies to advance knowledge on fungal bacterial interactions in the context of oral health. The first step (left panel) is to conduct longitudinal clinical studies in humans characterizing the fungal and bacterial components of the microbiome throughout progression and resolution of oral diseases. These observational studies should lead to identification of potential positive (top panel, fungus A and bacteria B) or negative (top panel, fungus A and bacteria A) interactions between bacteria and fungi. Selected interactions need to be tested in disease-relevant experimental models to confirm their importance to disease progression and dissect mechanisms mediating the interaction. These studies could then be used to develop strategies to interfere with fungal-bacterial interactions. Such strategies need to be tested in experimental model systems and eventually in humans.

"OMICS" TECHNOLOGIES REVOLUTIONIZED CHARACTERIZATION OF BACTERIAL AND FUNGAL ORAL COMMUNITIES

A variety of "omics" approaches, particularly those powered by high throughput DNA sequencing accelerated knowledge on the composition of oral microbial communities. Parallel sequencing of 16S rRNA-based amplicon libraries allows rapid identification of bacterial species present in oral samples in what has become a relatively straight forward process (Diaz et al., 2012a). The availability of curated 16S rRNA sequence databases for oral taxa, such as the Human Oral Microbiome Database (HOMD), facilitates assignment of species level taxonomies to short sequence reads (Dewhirst et al., 2010). We use the 16S rRNA hypervariable regions V1 and V2 as a tool to survey the oral bacteriome. Using this 16S rRNA region, it is possible to obtain species level taxonomic identities with almost the same accuracy as when using full length 16S rRNA gene sequences. For instance, if we download the 830 full length 16S rRNA sequences from the HOMD and classify each sequence using Mothur's version of the Ribosomal Database Project classifier, with a bootstrapping cutoff of 80% (Schloss and Westcott, 2011), and using the same HOMD as template, we are able to correctly assign ~92% of sequences to their respective species. Those sequences for which a species level taxonomy is not possible belong to species undistinguishable by their 16S rRNA sequences (e.g., *Streptococcus mitis* and *Streptococcus oralis*) or to organisms for which taxonomies still need clarification (e.g., *Peptosptreptococcacceae*, *Alloprevotella* spp.). When using only the V1-V2 regions, we are able to correctly assign ~90% of HOMD sequences to species, in close agreement to results using full length 16S rRNA. It is thus possible to perform accurate species-level taxonomic surveys of oral communities using partial V1-V2 16S rRNA amplicons.

Using high throughput sequencing of 16S rRNA gene fragments, our group and others have defined the complex shifts in subgingival bacterial communities associated with periodontitis, an inflammatory condition of the supporting structures of teeth (Griffen et al., 2011; Abusleme et al., 2013). These studies have shown that despite great inter-subject variability in microbiome composition, dozens of species are consistently associated with periodontitis. Similarly, the bacterial microbiomes associated with caries have been characterized revealing a complex community in which the acidogenic microorganism *Streptococcus mutans* becomes abundant in most, but not all, caries-associated microbiomes (Gross et al., 2012). Moreover, high throughput sequencing is being used to evaluate community functions in health and disease, by comparison of shifts in the metagenomes and metatranscriptomes of plaque samples (Belda-Ferre et al., 2012; Duran-Pinedo et al., 2014; Jorth et al., 2014). These studies, however, have targeted only the bacterial component of the microbiome.

In contrast to the oral bacterial microbiota, knowledge on the fungal microbiota is limited. Our group has used high throughput sequencing of internal transcribed spacer 1 (ITS1) amplicon libraries to characterize the fungi present in oral samples. As with bacterial amplicons, the actual process of sequencing is relatively

clear-cut, but there are three special challenges associated with the mycobiome (Dupuy et al., 2014). First, some fungal cells are notoriously difficult to break open, so we have adopted a relatively harsh bead-beating method that utilizes a very high density zirconia bead. We suspect this improvement allowed us to be the first to capture the widespread and abundant presence of species from the genus *Malassezia* in the healthy human mouth. A second challenge is to improve the legitimacy and accuracy of taxonomic assignments. Using the curated Fungal Metagenomics Project database to analyze our sequence datasets, we have empirically developed a BLAST E-value match statistic (10^{-42}) that reduces spurious assignments and improves the likelihood of identification of biologically relevant fungi. The third challenge, and one likely to be of considerable significance to understanding fungal involvement in oral health and disease, are the fungal-specific problems in binary names and phylogenetic classifications. Fungi provide the most abundant and widespread examples of organisms with multiple names, often involving different genera, lacking nomenclature guidelines. Three factors contribute to autonomous naming: independent isolation of the same fungus from different environments, dimorphic forms, and the presence of asexual (anamorph) and sexual (teleomorph) pairs. With respect to the oral cavity, the anamorph and teleomorph pairs of many *Candida* and *Pichia* species provide instructive examples. In our studies, *Cyberlindnera jadinii* (from NCBI) has been exclusively represented by its synonym, *Pichia jadinii*, which has the anamorph name of *Candida utilis*; in fact, anamorphic genus *Candida* names exist for each of the teleomorphic *Pichia* species we have found in the mouth to date. In the example mentioned, we have decided to use *Candida* as the priority genus referring to these sequences as *Candida utilis* (*Pichia jadinii, Cyberlindnera jadinii*). There is, however, an urgent need to develop a curated database for oral fungi with consensus nomenclature to link previous and current clinical studies.

Using the aforementioned improvements, we have examined the salivary mycobiome in healthy individuals via ITS1 sequencing (Dupuy et al., 2014). Our results are in good agreement with the only similar study (Ghannoum et al., 2010) in identifying consensus mycobiome members *Candida/Pichia, Cladosporium/Davidiella, Alternaria/Lewia, Aspergillus/Emericella/Eurotium, Fusarium/Gibberella, Cryptococcus/Filobasidiella,* and *Aureobasidium.* Our study, however, was the first to identify *Malassezia* species as prominent commensals. We have now extended our analysis to dozens of samples from healthy individuals that were collected in different clinical or research environments, and have confirmed widespread presence of *Malassezia* species in the mouth. The role of *Malassezia* spp. in oral homeostasis, however, remains unknown.

USING "OMICS" INFORMATION TO IDENTIFY CANDIDATE FUNGAL-BACTERIAL INTERACTIONS IMPORTANT TO ORAL HEALTH

Microbiome profiles can be used to explore co-occurrence and co-exclusion patterns in oral communities. Associations inferred from this analysis could be used to generate hypotheses regarding synergistic or antagonistic interactions among fungi and bacteria. Several approaches to this analysis have been proposed. Duran-Pinedo et al. (2011) used weighted correlation network analysis to identify associations between bacterial species in subgingival plaque. In a proof of principle experiment, these authors demonstrated that correlation network information could improve the growth of uncultivated taxa in laboratory media. Several *Prevotella* spp. were identified as candidate growth partners for the uncultivated *Tannerella* sp. HOT286, based on direct associations in microbial network modules. Using the helper *Prevotella* spp. in a co-cultivation approach, the authors were able to enrich for *Tannerella* sp. HOT286 in solid laboratory media.

More recently, Faust et al. analyzed the 16S rRNA-based microbial profiles of more than 5000 samples from healthy individuals to infer a bacterial interaction network, addressing the methodological limitations of using simple correlation coefficients such as Pearson's or Spearman's to analyze organismal associations from relative abundance data (Faust et al., 2012). Since relative abundance measures are dependent on each other and the increase in one organism is always accompanied by a decrease in others, these authors devised a series of analytical approaches to account for the compositionality of the data, inferring significant inter-species associations at different body sites.

The only publication to date that has used microbial 16S rRNA and ITS profiles to explore co-occurrence patterns among fungal and bacterial members of oral communities is that by Mukherjee et al. (2014). These authors report on a series of pairwise Spearman's correlation tests for bacterial and fungal genera present in oral rinse samples and then explore an antagonistic relationship between two oral fungi, *Candida* and *Pichia*. Validation of the fungal-bacterial correlations in larger cohorts and experimental evidence demonstrating their significance are still required.

Amplicon-based profiles have also been correlated to total and taxon-specific loads in oral samples. Kraneveld et al. combined 16S rRNA gene profiling with real time qPCR measurements to explore the relationship between *Candida* load, bacterial load and the bacterial microbiome composition of saliva in elderly subjects (Kraneveld et al., 2012). After comparison of the 16S rRNA to *Candida* ITS qPCR ratios, it was seen that in most subjects bacteria outnumbered *Candida*. However, in one subject *Candida* appeared at much higher levels than bacteria. Interestingly, the authors found that in samples with high *Candida* load, there was an increase in relative abundance of saccharolytic species from the genera *Streptococcus*, *Lactobacillus*, and *Scardovia*, among others, suggesting a relationship between an acidogenic flora and *Candida*.

No human clinical study to date has investigated associations among bacteria and fungi in a longitudinal manner simultaneously evaluating disease progression or therapy outcomes. Such longitudinal and/or interventional studies could reveal whether oral diseases are associated with disruption of fungal-bacterial relationships. For instance, the incidence of candidiasis in non-oral mucosal compartments such as the vaginal tract is associated with antibiotic intake and it is therefore believed to be a consequence of disrupting the bacterial microbiome (Maraki et al., 2003; Xu et al., 2008). Indeed, evidence from animal models suggests that in the gut *Candida* interacts with the resident bacterial

microbiome potentially influencing host-microbiome homeostasis (Mason et al., 2012a,b). Using a rodent model exposed to a cephalosporin antibiotic, Mason et al. (2012a,b) demonstrated that an intact bacterial flora is essential to prevent *Candida* colonization of the lower gastrointestinal tract. In turn, *C. albicans* colonization of microbiome-perturbed mice promoted sustained gut dysbiosis, preventing the regrowth of lactobacilli, which are presumably associated with gastrointestinal health, while allowing establishment of *Enterococcus* spp. at higher levels than those present prior to antibiotic treatment. Moreover, *in vitro* evidence suggests that *C. albicans* virulence may be modulated by the bacterial co-colonizing flora. For example, *Pseusomonas aeruginosa*, known to coexist in the cystic fibrosis lungs with *C. albicans,* has been demonstrated to affect yeast to hyphal transition and also biofilm formation, potentially limiting *C. albicans* to a commensal state of growth (Morales et al., 2013). In contrast to non-oral mucosal sites, the oropharynx has been demonstrated to be more resistant to *Candida* overgrowth than the lower GI tract and vagina following antibiotics (Maraki et al., 2003; Kim et al., 2014). It is not clear, however, if this is due to lack of profound perturbation of the oral bacteriome following antibiotic intake, or perhaps because of less dependency between fungi and bacteria in the mouth. Longitudinal human evidence is thus required on possible changes in global bacterial and fungal profiles during development of oral candidiasis. Similarly, although recent *in vitro* and animal models have suggested a possible role for *Candida-Streptococcus mutans* synergism in the pathogenesis of caries (Falsetta et al., 2014), longitudinal human studies are needed to evaluate the bacterial and fungal microbiome components simultaneously during the progression of this disease.

GOING BACK TO THE BENCH TO DISSECT MECHANISMS MEDIATING RELATIONSHIPS IN MODEL SYSTEMS

Although co-occurrence and co-exclusion patterns inferred from microbial profiles may be used to generate hypotheses, it should be noted that these associations could simply indicate microorganisms with similar nutritional requirements or niche preferences and may not represent direct mutualism or antagonism. Ultimately, the consequences of fungal-bacterial interactions identified clinically require testing in relevant model systems. Our group has used several *in vitro* and animal models to explore the consequences of a fungal-bacterial interaction likely to occur in the human oral cavity.

The genus *Streptococcus* is highly abundant at oral sites, with Mitis group streptococci (MGS) being the most numerically dominant (Dewhirst et al., 2010; Diaz et al., 2012a). MGS, principally represented by *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus sanguinis*, and *Streptococcus mitis* colonize both teeth and oral mucosal surfaces (Frandsen et al., 1991; Diaz et al., 2006, 2012a). An overgrowth of *Candida* on mucosal surfaces is associated with the appearance of white detachable lesions commonly known as oral thrush (Lalla et al., 2013). Although no evidence from human longitudinal studies of oral candidiasis is available, several lines of evidence point to MGS as potential partners for *Candida albicans*, the most common *Candida* species associated with thrush. *S. oralis* and *C. albicans* are frequently co-isolated from the sputum of antibiotics-treated symptomatic cystic fibrosis patients (Maeda et al., 2011). Furthermore, *C. albicans* and *Streptococcus pneumoniae*, a non-oral MGS with a high degree of genetic and phenotypic relatedness to *S. oralis* (Johnston et al., 2010; Denapaite et al., 2012) have been implicated in pulmonary infections (Yokoyama et al., 2011). Recent evidence has also shown that introduction of *C. albicans* in the intestinal mucosa of antibiotics-treated mice leads to a preferential re-colonization by enterococci (Mason et al., 2012a) and streptococci (Filler, personal communication). Characterization of the cellular composition of thrush lesions in humans is not available, and therefore animal models have been used to investigate the pathophysiology of this condition. Using a murine model of oropharyngeal candidiasis, we showed that thrush-like lesions are formed by densely packed *Candida* cells surrounded by indigenous murine bacterial cocci (Dongari-Bagtzoglou et al., 2009). Furthermore, microscopic studies have revealed corn-cob-like structures formed by *Candida* and streptococci in the mouth of humans (Zijnge et al., 2010). *In vitro* studies have also shown that *Candida* and streptococci physically interact via specific adhesin-receptor mediated binding forming biofilm structures on abiotic surfaces (Silverman et al., 2010). Collectively this evidence supports the idea that *Candida* and streptococci may form a potentially mutualistic partnership.

To begin to study interactions between *C. albicans* and MGS members in models relevant to oral disease we developed an *in vitro* organotypic mucosal model that incorporates salivary flow and also developed an oral polymicrobial infection mouse model. Using these models we showed that when *C. albicans* is co-inoculated with *S. oralis* on mucosal surfaces, streptococcal mucosal biofilm formation is enhanced (Diaz et al., 2012b; Xu et al., 2013). The next logical question to investigate was the role of streptococci in the progression of oropharyngeal candidiasis.

Despite the fact that oral MGS have been traditionally considered avirulent commensals, recent experimental evidence is unraveling a more "sinister" role for these cocci as accessories to primary pathogens in mucosal infections. Using a mouse model of oral infection we recently provided evidence for the role of MGS as accessory pathogens in oropharyngeal candidiasis (Xu et al., 2013). Two MGS species were tested (*S. oralis* and *S. gordonii*) and neither showed virulence on their own, even when animals were immunocompromised and inoculated with a high number of organisms. However, when co-inoculated with *C. albicans*, *S. oralis* (but not *S. gordonii*) triggered increased frequency and severity of oral lesions, and greater weight loss. Oral co-inoculation with both organisms also triggered an exaggerated mucosal inflammatory response. The majority of the immune regulatory genes upregulated in co-infected animals belonged to the categories of chemotaxis response, neutrophilic response, cytokine activity, and phagocytosis. Interestingly, strong induction of multiple neutrophil-activating cytokines (IL-17C, CXCL1, MIP-2/CXCL2, TNF, IL1α, IL-1β) with concomitant increased neutrophilic infiltration was observed in co-infected animals. Because increased *S. oralis* mucosal colonization in the presence of *C. albicans* aggravated mucosal infection, it is conceivable that like many opportunistic pathogens, a critical mass of this species, reached only in the presence of *Candida*, is needed to induce

pathology. These results dispute the long held belief that the commensal bacterial flora protect the host against oral candidiasis (Liljemark and Gibbons, 1973).

In vitro biofilm models and rodents have also been recently used to study the interaction of *C. albicans* and *Streptococcus mutans* in the context of dental caries (Gregoire et al., 2011; Falsetta et al., 2014) As seen with MGS, *S. mutans* biofilm formation is enhanced in the presence of *C. albicans*. This interaction appears to be mediated by extracellular polysaccharides. Using a rat caries model, this group also demonstrated that co-inoculation of *C. albicans* and *S. mutans* increased the oral infection burden for both organisms and produced more severe caries lesions than in mono-infected animals (Falsetta et al., 2014).

Evidence on the potential synergy between *Candida* and streptococci from experimental models highlights the need to understand fungal bacterial relationships in the context of human disease. Although human evidence confirming the role of fungal-bacterial interactions in the progression of oropharyngeal candidiasis and caries are still required, it is clear that such fungal-bacterial relationships should be considered as potential preventive and therapeutic targets.

CONCLUSIONS

A microbial community has emergent properties, that is, community characteristics cannot be inferred by studying its components separately. In this respect the advent of "omics" approaches will facilitate understanding of microbial communities in humans, using systems biology approaches. We argue, however, that such "omics" methods need to be coupled with adequate *in vitro* and *in vivo* models allowing mechanistic proof for interactions and relationships observed *in vivo* (see **Figure 1**). Laboratory models can also be used to develop interventional strategies to interfere with fungal-bacterial interactions important in disease development. The development of more complex polymicrobial laboratory models, however, is desirable. Ultimately, proof that interference with fungal-bacterial interactions could serve as an interventional tool for disease needs to be obtained in the complex human ecosystem.

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Advancements toward a systems level understanding of the human oral microbiome

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Oral microbes represent one of the most well studied microbial communities owing to the fact that they are a fundamental part of human development influencing health and disease, an easily accessible human microbiome, a highly structured and remarkably resilient biofilm as well as a model of bacteria-bacteria and bacteria-host interactions. In the last 80 years since oral plaque was first characterized for its functionally stable physiological properties such as the highly repeatable rapid pH decrease upon carbohydrate addition and subsequent recovery phase, the fundamental approaches to study the oral microbiome have cycled back and forth between community level investigations and characterizing individual model isolates. Since that time, many individual species have been well characterized and the development of the early plaque community, which involves many cell–cell binding interactions, has been carefully described. With high throughput sequencing enabling the enormous diversity of the oral cavity to be realized, a number of new challenges to progress were revealed. The large number of uncultivated oral species, the high interpersonal variability of taxonomic carriage and the possibility of multiple pathways to dysbiosis pose as major hurdles to obtain a systems level understanding from the community to the gene level. It is now possible however to start connecting the insights gained from single species with community wide approaches. This review will discuss some of the recent insights into the oral microbiome at a fundamental level, existing knowledge gaps, as well as challenges that have surfaced and the approaches to address them.

Keywords: oral microbiome, metagenomics, metatranscriptomics, stable isotope probing, single cell genomics

INTRODUCTION

Oral biofilm communities constitute dynamic, multiple-species metabolic networks with a multitude of interconnected functions (**Figure 1**). The species types, abundance, and activities of microbes are a function of the environmental (physical, chemical, and biological) parameters, including the carbon and other nutrient resources available. Oral communities may exhibit large and rapid changes in composition and activity both temporally and spatially and are developmentally dynamic with the human host (Xu et al., 2014). These complex, non-equilibrium dynamics are consequences of several factors, including the temporal frequency of host and diet, the rapid response to changes in pH, bacteria-bacteria interactions and on a larger time frame, genetic mutations and horizontal gene transfer that confer new properties to strains. Understanding the combination of host and environmental factors that drive the overall balance of biofilm communities represents one of the grand challenges in microbial ecology. Unfortunately, most of what is known about biofilm function has been extrapolated from mono-species culture studies. This is largely because the methods available for biofilm analysis have lacked the sensitivity and/or resolution required to unravel the complexity of these mixed species biofilms. In fact, until recently, the approaches being used lacked sufficient ability to capture the behavior of even known species within a background of a mixed community.

Biofilm structure, function and microbial species composition are all areas that have been and continue to be addressed, with the goal of enabling an understanding of the species interactions that ultimately govern biofilm communities. This is information that is critical to enhancing beneficial biofilms as well as combatting harmful ones. Recent development of advanced methods to be discussed here, in part has allowed us to begin to advance the study of multiple species biofilms, and begin to ask "who is there?", "who is active?" and "what processes are active?" In addition to having significant health related relevance, dental plaque is one of the best-described microbial communities (Marsh and Bradshaw, 1995; Kolenbrander, 2000; Kolenbrander et al., 2007; Kuramitsu et al., 2007; He and Shi, 2009; Palmer, 2009, 2014; Kuboniwa et al., 2012) and the breadth of fundamental research cannot be covered in this review. It is clear that oral plaque presents a very convenient system for demonstrating the species interactions and functional analysis of multi-species communities in general (Foster et al., 2003; Kolenbrander et al., 2007). For example, an oral biofilm on enamel for example is a much better characterized system than a natural community in marine or soil influencing other mineral surfaces in terms of: (1) the

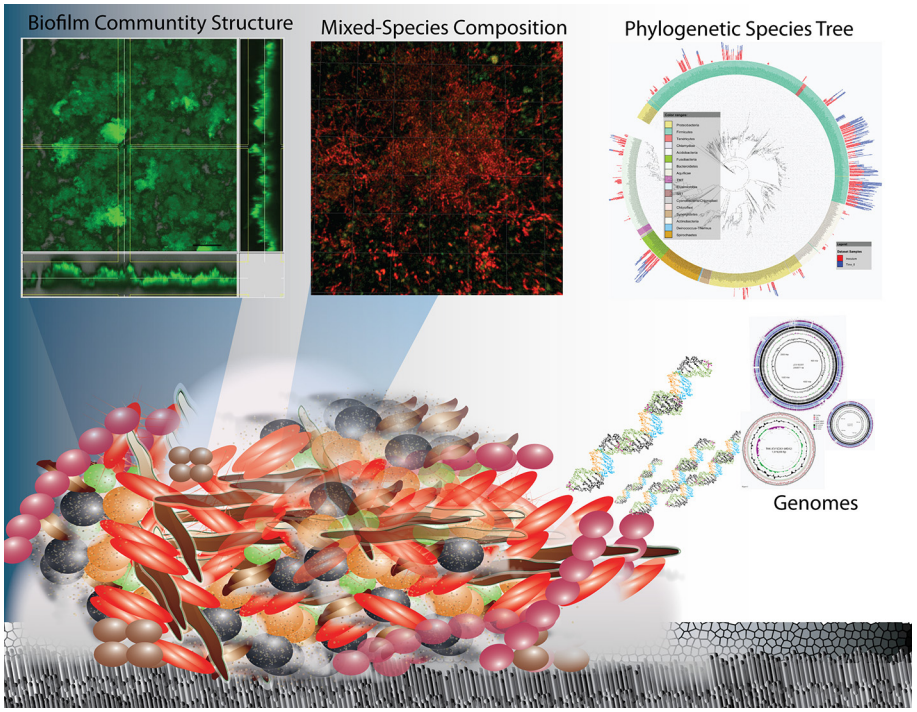

FIGURE 1 | Overview schematic representation of the structure and complexity of oral biofilms on enamel surfaces in terms of physical heterogeneity and species diversity. Research on oral biofilms has encompassed biofilm structure and function at the macro-scale to the species diversity through 16 rRNA sequencing and their genetic potential (whole genome sequencing and metagenomics) and is now progressing to the functional activity at the molecular level (metatranscriptomics).

biochemical reactions leading to dissolution; (2) the identity of species present; (3) the number of genomes available; and (4) the ease of conducting biological experiments.

Processes in the oral cavity related to human disease are predominately driven by reactions occurring within complex microbial biofilm communities in contrast to a few diseases that are the result of a single species. Most studies have been predominately conducted on model systems containing single species which greatly advance our understanding. Bacteria within oral biofilms have been described as "good" (i.e., protective) common components of healthy microbiomes (He et al., 2009, 2010, 2014), and also "bad" as in the case of *Streptococcus mutans* with many falling between these designations. Dental caries is generally now considered a polymicrobial disease that arises when there is dysbiosis and the communities shift metabolism in harmful ways. Under such conditions, the antagonistic biofilms often display enhanced resistance to antibiotics, and as such become the etiological agents of many serious human diseases, including cystic fibrosis, periodontitis, otitis media (inner ear infections), and bacterial endocarditis, to name a few. Tooth decay (dental caries), which is the loss of enamel that is composed of the mineral hydroxyapatite (HAP), is one such polymicrobial mediated process that is thought to be caused by a shift in biofilm populations from "good" to "bad." The shift to more acidogenic (acid generating) and aciduric (acid tolerant) species is thought to drive demineralization of the HAP crystals through an increase of acidic end products of fermentation. The physical and ecological model (Marsh, 1994) of this process is described briefly in **Figure 2**. This reproducible pH response observed after a sugar rinse has been acknowledged for nearly 80 years and is defined as the Stephan Curve (Stephan and Miller, 1943). This rapid cycling of pH has been well documented both *in vivo* and within *in vitro* collected plaque. Early research shows that this cycling is present within individual species to varying degrees (Kleinberg, 2002). Having defined a physiological and ecological model of supragingival plaque in relation to enamel demineralization has driven testing of these hypotheses. Despite many years of research however, demineralization of enamel remains enigmatic in terms of governing critical activities that occur within the diverse community.

The study of individual oral species and now entire communities in the oral cavity has benefited greatly from recent approaches based in genomics and bioinformatics. Many hurdles still remain however and overcoming these will rely on technological and experimental advances that disentangle the immense complexity of a multispecies biofilm. This review mainly covers the supragingival bacteria involved with dental caries and the resultant demineralization of enamel which, due to the reasons mentioned above, is most well described. Many of the challenges are also applicable to periodontal disease and other diseases related to the human microbiome. Understanding the role of bacteria in caries is inherently and extensively interdisciplinary, involving microbiology, molecular biology, genomics, proteomics, and metagenomics, as well as microbial physiology

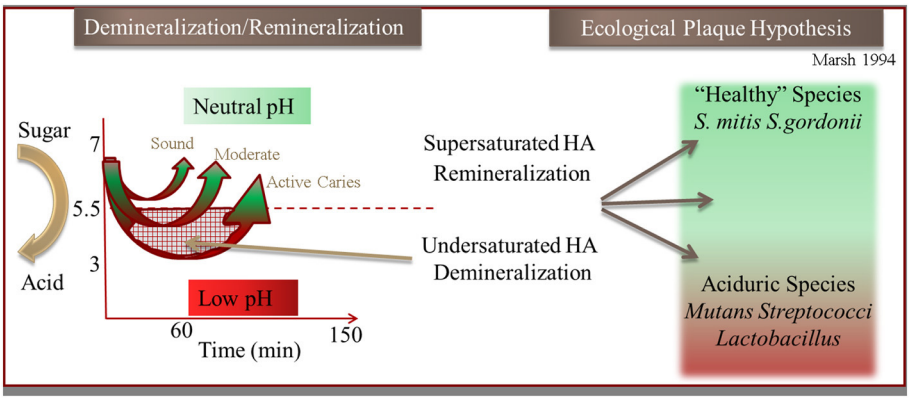

FIGURE 2 | Illustration of the hydroxyapaptite (HA) demineralization and remineralization process that occurs through the pH cycling ("Stephan Curve") in relation to the Ecological Plaque Hypothesis (adapted from Marsh, 1994).

and biochemistry. This work has involved the development and use of methods for the study of the structure and function of these complex biofilms on one hand, and for the physiology and genomic characterization of individual community members on the other. Both top-down and bottom up approaches are clearly needed to more fully understand the abiotic and biotic factors that contribute to the fundamentally important process of tooth decay.

CURRENT KNOWLEDGE OF BACTERIAL METABOLIC PROCESSES LEADING TO DEMINERALIZATION

It is clear that demineralization exhibits a strong correlation with biofilm induced pH reduction, but in reality, a detailed understanding of the metabolic processes responsible for pH cycling (**Figure 2**) is still lacking. Most of what is known about bacterially mediated demineralization in the oral cavity comes from the research on a few model organisms. It is commonly reported that lactic acid is the organic acid produced by the community and that lactic acid accumulation at the biofilm-enamel interface is responsible for the large pH shift leading to enamel demineralization. This is a very simplified view since only a few studies have addressed which organic acids are produced during the metabolism of the common sugars (glucose and/or sucrose) under conditions found in a biofilm. None have been able to quantify the absolute concentration of multiple organic acids in live biofilms, especially non-invasively in a temporally or spatially-resolved manner. The metabolite profiles of a supragingival oral community *in vivo* under "diseased" (low pH) and "healthy" (neutral pH) environmental conditions are therefore unknown and represent a challenge. Furthermore, we know that there is a large diversity of species with varying metabolic capacities encoded in their genomes that could modulate pH.

A number of studies support the contribution of other organic acids toward demineralization even in the model species *S. mutans*. It has been shown early on that *S. mutans* given excess glucose exclusively produced lactic acid under aerobic conditions (Yamada and Carlsson, 1975; Yamada et al., 1985). Under anaerobic conditions lactate, formate, acetate, and ethanol are formed, with lactate making up less than 50% of the total acids produced. Since biofilms are typically stratified with respect to oxygen concentration, these findings suggest that the outermost biofilm layer (where oxygen concentrations are higher) would possibly present high levels of lactic acid metabolites while internal regions of the biofilm that are closer to the enamel surface and exposed to reduced oxygen tensions would more likely give rise to other acids in combination with lactate. Furthermore, overall higher yields of acid occurred when cells were grown anaerobically rather than aerobically with acetate and formate being the dominant acids present (Yamada and Carlsson, 1975; Yamada et al., 1985). An NMR study of perchlorate extracts of oral biofilms demonstrated the utility of ^{1}H-NMR for the simultaneous analysis of ∼30 chemical constituents, including lactate and other corrosive organic acids (Silwood et al., 1999). Their results indicated that lactate, acetate, pyruvate, propionate, formate, and *n*-butyrate are produced in abundance, with acetate and formate being produced at higher concentrations than lactate. Considering the larger dissociation constants of these organic acids, they concluded that formic and pyruvic acids contribute significantly to the decreased pH values. The authors of this NMR study stated that previous studies of carious lesions have failed to detect and therefore consider the contribution of formic and pyruvic acids to demineralization of tooth surfaces (Silwood et al., 1999). While supporting the hypothesis that acids other than lactic acid are likely to be important players in cavity formation, this approach could not differentiate between acids produced extracellularly where they can interact with surfaces from those retained within the cytoplasm of the cells. This information is essential for determining which acids are most important in demineralization processes.

Recently, our laboratory has conducted experiments to gain insight into the spatial and temporal dynamics of metabolism. Novel NMR based metabolite measurements of sugar metabolism were performed in a non-invasive, real-time manner on active oral biofilm communities including, a *S. mutans* biofilm model with temporal and spatial resolution (McLean et al., 2008) as well as plaque samples derived from healthy children (McLean et al., 2012). These types of non-invasive measurement approaches combined with other destructive measures are essential to gain

an understanding of the metabolic processes that are responsible for cavity formation. Further efforts to obtain the full suite of untargeted metabolite profiles temporally and spatially will help reveal the pathways and processes active at low pH and also during the rapid pH recovery phase which is important for a "healthy" functioning community.

DYNAMIC METABOLIC AND POPULATION SHIFTS LEADING TO SUSTAINED DEMINERALIZATION AND THE ECOLOGICAL PLAQUE HYPOTHESIS

Possibly the best example in the development of overarching hypotheses governing the shift in a microbial population from a healthy (balanced neutral pH) to a "diseased" state (extended periods of low pH) comes from extensive studies of the oral cavity, beginning with the specific plaque hypothesis developed by Loesche (1976, 1979). This hypothesis implicated that only a few species were responsible for caries. *Streptococcus mutans,* identified very early as a major player in the onset of caries, has been the primary subject of most caries studies. With further investigations it became evident that many other bacteria termed "cariogenic or oral pathogens" such as *Streptococcus sobrinus* and *Lactobacillus* spp. exhibit low pH metabolic behavior similar to that of *S. mutans.* The non-specific plaque hypothesis developed years later (Theilade, 1986), implicated the microbial community as a whole as being responsible for caries. Marsh and colleagues later developed the "ecological plaque hypothesis" (Marsh, 1994) stating in essence that oral caries and periodontal diseases arise as a result of environmental perturbations that lead to a shift in the balance of the resident microflora. Key features of this hypothesis are that (a) the selection of "pathogenic" bacteria is directly coupled to changes in the environment; and (b) diseases need not have a specific etiology, any species with relevant traits can contribute to the disease process. Thus, the significance to disease of newly discovered species can be predicted on the basis of their physiological characteristics. For caries, the environmental perturbation arises from the intermittent introduction of dietary sugars during feeding leading to cycling of pH (Stephan, 1945). If the pH remains low for sustained periods, a shift in the bacterial populations to more aciduric organism is thought to occur (Marsh and Bradshaw, 1997; Kleinberg, 2002). This was documented to some extent through laboratory culturing studies in chemostats using defined mixed communities. It is envisioned that under disease conditions *in vivo*, the low pH would drive the dissolution of calcium and phosphate in the hydroxyapatite crystalline structure of the tooth and ultimately lead to cavitation. Cariogenic bacteria are then thought to thrive under these acidic conditions, increasing in proportion and worsening the diseased state. This has proven extremely difficult to validate *in vivo*.

From numerous 16S rRNA gene profiling and clinical investigations it is clear however that certain acidogenic and aciduric species such as *S. mutans* and *Lactobacillus* spp. are highly correlated with active caries. There are however, many other species that are likely to be relevant as evidenced by the diverse microbial populations present in caries in young children that include *Actinomyces*, *Fusobacterium*, *Porphyromonas*, *Selenomonas*, *Bacteriodetes*, and *Haemophilus* (Corby et al., 2005). Through Denaturing Gradient Gel Electrophoresis (DGGE) profiling at least 30 species were found in active caries sites including *Gemella*, *Kingella*, *Leptotrichia*, *Streptococcus,* and *Veillonella* (Li et al., 2007). More recent high throughput sequencing studies are further supporting the diversity of bacteria that may be involved with caries through association studies (Tanner et al., 2011; Gross et al., 2012; McLean et al., 2012). Clearly, substantial evidence is available to support the hypothesis that cariogenic activity of an oral biofilm could be impacted by multiple members of the community.

Overall, the contributions of each species to the healthy and diseased state still remain largely unknown. For example, while the properties of many of the identified cariogenic bacteria such as *S. mutans* are known in pure culture (Loesche, 1986) as well as differences in strains (De Soet et al., 2000), knowledge of their physiological and metabolic behaviors in a diverse multi-species dental biofilm is scarce. It is clear from investigation in mono- and dual-species model systems that *in-vivo* characteristics can be greatly impacted by other members of the community. Commensal bacteria in dental plaque biofilms may impact the processes of acid tolerant species (cariogenic pathogens) (Takahashi and Nyvad, 2008). This can be accomplished indirectly by modulating the activity of cariogenic species as well as impact the pH drop by the production of alkaline byproducts such as ammonia from arginine (Takahashi, 2003). It has also been shown that it is possible to impact virulence in a more direct manner such as the example of *Streptococcus sanguinis* inhibiting *S. mutans* growth through the production of H_2O_2(Kreth et al., 2005). Linking the functions observed in mono-species cultures to their activities within a population is a challenge that is now technically possible with advances in sequencing and bioinformatics there are still fundamental gaps yet to be addressed which is the subject of the following sections.

CURRENT NEEDS TO ADDRESS THE POLYMICROBIAL PROBLEM

The oral microbial system is an ideal system to study the driving forces influencing homeostasis and dybiosis. In order to gain knowledge to support or refute the hypothesized mechanisms behind the ecological plaque hypothesis for example, one needs to know all the players and be able to track their behavior. Since most microbes remain uncultivated, little is known about these species except for their 16S rDNA sequence. Furthermore, although many model bacteria that are known to be one of the species associated with a particular condition *in vivo,* these bacteria have only typically been characterized in the laboratory as pure cultures. Validating whether these observed laboratory characteristics are actually maintained *in vivo* in the presence of a mixed microbial community is a challenge and this is where the techniques have been lacking. Specifically, major outstanding questions in the study of mixed microbial communities include:

1. What is the behavior (metabolism) and gene expression of known model species when they are active within a mixed species microbial community?
2. What is the role of currently uncultivated organisms and their contribution to the overall function of the community?

3. How does a stable microbial community shift to an undesirable state? (which species, metabolic pathways, and genes are involved?) Can this species shift be predicted?

But why has it been so difficult to move from the study of single-species to the study of natural, mixed-species communities? In addition to the issue of unknown (uncultivated) taxa, there are the simple physical problems related to the size of microbes and microbial communities. Biofilm communities are often only a few to 100 μm in height and are composed of individual members at the micrometer size range that are in close contact with one another. Thus, the challenges in sensitivity and resolution are great, and the development of appropriate tools for the study of biofilms in a minimally invasive manner has moved slowly. Furthermore, while the questions that need to be answered are easy to phrase, the pathway to answering them is not easy. There are a few major scientific challenges that must be met before it will be possible to move confidently from the study of single to multi-species biofilm studies which are briefly stated here and covered in more detail in the following sections:

1. **The need for microbial genomes as references for community based studies.** In order to more fully grasp microbial taxonomic and gene diversity as well as to provide a means to assign metagenomic (DNA) and metatranscriptomic (mRNA) reads to a given species, reference genomes are critical. The rapid growth of metagenomic and metatranscriptomic sequencing has revealed a need for more reference genomes in order to be able to assign reads to genes and/or bacterial species and therefore identify possible taxon-related function within a given biofilm. As reference genomes continue to grow as they have in the last 5–10 years with advanced approaches, the complete inventory of genes and their linkage with a particular species/strain within a given microbiome may ultimately be possible to determine.
2. **The need for tools to address the large fraction of uncultivated species.** While many new microbes can be identified through 16S rDNA gene based diversity analyses, further research on many of these microorganisms is hampered by an inability to uncover their culture requirements. In the absence of a culture, physiological inferences can be made through the genome of an uncultivated species. Further advances on culture methods as well as methods such as single cell genomics capture a representative genome are needed to make substantial headway in this area given the vast amount of known uncultivated diversity present.
3. **The need for mixed-culture laboratory model biofilm communities.** Understanding individual species function, metabolism and gene expression profiles in a biofilm is a necessary step in the study of polymicrobial processes. Importantly, multi-species models that are reproducible and stable will allow for hypothesis testing and functional validation of observations made *in vivo*. Models that contain uncultivated phylotypes (those species only known by their 16S rRNA gene sequence) are indeed more comprehensive and valuable. Once again, it should be noted that the need for cultivated strains and reference genomes from these model systems is key in order to more fully understand the dynamics within laboratory models and the role uncultivated species play.
4. **The need for methods that enable species level resolution of function within biofilm communities.** Overall, it is likely that model species modify their behavior in the presence of other community members. How (and how much) the behavior of a given microbe changes in the presence of the other members of a complex biofilm community is not presently known. This knowledge gap is a result of the inability to track the behavior of many individual species. There are few approaches available to understand the behavior of cultured isolated organisms when they are put back in a complex community. A number of exciting new approaches predominately based on deep sequencing technologies have allowed us to ask questions about "who" is there and "what" are they doing within a diverse community. The ability to monitor biological functions and link this observed activity to the identity of the species responsible is an area that is just starting to become available through techniques such as nucleic acid base Stable Isotope Probing (SIP) and more recently metatranscriptomics.

CHALLENGES TO PROGRESS ON COMMUNITY LEVEL MICROBIOLOGY: THE UNCULTIVATED AND UNKNOWN MAJORITY

With the increasing advances in DNA sequencing technologies, combined with the reduction of sequencing costs, access to the microbial world has greatly expanded to reveal an unprecedented microbial diversity across nearly every environment. Pioneering large scale environmental shotgun sequencing with the Sargasso Sea pilot study (Venter et al., 2004) and the larger Global Ocean Sampling (GOS) expedition (Rusch et al., 2007), focused on marine surface waters. Recently the Human Microbiome Project (HMP) (Turnbaugh et al., 2007; Human Microbiome Jumpstart Reference Strains et al., 2010; Consortium, 2012a,b) efforts have revealed remarkable microbial diversity within and on the human body. Initial metagenomic HMP efforts also made it painfully obvious that there were major gaps in terms of the number of available reference genomes. Reference genomes for the oral cavity are critical for capturing species diversity, gene content (metagenomics), gene expression profiles (metatranscriptomics), expressed proteins (metaproteomics) and small molecules (meta-metabolomics). Without annotated genes from reference genomes to assign reads and proteins to, there is no taxonomic information obtained. These are referred to as "orphan reads" and currently a large proportion of sequences from microbial community studies fall within that category.

Depending on the environment being studied, only a small percentage of the microbes visible under the microscope are likely to be easily domesticated in the lab. It has been dubbed the "great uncultivated majority" (Whitman et al., 1998), "dark matter of life," and "microbial dark matter," which includes microbes and even entire divisions of bacterial phyla that have evaded cultivation. Many candidate phyla for example have yet to have a single representative whole genome sequence. Since the realization of this missing diversity in culture attempts (Staley and Konopka, 1985), estimates now indicate only 1–10% of known bacterial species (Rappe and Giovannoni, 2003) are thought to

be currently cultivated. Fortunately, great progress is being made for some bacterial communities; for example, roughly half of bacterial species within the human oral cavity have been cultivated (Dewhirst et al., 2010). Since this vast majority of bacteria in the environment as well as those associated with the human microbiome have eluded standard culturing approaches, their physiology and their gene content are unknown. In the absence of culture-based physiological analyses, the functional roles of these uncultivated species remain mysterious despite their apparent correlations with important processes. These problems have become the limiting step in studying ecology-based community activities. In the "best of all worlds," reference genomes would be obtained, then used to link function to phylotype for uncultivated microbes. Eventually the information could be used to guide successful cultivation of these abundant or rare uncharacterized microbes.

CURRENT KNOWLEDGE ON THE SPECIES IN ORAL BIOFILMS

Through isolation/culture and culture-independent methods, the species present as attached cells in biofilms within the oral cavity have been estimated to comprise a diverse community of more than 700 phylotypes inclusive of bacterial and archaeal domains, although less than 100 phylotypes are found in a typical individual (Dewhirst et al., 2008). The most comprehensive database for 16S and genomic data for the oral cavity is the Human Oral Microbiome Database (HOMD; www.HOMD.org) (Dewhirst et al., 2008). To date, from this curated database, there are 691 total taxa (98.5% similarity in 16S rRNA), 344 named taxa, 112 cultivated but unnamed taxa, and 232 uncultivated taxa (phylotypes). The oral cavity is one of the most well covered microbiomes to date with a total of 392 taxa that have at least one reference genome with the total genomes across the oral cavity approaching 1500 (Human Microbiome Jumpstart Reference Strains et al., 2010). The HMP initiative (http://commonfund.nih.gov/hmp/index) leveraged the recent advances in genomics to allow for a far more comprehensive survey of the microbial species and their associated genes present in the oral cavity both through the generation of hundreds of new reference genomes from cultivated oral strains to deep metagenomic sequencing of human subjects(Consortium, 2012b). Many databases cover the extensive data produced from nine distinct sites in the oral cavity from the roughly 230 healthy western volunteers to serve as a baseline for a "healthy" human microbiome. The 16S rRNA and shotgun datasets as well as assemblies are available on a number of public databases which house comparative metagenomic tools. These include the HMP Data Analysis and Coordination Center (http://www.hmpdacc.org/), the Integrated Microbial Genomes Human Microbiome Project (https://img.jgi.doe.gov/cgi-bin/imgm_hmp/main.cgi), the JCVI METAREP (http://www.jcvi.org/hmp-metarep/) to name a few.

SINGLE CELL GENOMIC SEQUENCING: CAPTURING REFERENCE GENOMES OF RARE AND UNCULTIVATED MICROBES

Given the number and diversity of taxa found within biofilms, it becomes important to know the members of these communities (and their activities) at a higher level of resolution than allowed by the most commonly used detection and identification methodologies. Determining what species and what genes are present are some of the initial strategies. Culture-independent surveys using the 16S rRNA gene as a marker are currently the most widely used approach however genetic strain differences reflecting potential different metabolisms and phenotypes are often difficult to resolve due to this gene being highly conserved amongst many bacterial strains. Quantitative PCR and direct culturing are focused on either a handful of predetermined species or what can be readily cultivated which we already know to be only a minor portion of the species in any given environment. Metagenomic is limited with regard to accurately predicting taxonomic affiliation at the species or strain level from highly diverse and complex datasets with short read sequencing technology. In addition, whole genome comparative genomic studies on the evolution and transmission of a low abundance organism of interest that resides in a microbial community requires substantial amounts of DNA or a cultured strain from the community which often cannot be obtained.

Sequencing from single bacterial cells, first achieved in 2005 (Raghunathan et al., 2005). This breakthrough was enabled by the development of the MDA reaction (Dean et al., 2001, 2002), which can amplify a single genome copy more than a billion fold enabling sequencing of DNA from very low (femtogram) levels (about the amount of DNA in a single bacterial cell). Bacteria that have not been cultivated by conventional culturing techniques are currently the central target of single-cell genomics (Lasken et al., 2005; Raghunathan et al., 2005; Hutchison and Venter, 2006; Ishoey et al., 2008; Lasken, 2012) The recent advancements in DNA sequencing of single bacterial cells has accelerated the study of uncultivated microbes (Lasken, 2012), providing genomic assemblies for species previously known only from 16S rRNA clone libraries and metagenomic data (Marcy et al., 2007; Podar et al., 2007; Binga et al., 2008; Eloe et al., 2011; Youssef et al., 2011; Dupont et al., 2012; McLean et al., 2013a; Nurk et al., 2013b; Rinke et al., 2013). Using these approaches, the so-called "dark matter of life" which represents uncultivated microbes and even entire divisions of bacterial phyla (candidate divisions and candidate phyla) are slowly being revealed with assembled genomes. The single cell sequencing approach has had a number of notable successes allowing full and partial recovery of genomes from many elusive Candidate bacterial groups at the phylum level including but not limited to; oral TM7 (Marcy et al., 2007), oral SR1(Campbell et al., 2013), and TM6 (McLean et al., 2013a) from a drinking water distribution system.

A new high throughput and highly automated platform was recently reported for sequencing and assembly of single cell genomes of bacteria (McLean et al., 2013a) and viruses (Allen et al., 2011) (**Figure 3**). The workflow consists of: (1) delivery of single bacterial cells (single cell genomics) or small pools of cells (mini-metagenomics) (McLean et al., 2013a) into 384 well microtiter plates by Fluorescence Activated Cell Sorting (FACS); (2) use of a robotic platform to perform 384 well automated cell lysis and amplification of DNA by the (MDA) method (Dean et al., 2001, 2002; Hosono et al., 2003) to create libraries of genomic DNA derived from single cells; (3) PCR and cycle sequencing of 16S rRNA genes to profile the taxonomy

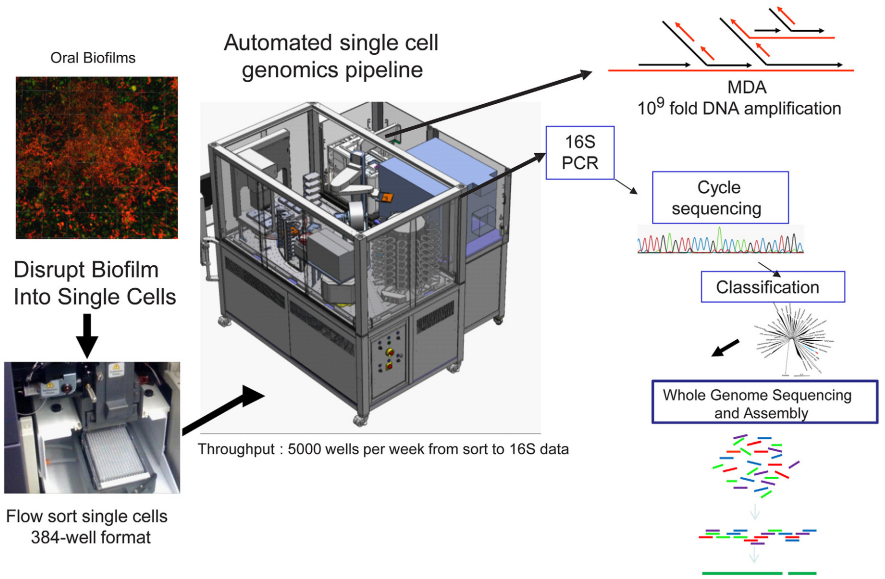

FIGURE 3 | Workflow of the high-throughput Single Cell Genomics Platform at the J. Craig Venter Institute. Custom integrated Agilent Technologies BioCel 1200 liquid handling automated platform for high throughput single cell genomics. The BioCel platform allows processing of more than 5000 single cells per week through a multi-stage protocol that includes multiple displacement amplification (MDA) of DNA, MDA dilution and 16S rDNA PCR. After classification of 16S rRNA gene sequences, candidate genomes can be deeply sequenced followed by assembly and annotation of generated contigs. Modified from McLean et al. (2013b).

and diversity of the libraries; (4) selection of candidate amplified genomes for whole genome sequencing; and (5) sequencing and assembly of selected genomes using assembly tools designed specifically for MDA amplified single cells (Chitsaz et al., 2011; Bankevich et al., 2012). This system was applied to diverse and difficult sample types such as environmental biofilms (McLean et al., 2013a,b; Nurk et al., 2013a) which enabled the recovery of oral pathogens from a hospital sink biofilm (McLean et al., 2013b). This recent work represented only the third genome of the globally important pathogen *P. gingivalis* at the time (McLean et al., 2013b). The validation of this technique marks a new opportunity to capture pathogen genomes from environmental samples which may enable pathogen transmission between the environment and host to be better understood. Single cell genomic techniques are rapidly expanding the reference genomes available for oral and other body sites from the human microbiome through such high throughput platforms (Lasken, 2012). In particular many novel oral and gut bacterial genomes of varying finished quality will soon become publically available as part of the HMP effort. Many of these were chosen from the "100 most wanted" list of bacteria (Fodor et al., 2012) that reside in the human body but represent phylogenetic branches that do not have representative genomes. Recently, several uncultivated and difficult to cultivate oral bacteria such as members of the Tanerella genus [67], the Deltaproteobacteria [68] have now been sequenced with this approach. There are some 30 or more recognized candidate phyla still without a single representative cultivated member. Overall the impact of single cell genomics is immense as genomes from species that have so far eluded standard cultivation approaches are being captured. Not only are these providing interesting insights into novel metabolisms(McLean et al., 2013a; Rinke et al., 2013) but they are also being used as reference genomes to recruit DNA and RNA reads from other global sequencing studies(Rinke et al., 2013), ultimately providing a more comprehensive community level understanding of microbiomes.

LINKING FUNCTION TO PHLYOGENY

As discussed earlier, determining which microbes are responsible for metabolizing substrates in a mixed microbial community is one of the biggest challenges in microbial ecology. SIP (Boschker et al., 1998; Radajewski et al., 2000) methods offer great potential to identify the cultivated and uncultivated microorganisms that metabolize and assimilate specific substrates in lab and field samples, and to identify metabolic networks that define functional microbial communities. Given what little is known about the metabolism of uncultivated bacteria and even metabolisms of known bacteria in the context of a diverse species background, the application of SIP to dental plaque holds considerable promise for meeting this challenge. Recent efforts in our laboratory have combined nucleic acid based SIP with the previously mentioned *in situ* non-invasive Magnetic Resonance Spectroscopy (MRS) to link real-time organic acid production measurements and the specific bacterial species active in oral biofilms (McLean et al., 2012) (**Figure 4**). The experimental procedure involved incubating plaque samples from healthy juvenile human subjects with isotopically labeled carbon sources (^{13}C-glucose or ^{13}C-lactate) in a defined minimal medium under various pH and buffering conditions. The temporal metabolite profiles of these live samples were monitored by inserting the biofilms into the NMR and performing spectroscopy with ^{1}H MRS. The study was based

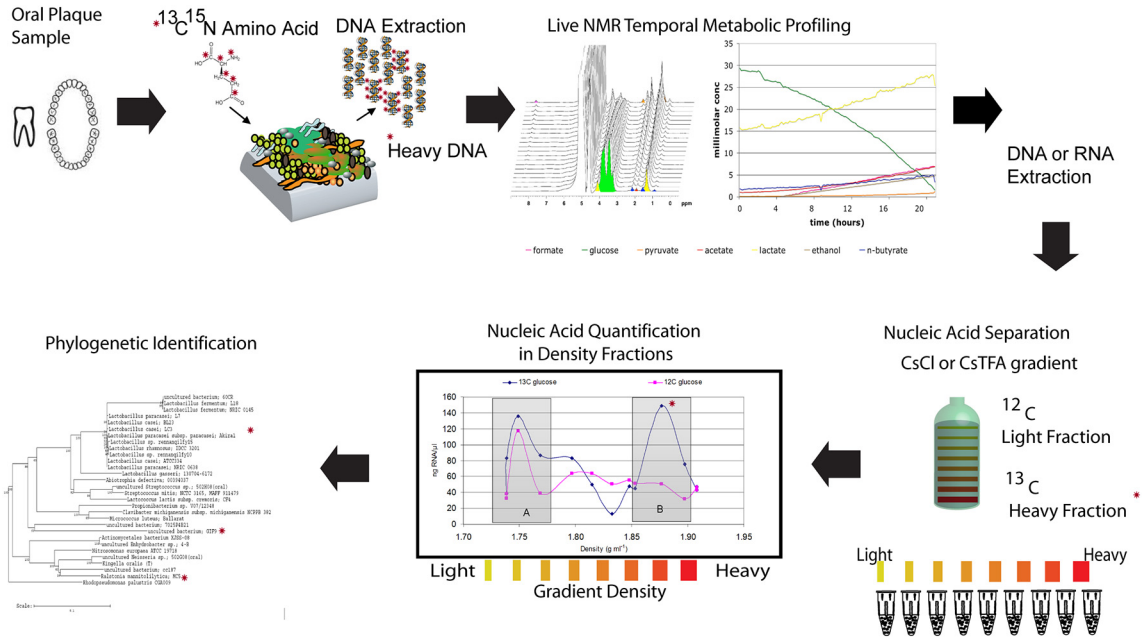

FIGURE 4 | Illustration of a NMR-SIP experimental procedure. Combining nucleic acid based Stable Isotope Probing (SIP) with *in situ* non-invasive Magnetic Resonance Spectroscopy (MRS) to link real-time organic acid production measurements and the specific bacterial species active in oral biofilms. The experimental procedure involves incubating plaque samples from healthy juvenile human subjects with isotopically labeled carbon sources (^{13}C-glucose or ^{13}C-lactate) in a defined minimal medium under various pH and buffering conditions. The temporal metabolite profiles of these live samples can then be monitored by inserting the biofilms into the NMR and performing spectroscopy with ^{1}H MRS followed by nucleic acid isolation and SIP to determine the active species (modified from McLean et al., 2012). The * indicates a heavy isotope labeled carbon or nitrogen that can be tracked in each panel.

on the working hypothesis that a low pH environment simulates the time at which the dissolution rate is highest and only those bacteria that can tolerate and continue to metabolize ^{13}C-glucose (and byproducts) will be detected in the heavy labeled isotope fractions. Using this novel application, we demonstrated that this approach allows reconstruction the community interactions by identifying potential acid active species (including uncultivated species) under a set of conditions that were relevant to the enamel (hydroxyapatite) dissolution (McLean et al., 2012). For example, our initial findings through the use of SIP confirmed that species other than the model species of mutans streptococci are metabolizing at low pH. Specifically, Lactobacilli (which are known cariogenic species) are highly active at pH 5.5 and also pH 4.5 within intact plaque. Additionally through the use of ^{13}C-labeled lactate, SIP gave some indication of the diversity of species able to metabolize lactate and byproducts. In the future, addressing these types of outstanding questions with the use of advanced methods to link phylogeny with function will enable assigning key functions to both known and uncultivated species thereby building the overall knowledge base.

ADVANCED BIOFILM MODELS: ENABLING CONNECTION BETWEEN SINGLE AND MIXED SPECIES APPROACHES

Most model systems for biofilms have utilized single-species systems, with the goal of understanding the various processes that occur during the "life cycle" of a biofilm formed in the laboratory. Model systems can drive technological advances since they provide a test bed for new technologies and approaches. They have proven to be valuable for the elucidation of the fundamental aspects of oral microbial biofilm formation. In general, growth models attempt to mimic *in situ* conditions as much as possible and to control input and environmental parameters so that cell-cell interactions can be understood. While some may question the relevance of this approach for understanding *in situ* biofilms, it is a necessary step between studying individual members and directly sampling and interpreting the highly complex, uncontrolled environment that the human oral microbiome represents.

There are considerable difficulties inherent in the development of a multi-species biofilm model system. A range of approaches and microbial communities of varying complexity has been utilized, with different uses, strengths, and limitations. Undoubtedly, each is a compromise between the actual microbiome conditions and the simplification and controllability necessary to gain meaningful, useful results in the laboratory. The model system thus tend to be limited in the range of uses, with potentially high variability, and difficult in terms of the interpretation of results (Sissons, 1997). The further development of existing model systems and the development of new complex multi-species systems that are particularly well suited to address fundamental questions of biofilm community structure and function are truly needed. With such systems, we can begin to address some of the issues specific to the study of biofilms, including: (1) temporal and spatial heterogeneity in environmental parameters; (2) spatial heterogeneity in growth rates; (3) small sample sizes; and

importantly, (4) fast dynamic temporal changes in metabolites and gene expression.

The challenge of establishing oral biofilm models that attempt to approach the complexity of the species seen *in vivo* is an area that has seen significant progress. These range from using defined mixtures of 10 or more species (Bradshaw et al., 1989, 1994, 1996) as well as using plaque or saliva inoculum and various media formulations including real and simulated saliva (Palmer et al., 2006; Kolenbrander et al., 2010). Recently, efforts to develop a stable, highly diverse mixed microbial *in vitro* biofilm model of the oral cavity was achieved through iterative manipulation of media components and monitoring the species diversity compared to a pool of saliva from healthy subjects (Tian et al., 2010). Edlund et al. (2013) describe how this multi-species model system was developed and investigated. The general outline of the easily employed model is shown in **Figure 5**. We discovered that there was remarkable reproducibility of species occurrence and even abundance between biofilm in each well of a 24 well plate, between batches and even between laboratories with independent media batches. Although surprising, it explained the highly repeatable pH profiles after carbohydrate addition nearly identical to the Stephan pH profiles (Stephan and Miller, 1943) (**Figure 1**) seen from early studies in the 1940s and documented in nearly every *in vivo* and *in vitro* plaque sample to date. In light of the recent HMP studies which documented a high taxonomic variability between individuals but a highly similar functional genomic content (Consortium, 2012b), this stable physiology is likely a consequence of this encoded metabolism and is probably specific to the conditions in each body site. The use of a laboratory model system with functional and species reproducibility maintaining a highly complex bacterial diversity that also supports the growth of otherwise uncultivated species is desirable as it can be manipulated and studied over a longer period of time in a controlled environment. A validated system that fulfills these criteria and that can be readily used for example to target changes in taxa, regulation of metabolic pathways and signaling molecules by using next generation sequencing (NGS) and "omics" methodologies (SIP, metatranscriptomics). Specifically such a model system will help facilitate experimental approaches that seek answers to questions related to the roles each bacteria plays in the overall structure and function of the human oral microbiome.

CAPTURING GENE EXPRESSION OF ENTIRE COMMUNITIES: METATRANSCRIPTOMICS

NGS technologies have provided a new way to assess the gene expression (transcription activity) of bacteria, predominately referred to as RNA-seq (Mader et al., 2011; Pinto et al., 2011). The strength of this methodology relies on the very large number of sequence reads generated with NGS platforms. This enormous quantity of reads generated; now allow the number of expressed transcripts (mRNA) to be determined by mapping reads to reference genomes or assembled *de novo*. Uniquely, RNA-seq also permits the quantification of novel transcripts such as small RNAs within intergenic regions that might not have been previously predicted and targeted by microarrays or qRT-PCR primers. Furthermore, the decreasing costs associated with RNASeq in comparison to conventional DNA-microarray hybridization techniques, justifies the use of this approach. Until recently however, techniques used to characterize gene expression in more complex natural microbial communities (Shi et al., 2009) have been challenged by the overwhelming genetic diversity and metabolic complexity of these consortia (Shi et al., 2009; Moran et al., 2013). The working hypothesis for metatranscriptomics applied to microbial communities is that transcripts associated with the active genes responding to each stage of the described interactions will be more highly abundant. Specifically, metatranscriptomic analyses can be applied to communities in a number of defined interactions to delineate the expressed genes thereby moving closer to the real functions of the bacteria under specific conditions. Species-specific genes in oral bacteria that are up-regulated in response to specific conditions such as low pH for example would ideally give insight into the mechanisms that bacteria use for acid tolerance and maintenance of cytoplasmic pH. Specifically tagging and tracking these genes or the cell response within a species is then possible, as recently shown for tracking extracellular pH with *S. mutans* (Guo et al., 2013).

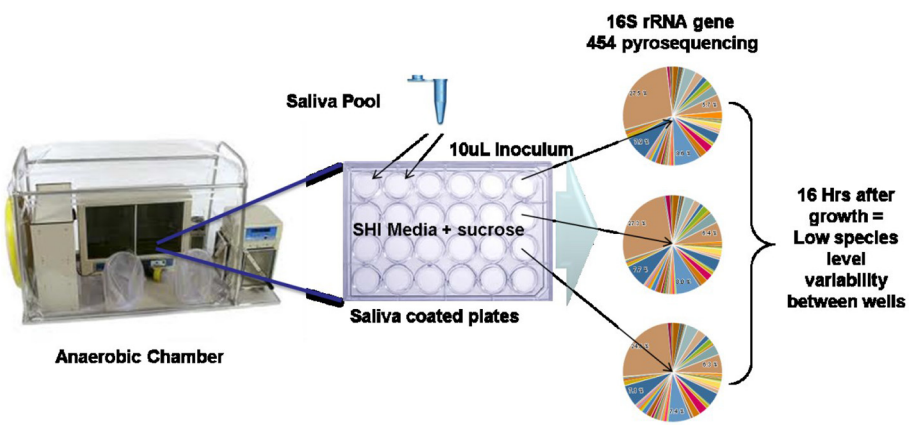

FIGURE 5 | *In vitro* oral biofilm model system (Edlund et al., 2013). Schematic of *in vitro* model system growth and 16S rRNA gene sequencing results indicating high reproducibility of taxonomic abundance and carriage between wells.

GENERAL ANALYSIS OF GENOMIC AND METATRANSCRIPTOMIC DATASETS

Although many groups approach the collection and analyses of global mRNA datasets, the generalized workflow for metatranscriptomics involves mapping reads onto reference and/or assembled genomes/metagenomes using such short read mapping tools. The counts for each genomic region can then be extracted and tabulated. Comparative gene expression analyses between sequencing libraries can be performed using tools and approaches developed to handle the dynamic range of RNA-seq datasets as opposed to methods developed for microarrays. Importantly, to determine the significance of the genes expressed, approaches for normalization of the data built into these processing tools and the statistical tests used within are being developed and tested. Gene transcription boundaries, regulatory regions and expression of small RNA can be analyzed in detail by using more sophisticated approaches once they are identified in the genome of interest. In addition, *de novo* assembly of the transcripts can then be performed which allows coding regions to be determined from the community that could assemble into new and novel genes. These open reading frames (ORFs) can be annotated and compared to existing genomes representing closely related bacterial genomes. Analysis of the metatranscriptomic data ideally allow identification of both structural and regulatory genes coding for the molecular mechanisms involved in bacterial physiology.

In general, the types of functional analyses approaches looking at global metatranscriptome data include:

1) **Comparative metatranscriptomics**. Differential expression (DE) of genes or orthologous clusters of genes in regards to a reference condition or temporal change in expression patterns.
2) **Functional Analyses**. Annotation information such as enzyme commission (EC) number assignments, COGs and hidden Markov models (HMMs) can be assigned to reads that assembled into partial or full genes and used to construct metabolic pathways to the extent possible to gain new insights into metabolic pathways present in a community. EC number assignments, for example, can be used to populate KEGG metabolic maps and can be enhanced with expression level information. The assignment of EC numbers, COGs, and HMMs can then be used to perform statistical analyses to determine over-representation of pathways/processes in the transcriptomes from each sample relative to one another.
3) **Phylogenetic profiling.** Patterns of up and down-regulated gene families or proteins in different genomes can be compared. Organisms sharing a particular expression pattern for example can be considered functionally similar thus indicating possible synergistic or competitive interactions.

The transcriptome of oral bacterial communities are being explored both in terms of the potential differences in expression between health and disease in caries (Peterson et al., 2013; Benitez-Paez et al., 2014), periodontal disease (Duran-Pinedo et al., 2014; Jorth et al., 2014) and defined mixed communities containing sequenced oral bacteria (Frias-Lopez and Duran-Pinedo, 2012). There are many technical and bioinformatics based challenges still associated with interpretation of microbial gene expression patterns in mixed species biofilm communities. One of the major concerns that needs to be addressed is the existence of phylogenetically closely related strains in the community: the identification of a unique read to a given strain can accomplished only when a reference genome of that strain is available. For the present, in most microbiome communities, a great majority of reads do not map to any known reference genome or may be overlapping with a closely related genome. Using large databases of reference genomes enable read counts across multiple genomes to be divided to attempt to account for highly conserved genes present in multiple genomes.

Another large confounding parameter is the change in a given genome abundance between two samples that can skew differential gene expression when comparing samples sets such as between healthy vs. disease within *in vivo* collected samples. For example, if particular species abundance increases the number mRNA copies will increase and thus appear to be a differentially expressed gene between the sample sets. This is somewhat more tractable when studying controlled model systems that may not suffer from the large intra- and inter-personal taxonomic variance that can occur in samples collected directly from human subjects. Efforts to correct for this abundance change are not a straight forward task and likely will not be routinely implemented until further development, testing and validation. Given the discrepancy between 16S rRNA gene abundance and abundance measures using read mapping to a particular genome strain (using marker genes), the act of building out the reference genome with single cell genomics and standard cultivation approaches will greatly aid this effort. Future research including more fundamental approaches to validate metatranscriptomic data will prove the utility of this technique ultimately providing useful transcript biomarkers of health and disease.

SUMMARY AND FUTURE DIRECTIONS

Much of what is known about all of the cultivated and sequenced oral bacterial species to date has been derived from pure culture approaches and laboratory experimentation, which likely does not reflect their actual behavior in complex microbial communities. Furthermore, as mentioned, roughly half of species identified through culture independent methods in the oral cavity are still only classified as uncultivated phylotypes. Many of these species have been found in deep cavities and therefore possibly linked to demineralization processes however their contribution to diseased states has not yet been established. Even low abundance members of a microbial community cannot be dismissed as inconsequential and in fact may express key properties that upset the balance and shift the metabolism of the community which for example, is the current belief with *Porphyromonas gingivalis* (Darveau et al., 2012; Hajishengallis et al., 2012). With the high level of effort to characterize the diversity and metabolic capacity encoded in the genomes of isolated bacteria associated with the oral cavity, a logical (and very important) next step is to increase our limited understanding of the process through community level physiological and molecular based studies. Such insights will help find new solutions to modulate the activity of the communities and steer them toward a healthy state. The huge

challenges remaining, such as the vast uncultivated species and the lack of reference genomes currently limit this understanding. Capturing genomes of yet-to-be cultivated species will serve not only to gain insight into their potential physiology but will enable verification of this predicted metabolism using sequencing based approaches such as metatranscriptomics to measure the expression of genes while they are within the community. Methods such as single cell genomics to capture genomes from biofilms as well as innovative cultivation strategies such as the stable domestication from human communities to *in vitro* communities are key. Ultimately, once more genomes become available, we can apply concomitantly, the arsenal of approaches as described earlier including non-invasive imaging and metabolic analysis methods followed downstream by such tools as SIP and the expression profiling of all species. Notably, the involvement of virus component and the host responses add to the already enormous challenges. Logically, these techniques are best applied in more controlled and reproducible mixed species model systems first to gain baseline information and build solid databases of information. As the knowledge, technology and capabilities evolve, these can be more confidently applied to *in vivo* samples.

In future oral microbiome studies developing from these advancements, a particular emphasis can be placed on the discovery of low pH metabolism, low pH adaptations and organic acid production most relevant to mineral (hydroxyapatite) dissolution process as well as the metabolisms/species that are linked to healthy pH recovery phase. Overall the combination of these approaches on oral microbial systems of interest will reveal species (cultured and uncultured) involved in disease related processes and provide new insights into specific species, genes/domains, gene products, and metabolic pathways that define the synergistic and competitive contributions to both health and disease.

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Effect of an oxygenating agent on oral bacteria *in vitro* and on dental plaque composition in healthy young adults

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Oral bacteria live in symbiosis with the host. Therefore, when mouthwashes are indicated, selective inhibition of taxa contributing to disease is preferred instead of broad-spectrum antimicrobials. The potential selectivity of an oxygenating mouthwash, Ardox-X® (AX), has not been assessed. The aim of this study was to determine the antimicrobial potential of AX and the effects of a twice-daily oral rinse on dental plaque composition.

Material and methods: *In vitro*, 16 oral bacterial strains were tested using agar diffusion susceptibility, minimum inhibitory and minimum bactericidal concentration tests. A pilot *clinical study* was performed with 25 healthy volunteers. Clinical assessments and microbiological sampling of supragingival plaque were performed at 1 month before the experiment (Pre-exp), at the start of the experiment (Baseline) and after the one-week experimental period (Post-exp). During the experiment individuals used AX mouthwash twice daily in absence of other oral hygiene measures. The microbiological composition of plaque was assessed by 16S rRNA gene amplicon sequencing.

Results: AX showed high inter-species variation in microbial growth inhibition. The tested *Prevotella* strains and *Fusobacterium nucleatum* showed the highest sensitivity, while streptococci and *Lactobacillus acidophilus* were most resistant to AX. Plaque scores at Pre-exp and Baseline visits did not differ significantly ($p = 0.193$), nor did the microbial composition of plaque. During a period of 7-days non-brushing but twice daily rinsing plaque scores increased from 2.21 (0.31) at Baseline to 2.43 (0.39) Post-exp. A significant microbial shift in composition was observed: genus *Streptococcus* and *Veillonella* increased while *Corynebacterium, Haemophilus, Leptotrichia, Cardiobacterium* and *Capnocytophaga* decreased ($p \leq 0.001$).

Conclusion: AX has the potential for selective inhibition of oral bacteria. The shift in oral microbiome after 1 week of rinsing deserves further research.

Keywords: microbiome, selective inhibition, oxygenating agents, antimicrobials, Ardox-X®-technology

INTRODUCTION

Dental plaque biofilm is part of the oral microbiome that co-evolves in symbiosis with the human host (Marsh, 2012). Recently the importance and beneficial role of the oral microbiome in maintaining oral and general health has been brought forward (Marsh, 2012; Hezel and Weitzberg, 2013). On the other hand, undisturbed dental plaque accumulation is associated with an enhanced host inflammatory response and gingival inflammation (gingivitis) (Lee et al., 2012). Gingivitis is known to be associated with the onset of periodontitis (Schatzle et al., 2003), therefore the importance of maintaining gingival health is well understood.

Although regular mechanical plaque removal is recommended for prevention of periodontal diseases, the quality of self-performed mechanical plaque removal may not always be sufficient (Hioe and van der Weijden, 2005). When this fails or cannot be optimally maintained, for instance in physically or mentally disabled populations, a chemical approach, such as the use of an antimicrobial mouthwash, can be an alternative or an adjunct.

Anti-plaque agents should not eradicate the oral microbiota. Instead, they should maintain the microbiota of the mouth at the level and composition that is compatible with oral health, this way preserving the beneficial functions of resident microbes (Marsh, 2012; ten Cate and Zaura, 2012). This requirement is not met by so-called broad spectrum antimicrobial agents such as chlorhexidine (CHX). Interestingly, oxygenating mouthwashes containing peroxoborate are able to reduce the dental plaque amount and retard the colonization and growth of anaerobes (Wennstrom and Lindhe, 1979; Binney et al., 1992; Moran et al., 1995) and Gram-negative bacteria (Hernandez et al., 2013). Gram-negative

anaerobes are generally associated with oral infections (e.g., periodontitis, peri-implantitis, endodontic infections).

Among oxygenating agents, boron-derived compounds such as sodium perborate (peroxoborate) generate active oxygen in aqueous solutions. This characteristic is the basis for their use as bleaching agents in detergents, cleaning products and cosmetic preparations, as well as a preservative in eye drops (Safety, 2010). In clinical dentistry, boron-derived compounds are used as a bleaching agent for teeth and as an adjunct to CHX to counteract extrinsic staining of the tongue and tooth surfaces (Dona et al., 1998; Grundemann et al., 2000; van Maanen-Schakel et al., 2012; Feiz et al., 2014).

Ardox-X® technology (AX) was introduced to the market and promoted as a teeth whitening, anti-microbial, anti-fungal and anti-inflammatory compound (NGen Oral Pharma, www.ngenpharma.com)[1]. According to the manufacturer, the AX compound is formed by chemical complexation of peroxoborate with specific carriers such as glycerol and cellulose. This produces sodiumperborate-1,2-diol-glycerol/cellulose-ester adducts, i.e., single-reaction products containing all the atoms of all components. The manufacturer considers this to be a distinct molecular compound that provides controlled release of active oxygen without generating hydroxyl radicals. However, the scientific evidence for the antimicrobial efficacy of this compound is scarce. So far, only one *in vitro* study has been published which showed that AX has an antimicrobial effect against polymicrobial biofilm (microcosm) grown on titanium surfaces (Ntrouka et al., 2011).

The aims of the current study were: first, to determine the antimicrobial effect of AX against oral bacteria *in vitro*; second, to evaluate *in vivo* the effect of AX containing mouthwash on the composition of undisturbed plaque accumulation in a one-week non-brushing model in healthy adults.

MATERIALS AND METHODS

IN VITRO STUDY

Bacterial strains (**Table 1**) were cultured on blood agar plates (Oxoid no 2, Oxoid, Basingstoke, UK) supplemented with 5% horse blood, 0.1% (w/v) haemin and 0.01% (w/v) menadione. For *Tannerella forsythia* Trypticase Soy Agar (TSA) (BBL, Beckton Dickson Microbiology Systems, Cockeyscille, MD) was used supplemented with 5% horse blood, 0.1% (w/v) N-acetyl muraminic acid (TSNAM plates), 0.05% (w/v) haemin, and 0.01% (w/v) menadione (van der Reijden et al., 2006). All strains except *Staphylococcus aureus* HG386 were grown in anaerobic atmosphere containing 80% N_2, 10% CO_2, and 10% H_2. *S. aureus* was grown aerobically.

For this study, the manufacturer provided different concentrations of AX in standard equivalent units (SE) in a range 1–20 SE, where 1 SE contains 0.27% (w/v) of sodium perborate (SP), as well as the AX blank (AX without sodium perborate; NGen Oral Pharma[1]; Curacao; van den Bosch, 2000, US patent number 6.017.515) that was used as negative control. As positive controls, two different concentrations of over-the-counter chlorhexidine (CHX) products were used: Perio Aid (0.12% CHX) (Dentaid, Barcelona, Spain) and Corsodyl (0.2% CHX) (GlaxoSmithKline, Zeist, the Netherlands).

Table 1 | Bacterial strains and their abbreviations used in the text.

Strain	Abbreviation
Actinomyces naeslundii ATCC 12104	*An*
*Aggregatibacter actinomycetemcomitans HG 683**	*Aa*
*Campylobacter rectus HG 963**	*Cr*
Fusobacterium nucleatum ATCC 25586	*Fn*
Lactobacillus acidophilus ATCC 4356	*La*
*Parvimonas micra HG 1179**	*Pm*
*Porphyromonas gingivalis K- HG 91**	*Pg K-*
*Porphyromonas gingivalis K1 HG 66/W83**	*Pg K1*
*Porphyromonas gingivalis K6 HG 1691**	*Pg K6*
*Prevotella intermedia HG 110**	*Pi*
*Prevotella nigrescens HG 70**	*Pn*
Staphylococcus aureus ATCC 2592	*Sa*
*Streptococcus mutans HG 708**	*Sm*
*Streptococcus sanguinis HG 1471**	*Ss*
Tannerella forsythia ATCC 43037	*Tf*
*Veillonella parvula HG 318**	*Vp*

**Clinical isolates.*

Agar diffusion tests were performed as described before (Clinical and Laboratory Standards Institute, 2009). For each strain, two blood agar plates were inoculated with 100 µl suspension of a single colony suspended in 5 ml phosphate buffered saline (PBS). The compounds were added in 5 mm holes punched in the agar. Five concentrations of AX (1.36; 1.9; 2.72; 4.08; 5.44%) together with a blank and two CHX concentrations were used. The blank and AX (2.72%) were included in each agar plate (twice per strain). Agar plates were incubated for 7 days at 37°C under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2). After incubation, plates were examined for growth inhibition. The inhibition zone around the holes was measured and expressed in mm from the edge to the nearest CFU.

Serial dilutions were used to determine the minimum inhibitory concentration (MIC) (Hecht, 2007) and the minimum bactericidal concentration (MBC) of AX. Two 24-wells plates were used per dilution set of 4 strains. The medium consisted of Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) supplemented with 0.1% haemin and 0.01% menadione (h/m). For *Tannerella forsythia* filter-sterilized N-acetyl-muraminic acid (NAM) to a final concentration of 0.1% (w/v) was added. The compound was filter-sterilized (0.2 µm pore size; 7 bar max Whatman, Germany). The initial dilution for the MIC was prepared using 0.5 ml AX at the highest concentration (54.4 g/l) in two-fold serial dilution series (range 40–40, 800 mg/l)

Inocula were prepared from a pure culture of each strain. A single colony was taken using a sterile cotton pick and suspended in 5 ml of PBS. Then 50 µl of suspension were dispensed into each labeled well. Plates were incubated for 7 days at 37°C under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2). After 1 week the MICs were determined by visual means. The procedure was performed in triplicate at different time points.

After MIC determination, 100-µl samples from the various dilutions were inoculated onto appropriate agar plates and

[1]http://www.ngenpharma.com (Accessed 08 April, 2014).

incubated for 7 days at 37°C under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2). The concentration at which growth was visibly inhibited was defined as the MBC.

PILOT CLINICAL STUDY

The study protocol was approved by the Medical Ethics Committee of the Academic Medical Center (AMC) of Amsterdam (NL37567.018.11) and registered at the Dutch trial register under the number NTR3145. The study followed the instructions based on the declaration of Helsinki. That statement acts as a starting point in subject recruitment.

Study population

Participation in this study was voluntary. Before enrollment all participants were given oral and written information about the products and the reason, aim, duration, demands of benefits and possible harm. After signing a declaration of informed consent, 26 systemically healthy participants meeting the inclusion criteria agreed to participate in the study.

All participants (non-dental students, ≥18 years) had to be dentate with at least 5 evaluable teeth per quadrant excluding prosthetic crowns. To include a population with high plaque scores at the start of the experimental period, participants were selected that had an overnight plaque score of 2 or higher as assessed according to Modified Quigley & Hein Plaque index (Paraskevas et al., 2007). Exclusion criteria were: oral mucosal lesions, orthodontic appliances, removable (partial) dentures, and overhanging margins of dental restorations (clinically assessed with a periodontal probe), the use of antibiotics during the last 6 months, Dutch Periodontal Screening Index (Mantilla Gomez et al., 2001) (DPSI) ≥3+ (periodontal pockets >5 mm with bleeding on probing and gingival recession), the use of medication possibly influencing normal gingival health, pregnancy and smoking.

Test compound

According to the manufacturer, the hydro-carbon-oxo-borate compound AX had the following ingredients: aqua, sodium lauryl sulfate, PEG-40 hydrogenated castor oil, sodium gluconate, cellulose gum, aroma, sodium citrate, magnesium sulfate, sodium perborate, sodium methylparaben, citric acid, sodium chloride, sodium fluoride, sodium saccharin (NGen Oral Pharma, www.ngenpharma.com[1]; van den Bosch, 2000, US patent number 6.017.515).

Study design

The study started with a pre-experimental appointment (1 month prior to baseline) during which dental plaque was scored and sampled with the intention to assess the consistency of collected plaque scores and microbiological data relative to the baseline. At baseline, dental plaque was again scored and sampled. In addition, the level of bleeding on marginal probing was assessed as a descriptive of the oral health status of the included subjects. After the baseline measurements, a professional prophylaxis was performed by a dental hygienist as described in detail by Slot et al. (2010) in order to start the experiment with equally clean teeth. Following the prophylaxis, a one-week non-brushing experimental period of undisturbed plaque accumulation was started. With respect to oral hygiene the participants were only allowed rinsing with the distributed mouthwash (AX). Each subject received an instruction form on how to use the intervention product and the first rinse was performed under supervision. Participants were instructed to rinse twice daily (morning and evening) for 1 min and not to rinse, drink or eat for at least 30 min thereafter. No other form of oral hygiene during the subsequent week was allowed, including chewing gum (Keukenmeester et al., 2014) or any xylitol containing sweets or gum (Soderling, 2009).

Clinical assessments

In the study a partial-mouth model (Bentley and Disney, 1995) was used. Two contra-lateral randomly chosen quadrants (www.random.org) served for the collection of dental plaque biofilm that was not disturbed by scoring or the disclosing solution (one in the upper and one in the lower jaw; Heijnsbroek et al., 2006; Van Leeuwen et al., in press).

The two opposing contra-lateral quadrants were used for the clinical plaque assessments. All teeth in each of the two quadrants were examined except third molars. Scoring was performed by two experienced examiners each responsible for scoring one clinical parameter (plaque or bleeding) separately. For plaque scores teeth were disclosed with a 1% erythrosine solution. Plaque was assessed at six sites per tooth on a six-point scale using the Quigley & Hein's plaque index (Quigley and Hein, 1962) as modified by Turesky et al. (1970) and further modified by Lobene et al. (1982), in which the absence or presence of plaque was recorded on a 0–5 scale (0 = no plaque, 5 = plaque covering more than two-thirds of the tooth surface) and described in detail by Paraskevas et al. (2007). At the baseline appointment the level of oral health was assessed in the two contra-lateral quadrants that had previously been sampled for supragingival plaque using the Bleeding on Marginal Probing (BOMP) score (van der Weijden et al., 1994a,b; Lie et al., 1998). Bleeding was elicited with a WHO-approved ball-ended probe (Ash Probe EN15, Dentsply International, York, PA, USA). The absence or presence of bleeding was scored within 30 s of probing on a scale of 0–2 (0 = non-bleeding, 1 = pinprick bleeding, 2 = excess bleeding).

Sampling procedure

Since it is imperative to characterize differences in microbial composition among specific oral locations, supragingival dental plaque was collected from the buccal sites of four pre-selected teeth being the same at all three assessments (first molar and canine, upper and lower jaw). Dental plaque was carefully collected by an experienced examiner with a sterile microbrush (Microbrush International, Grafton, USA) per tooth moving over the enamel surface from the mesial to distal curvature of the tooth crown along the gingival margin and tooth-surface border. The tip of each of the four microbrushes was clipped off and placed in a single vial containing RNAProtect Bacteria reagent (Qiagen, Hilden, Germany). Samples were coded, kept on ice until transfer within 2 h to the laboratory.

DNA extraction, amplicon preparation, and pyrosequencing

Of the 72 clinical samples belonging to 25 subjects, 6 samples were lost due to technical reasons. DNA was extracted

with the AGOWA mag Mini DNA Isolation Kit (AGOWA, Berlin, Germany) as described previously (Crielaard et al., 2011). Barcoded amplicon libraries of the small subunit ribosomal RNA gene hypervariable region V5–V7 were generated for each of the individual sample as described previously (Kraneveld et al., 2012), pooled and sequenced by means of the Genome Sequencer FLX Titanium system (Roche Molecular Diagnostics). The sequencing data was processed using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010) version 1.5.0. The reads were denoized using Denoiser version 1.3.0 (Reeder and Knight, 2010) and checked for chimeric sequences using UCHIME version 4.2.40 (Edgar et al., 2011). The results of the *de novo* and the reference-based approach were combined and reads marked as chimeric were removed. Sequences were clustered in operational taxonomic units (OTUs) at 97% similarity.

Statistical analyses

The statistical package SPSS software version 19.0 was used to perform statistical analyses. The effect of AX on bacterial strains in the agar diffusion assay was analyzed for each AX concentration relative to the effect of 0.2% CHX for each bacterial strain (Mann–Whitney test). Differences among strains per-compound were calculated using One-Way ANOVA and Tukey B *post-hoc* test.

For the clinical study, the mean plaque score and gingival bleeding score were calculated first per participant. Additionally the mean bleeding score at baseline for the sampled teeth was calculated. Plaque scores were tested for normality using the Shapiro–Wilk test. Non-parametric Wilcoxon Signed Rank test was performed to test for differences in plaque scores between the three visits: pre-experimental, baseline and post-experimental.

To normalize the microbial data for comparisons among different samples and to avoid the effect of variable sample size on the diversity analyses, a randomly subsampled data set of 850 reads per sample was created. This resulted in exclusion of additional five samples with less than 850 reads/sample. PAST software (Hammer et al., 2001) was used to calculate Shannon diversity index, which takes into account the abundance of each OTU, as well as the number of OTUs. The normality of the diversity index data was assessed using Shapiro–Wilk test. Paired samples *T*-test was used to compare the diversity indices between the three time points. OTU-significance paired samples *T*-test implemented in QIIME 1.5.0 was used to compare the abundances of OTUs at baseline and post-experimental samples. Only those OTUs that were present in at least 10 samples were included in the analyses, resulting in 75 comparisons. The *p*-values were corrected for these multiple comparisons using Bonferroni correction. *P*-values below 0.05 were considered statistically significant.

To visualize microbial profile data, principal component analysis (PCA) was used in PAST. The OTU abundances were log2 transformed to normalize the data distribution.

RESULTS

IN VITRO STUDY

Based on the size of the inhibition zone (mm), the efficacy of CHX was greater than that of AX at all concentrations tested and for all bacterial strains ($p < 0.05$). AX showed high variation in inhibition (**Figure 1**), which was statistically significant among various strains (**Table 2**). *Streptococcus mutans*, *Lactobacillus acidophilus* and *Streptococcus sanguinis* were the least affected (inhibition zone 0–0.3 mm), while *Prevotella nigrescens* (9 mm) and *Prevotella intermedia* (9 mm)—the most $p < 0.05$ (**Table 2**).

All tested strains were inhibited by CHX and the differences in inhibitory activity among strains were less pronounced than for AX (**Table 2**). *Porphyromonas gingivalis K-* was the most affected (14 mm) and *Staphylococcus aureus*—the least (6 mm). None of the strains were inhibited by the AX blank (0 mm).

For most strains the MICs and MBCs for AX were ≤638 mg/l SP. Except for *L. acidophilus*—2550 mg/l SP, *S. aureus* and *S. sanguinis*—1275 mg/l SP (**Table 3**). The MBCs and MICs for AX were nearly the same (**Table 3**).

PILOT CLINICAL STUDY

Of 26 participants initially enrolled in the study, one participant dropped out for a reason unrelated to the study (**Table 4**, **Figure 2**). The duration of overnight plaque accumulation assessed at the pre-experimental and the baseline visits ranged from 10 to 16 h with an average of 13 h (*SD* 2.9). At the baseline appointment, the mean level of gingival health of the participants, as assessed by Bleeding on Marginal Probing BOMP in two contra-lateral quadrants, was 1.15 (*SD* 0.33) (**Table 4**). The mean bleeding scores at the sampled teeth (total of 12 buccal sites from four pre-selected teeth) was 0.98 (SD 0.43), which corresponds to 51% bleeding (*SD* 23).

Plaque scores did not differ significantly between the pre-experimental visit and baseline ($p = 0.193$), while plaque scores increased significantly ($p = 0.014$) during a week without any additional oral hygiene measures but with twice-daily use of AX mouthwash (**Table 5**).

Compliance to the rinsing protocol was assessed by weighing the bottles at the baseline and 7 days later at the post-experimental visit. The difference was on average 140 g (±14 g), which implied on average 14 servings of 10 ml complying with the individual instructions for use given.

In total 19 participants provided evaluable microbiological data. The data of three participants were excluded due to the technical reasons in sample processing, and another three—due to low reads (<850) per sample in one of the samples after the filtering steps of the sequencing data. The remaining 54 samples had on average 3135 reads/samples (*SD* 1047). The total of 169,309 reads were classified into 15 phyla, with *Actinobacteria* (39% of the reads) and *Firmicutes* (31%) dominating the data, followed by *Proteobacteria* (19%), *Bacteroidetes* (7.5%), *Fusobacteria* (3.1%) and Candidate division TM7 (0.3%).

After subsampling at 850 reads/sample, the diversity and taxonomic comparisons among the three visits (pre-experimental, baseline and post-experimental) were performed. Shannon diversity index, taking into account the abundance of each OTU as well as the number of OTUs, significantly increased from pre-experimental to baseline visit from 2.67 (*SD* 0.29) to 2.79 (*SD* 0.29) ($p = 0.02$) and significantly decreased at post-experimental visit to 2.09 (*SD* 0.39) ($p < 0.001$).

Genera *Corynebacterium* (21% of reads) and *Streptococcus* (16–20%) dominated the pre-experimental and baseline samples. Both of these genera were significantly affected by the treatment

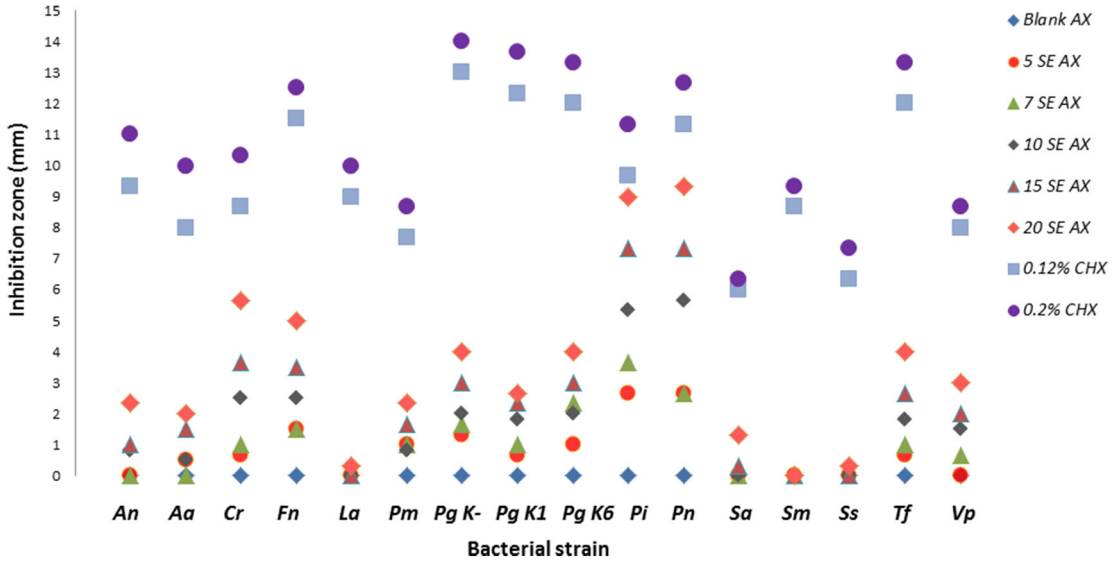

FIGURE 1 | Effects of Ardox-X®-technology (AX), Blank AX and Chlorhexidine (CHX) on inhibition of oral bacterial strains in agar diffusion assay. Inhibition zone size is expressed in mm (mean of triplicate experiment, except duplicate for *Aa* and *Fn*). The strains (**Table 1**) used were: *Pm, Parvimonas micra*; *Porphyromonas gingivalis (Pg K1). (Pg K-). (Pg K6); An, Actinomyces naeslundii; Fn, Fusobacterium nucleatum; Cr, Campylobacter rectus; Sa, Staphylococcus aureus; Aa, Aggregatibacter actinomycetemcomitans; La, Lactobacillus acidophilus; Vp, Veillonella parvula; Ss, Streptococcus sanguinis; Sm, Streptococcus mutans; Pi, Prevotella intermedia; Pn, Prevotella nigrescens; Tf, Tannerella forsythia.*

Table 2 | Results of the Agar Diffusion assay, performed in triplicate.

Strain	Concentration of Ardox-X® technology													
	0.2% CHX		0.12% CHX		5 *SE*		7 *SE*		10 *SE**		15 *SE*		20 *SE*	
	Mean (*SD*)	Diff.	Mean (*SD*)	Diff.	Mean (*SD*)	Diff.	Mean (*SD*)	Diff.	Mean (*SD*)	Diff.	Mean (*SD*)	Diff.	Mean (*SD*)	Diff.
An	11 (0)	cd	9 (1)	cdef	0 NA	b	0 NA	d	1 (1)	cd	1 (1)	cde	2 (1)	def
Cr	10 (1)	cdef	9 (1)	def	1 (1)	ba	1 (1)	cd	2 (1)	b	4 (1)	b	6 (1)	bc
La	10 (2)	cdef	9 (0)	def	0 NA	b	0 NA	d	0 NA	d	0 NA	e	0 NA	fg
Pm	9 (1)	def	8 (1)	fgh	1 (2)	ba	1 (1)	bcd	1 (1)	cd	2 (1)	bcde	2 (1)	def
Pg K-	14 (1)	a	13 (1)	a	1 (1)	ba	2 (1)	bcd	2 (1)	bd	3 (1)	bcde	4 (1)	cd
Pg K1	14 (1)	a	12 (1)	ab	0.7 (0.6)	ba	1 NA	cd	2 (0)	bd	2 (1)	bcde	2.7 (0.6)	de
Pg K6	13 (1)	ab	12 (0)	abc	1 (1)	ba	2 (1)	abc	2 (1)	bd	3 (1)	bcde	4 (1)	cd
Pi	11 (1)	bcd	10 (2)	bcde	2.7 (0.6)	a	4 (1)	abc	5 (1)	a	7 (1)	a	9 (1)	a
Pn	13 (1)	bac	11 (1)	abcd	3 (1)	a	2.7 (0.6)	abc	6 (1)	a	7 (2)	a	9 (2)	a
Sa	6 (1)	f	6 (0)	h	0 NA	b	0 NA	d	0 NA	d	0.3 (0.6)	de	1 (1)	efg
Sm	9 (1)	cdef	9 (1)	efg	0 NA	b	0 NA	d	0 NA	d	0 NA	e	0 NA	g
Ss	7 (1)	ef	6 (1)	gh	0 NA	b	0 NA	d	0 NA	d	0 NA	e	0 NA	fg
Tf	13 (1)	ab	12 (0)	abc	1 (1)	ba	1 NA	cd	2 (1)	bc	3 (1)	bcde	4 (1)	cd
Vp	9 (1)	def	8 (2)	fgh	0 NA	b	1 (1)	d	2 (1)	bc	2 (0)	bcde	3 (0)	de
*Aa***	10 (1)		8 (1)		0 NA		0 NA		1 (1)		1 (1)		2 (1)	
*Fn***	12 (1)		11 (1)		1 (1)		1 (1)		2.5 (0.6)		3.5 (0.7)		5 (1)	

*Mean size of the inhibition zones (mm) and standard deviation. Diff., Different letters indicate statistically significant difference among the different bacterial strains per-compound (within each column) ($p < 0.05$; ANOVA, Tukey B-test). *n = 6; **Excluded from analysis (n = 2); NA, not applicable; SE, standard equivalent units.*

period: *Corynebacterium* was reduced to 2% and *Streptococcus*—increased to 32% (**Figure 3**). Additionally, genus *Veillonella* showed significant increase from 2–3 to 12% after the treatment, while genus *Derxia* showed significant decrease from 3 to 0.7%, respectively (**Figure 3**). Genus *Leptotrichia* was nearly absent after the experimental period, while it constituted approximately 2% of the reads at the pre-experimental and baseline visits. Genus *Prevotella* was present at a very low proportion—between 1 and

Table 3 | MICs and MBCs of Ardox-X® technology for the 16 strains studied, expressed as sodium perborate (SP) concentration in the compound.

Bacteria strain	MIC		MBC	
	***SP* mg/l**	**Range**	***SP* mg/l**	**Range**
An	319	319–638	319	319–638
Aa	159	80–319	319	80–638
Cr	159	NA	638	319–638
Fn	159	159–319	159	159–319
La	2550	1275–2550	2550	1275–2550
Pm	638	638–1275	638	638–1275
Pg K-	319	NA	319	319–638
Pg K1	319	NA	638	NA
Pg K6	159	80–159	159	NA
Pi	80	40–80	80	80–159
Pn	80	80–159	80	80–159
Sa	1275	638–1275	1275	638–1275
Sm	638	638–1275	638	638–1275
Ss	1275	638–1275	1275	638–1275
Tf	319	159–319	319	159–319
Vp	159	NA	159	NA

Values are median (range) of experiment in triplicate. NA, not applicable due to equal values.

Table 4 | Subject demographics and their periodontal health.

N	**25**
Female/Male	17/8
Age in years, mean (SD)	21.5 (1.9)
DPSI*	
1	1
2	11
3–	13
BOMP**, mean (*SD*)	1.15 (0.33)

**Dutch periodontal screening index (Mantilla Gomez et al., 2001).*

***Bleeding on marginal probing (BOMP) at baseline (van der Weijden et al., 1994a,b; Lie et al., 1998).*

1.5% of the reads throughout the study and showed no significant effect of the treatment.

To identify the OTUs that contribute to the differences between the visits, an OTU-category significance test using paired samples *T*-test was performed, corrected for multiple comparisons (75) using Bonferroni correction. No OTUs differed significantly between the pre-experimental and baseline visits, while 10 OTUs differed between the baseline and post-experimental visit (**Table 6**). Two OTUs—OTU169, classified as *Veillonella*, and OTU113, classified as *Streptococcus* (blast: *Streptococcus sanguinis* SK1, 100% ID) increased. From the eight OTUs that showed a significant decrease, OTU197 was identified as *Streptococcus cristatus* (100% blast ID) and OTU183—as *Leptotrichia hongkongensis* (100% blast ID), while the remaining six OTUs (**Table 6**) could not be identified at species level.

Next, the microbiome profile data were ordinated by applying principal component analysis (PCA) (**Figure 4**). The first principal component (PC1) explained 27% of the overall variance among the samples and showed a clear separation of the pre-experimental (black dots, **Figure 4**) and baseline samples (blue dots, **Figure 4**) from the post-experimental samples (red dots in **Figure 4**). The second component explained 11% of the total variance and separated the samples belonging to subjects Nr 1 and Nr 31 from the rest (**Figure 4**).

DISCUSSION

The *in vitro* experiments of this study indicated that oxygen-releasing compound Ardox-X® technology (AX) selectively inhibits oral bacteria, with anaerobe Gram-negative species being the most sensitive. These promising findings were further tested *in vivo*, during a pilot clinical study with experimental period without any oral hygiene measures but twice-daily rinse with mouthwash containing AX. After a week of non-brushing, the plaque scores increased, while the microbial composition showed a shift toward compositionally less diverse plaque, dominated by primary colonizing genera *Streptococcus* and *Veillonella* compared to the *Corynebacterium* dominated plaque at the baseline.

It has been proposed that in order to study the effects of a mouthwash, a population with a high amount of plaque should be studied (Wennstrom, 1988). The study population therefore included individuals that proved to be good plaque formers at the screening visit. Moreover, it is known that the periodontal condition affects the rate of supragingival plaque forming (Hillam and Hull, 1977; Rowshani et al., 2004). Participants showed to have moderate gingivitis at baseline whereby 51% of the sampled sites were bleeding on marginal probing. The one-month interval between the pre-experiment assessment and baseline did not result in significant changes in plaque scores or in plaque composition of the study population. This is in line with previous studies, which have demonstrated the stability of the oral microbiome (Zhou et al., 2013). Microbial composition of the supragingival plaque in this gingivitis population 1 month before the experiment and at the baseline resembled mature plaque (Haffajee et al., 2009) and plaque associated with gingivitis (Huang et al., 2011). The major taxon in these samples was identified as *Corynebacterium*, a Gram-positive, facultatively anaerobe bacterium that resembles Gram-negatives with respect to the lipid layer in the cell membrane (John, 1984). *Corynebacterium* is associated with mature plaque and is found in dental calculus (Moorer et al., 1993).

The participants of the classical experimental gingivitis model (Loe et al., 1965; Theilade et al., 1966) received a prophylaxis and subsequently refrained from oral hygiene for 21 days inducing an acute stage of inflammation in otherwise healthy subjects. For the present study a seven-day model was chosen since the purpose was not to change the level of gingival health but to assess the effects on the microbial composition of undisturbed plaque. In the absence of oral hygiene, bacterial re-colonization increases after professional oral hygiene reaching or exceeding pre-prophylaxis levels at 2 days (Uzel et al., 2011) and *de novo* plaque formation reaches a stable microbial community between 4 and 7 days (Uzel et al., 2011). Higher diversity of mature

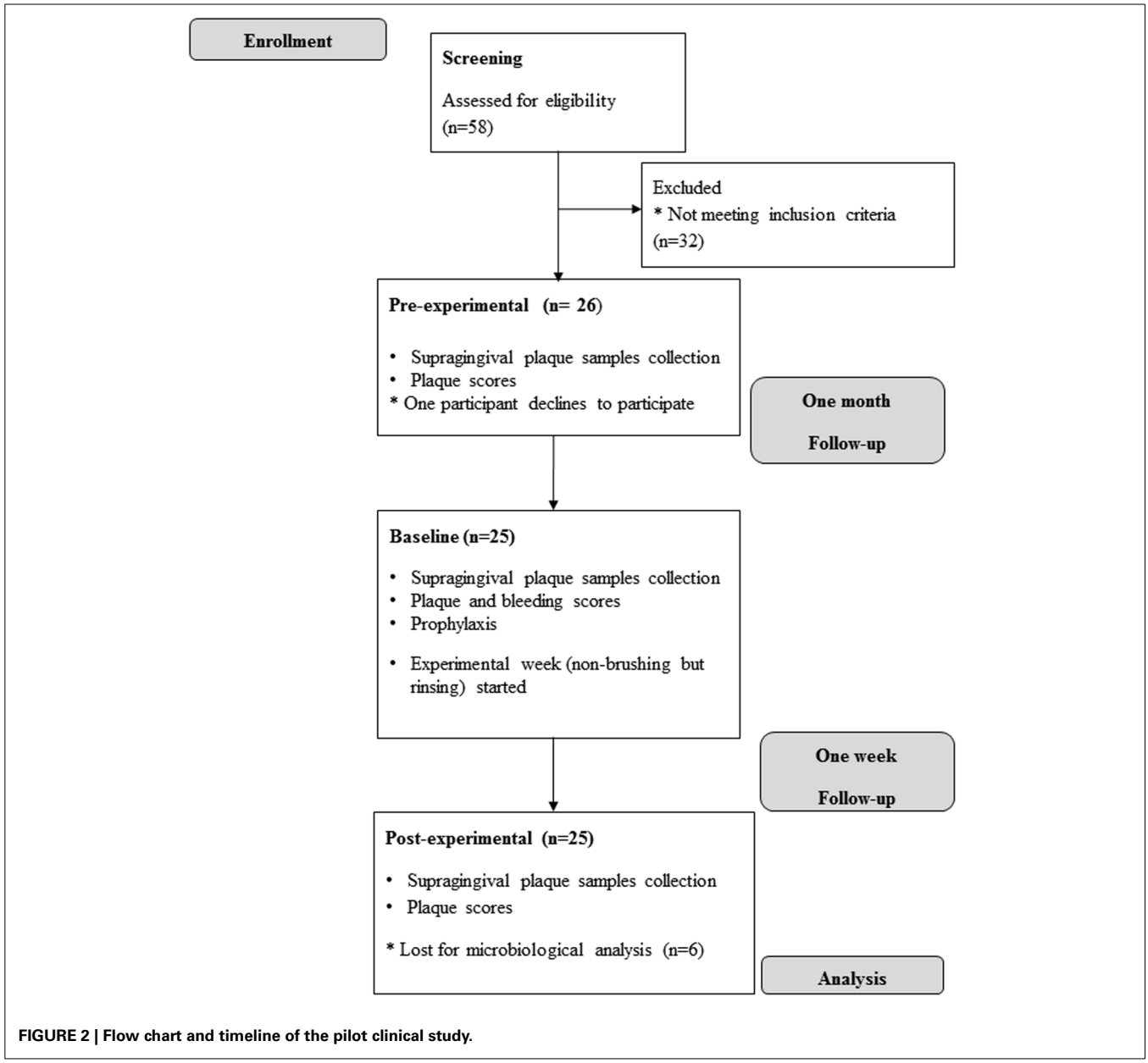

FIGURE 2 | Flow chart and timeline of the pilot clinical study.

Table 5 | Mean (SD) and range of Plaque index scores.

	Mean	Min	Max	*p-value**	
Pre-experimental	2.27 (0.34)	1.68	3.01	*0.193*	*Pre-experimental vs. Baseline*
Baseline	2.21 (0.31)	1.60	3.12	*0.014*	*Baseline vs. Post-experimental*
Post-experimental	2.43 (0.39)	1.63	3.26		

**Wilcoxon Signed Ranks test.*

supragingival plaque compared to younger plaque has been found in a recent experimental gingivitis study (Kistler et al., 2013). In the present study after the baseline assessment, the participants received a thorough professional oral hygiene and were not allowed to brush their teeth for 1 week. Instead, they were asked to perform a twice daily rinsing with AX-containing mouthwash. As expected, the plaque amount increased during the experimental period. The composition of the sampled plaque also changed impressively, whereby microbial diversity had decreased significantly, when compared to the pre-experimental and baseline visits. Genus *Corynebacterium* was considerably reduced, while streptococci, *Veillonella* and *Haemophilus*—all health-associated primary colonizers (Colombo et al., 2009; Simon-Soro et al., 2013)—dominated the post-experimental plaque.

In vitro diffusion and susceptibility tests showed that AX is highly selective in inhibiting oral bacteria. The Gram-negative anaerobes such as prevotellas, but also *Veillonella*, *Tannarella*, *Campylobacter*, *Fusobacterium*, and *Porphyromonas* were highly sensitive, while streptococci and lactobacilli, facultative anaerobe

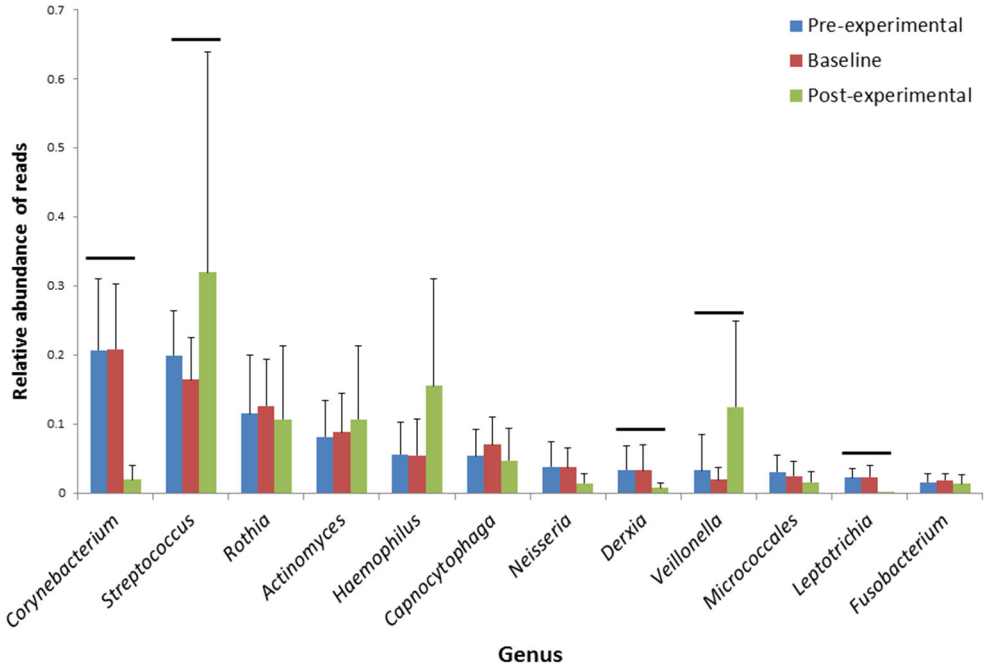

FIGURE 3 | Average proportions of major genera from dental plaque samples collected at pre-experimental, baseline and the post-experimental visit. Error bars—standard deviations. Horizontal lines indicate statistically significantly different proportions of reads of the respective genera between the post-experimental and the other two visits ($p < 0.05$, Wilcoxon signed ranks test, after Bonferroni correction for multiple comparisons). $N = 19$.

Table 6 | Significantly differently abundant OTUs between baseline and post-experimental visit and their abundance in plaque samples.

OTU#*	Number of reads (*SD*)		
	Pre-experimental	Baseline	Post-experimental
113.Streptococcus	155 (52)	128 (53)	258 (93)
120.Corynebacterium	153 (93)	158 (84)	13 (15)
169.Veillonella	28 (43)	16 (14)	106 (61)
16.Corynebacterium	23 (21)	20 (15)	3 (6)
183.Leptotrichia	11 (8)	9 (7)	0.05 (0.2)
251.Capnocytophaga	7 (7)	9 (7)	2 (3)
197.Streptococcus	7 (8)	6 (4)	0.2 (0.5)
245.Cardiobacterium	6 (6)	8 (6)	2 (2)

*Samples were randomly subsampled to 850 reads/sample. *OTUs that remained significant after OTU-significance Paired samples T-test, Bonferroni correction, $p \leq 0.001$; $N = 19$.*

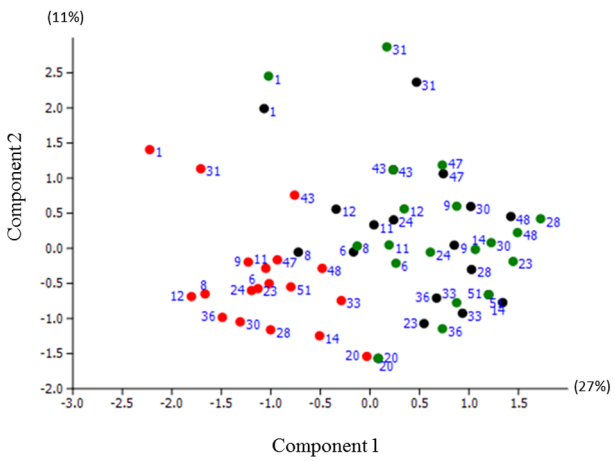

FIGURE 4 | Principal Component Analysis (PCA) plot of microbiome samples from pre-experimental visit (black dots); baseline of the experimental period (green dots) and post-experimental visit (red dots). The first component (PC1) explained 27% of the total variance, the PC2—11%. The same labels indicate samples that originated from the same individual.

Gram-positive bacteria, were not inhibited even by the highest dose of AX tested in the diffusion test. In the clinical samples genus *Prevotella* were found at very low levels throughout the study and no effect of AX was discernible. However, other Gram-negative taxa such as genera *Derxia* and *Leptotrichia*, as well as OTUs classified as *Cardiobacterium* (OTU#245) and *Capnocytophaga* (OTU#251) were significantly reduced during the experimental period. Unfortunately, *in vitro* tests did not include any genus *Corynebacterium* species which allows only for speculation whether this Gram-positive bacterium with the characteristics of Gram-negatives (John, 1984) was also highly susceptible to exposure to AX. Alternatively its nearly complete elimination from the post-experimental plaque samples could have other reasons for instance its growth could have

been inhibited due to ecological shifts in the entire community (Bradshaw et al., 1989). Another intriguing bacteria was *Veillonella*—anaerobe Gram-negative bacteria, associated with early supragingival plaque (Li et al., 2004; Haffajee et al., 2009). Although *Veillonella* was found to be susceptible to AX in *in vitro* testing, this genus showed a significant increase after the experimental period with twice-daily exposure to the AX mouthwash. This could be attributed to the "pioneering" function of this bacteria; it is found in healthy individuals, in young supragingival plaque, in similar proportions with streptococci (Keijser et al., 2008; Haffajee et al., 2009). Veillonellae are secondary fermenters—they consume lactic acid produced during glucose fermentation by streptococci (Keller and Surette, 2006; Periasamy and Kolenbrander, 2010) and produce other, weaker acids such as acetic and propionic acid. By doing so, the environmental conditions are created that promote growth of both of these genera (Bradshaw et al., 1989). The most likely explanation of the increase of genus *Veillonella* during the experimental period, although sensitive to direct exposure to AX *in vitro*, could be related to this ecologically beneficial relationship with streptococci.

The selective inhibition of oral bacteria by AX is of interest with respect to gingival and periodontal diseases, since infections associated with Gram-negatives would be selectively suppressed whereas the microorganisms regarded as more beneficial for periodontal health such as streptococci and lactobacilli would not. Several *in vitro* studies (Teughels et al., 2007; van Essche et al., 2012) and recent clinical studies (Iniesta et al., 2012; Teughels et al., 2013; Yanine et al., 2013) have suggested that these allegedly beneficial bacteria can cause antagonism toward Gram-negative bacteria.

In the agar diffusion assay, AX had significantly lower inhibitory effect than CHX. The activity of AX could have been limited to a short period right after its administration until the active component is broken down and oxygen is released. CHX is known to retain its activity for a longer time period after a single application *in vitro* (Carrilho et al., 2010). The only other study that has assessed the antimicrobial effect of AX, showed that the AX containing product had higher antimicrobial capacity than chlorhexidine toward monospecies bacterial biofilm and microcosm obtained from pooled saliva (Ntrouka et al., 2011).

So far, chlorhexidine (CHX) has proven to be the most effective antimicrobial agent in clinical dentistry and is considered as the "gold standard" disinfectant in dental research (Jones, 1997; Arweiler et al., 2001). Although it is widely used in periodontics and is among the most effective compounds preventing plaque formation (Addy, 1986), it has several side effects (Keijser et al., 2003; Gurgan et al., 2006) and therefore may result in poor rinsing compliance by patients (Addy and Moran, 1985; Cortellini et al., 2008; Van Strydonck et al., 2012). Optimizing anti-plaque agents, reducing their side effects while at the same time taking care that the oral microbiota are kept in balance with oral health has initiated interest in the development of other chemotherapeutical agents. Interestingly, AX showed selective inhibition of oral bacteria that may contribute to this demanding balance and deserves further investigation.

The lack of controls is a major limitation of this study that could have potential bias on the interpretation of this study outcomes. This pilot however indicates a potential rationale for more elaborate studies with a randomized clinical trial protocol that would include both a positive control such as CHX, and a negative control without any antimicrobial effects. The potential effect of AX on reductions in both the clinical manifestations of gingivitis and the inhibition of or reduction of plaque or plaque pathogenicity still needs to be demonstrated. For that purpose a 21 days experimental gingivitis model could be used or alternatively a 4-week trial among gingivitis subject as proposed by the American Dental Association in their Acceptance Guidelines of Chemotherapeutic Products for Control of Gingivitis (ADA, Acceptance Guidelines, 2008).

In conclusion, a mouthwash containing the oxygenating agent Ardox-X® technology showed potential for selective inhibition of oral bacteria. Twice-daily exposure for 1 week to this mouthwash resulted in a shift in the microbial composition toward a less diverse and less mature plaque. The clinical consequences of this shift in the oral microbiota need to be established.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00095/abstract

(1) seqs_rct_rep_set.fasta with the representative sequences of each OTU;

(2) otu_table_rct_not_subsampled.txt file with the OTU-table containing the list of OTUs and their respective taxonomical assignment and read abundances per each sample.

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Mouthguards: does the indigenous microbiome play a role in maintaining oral health?

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The existence of symbiotic relationships between bacteria and their hosts in various ecosystems have long been known to science. The human body also hosts vast numbers of bacteria in several habitats. Emerging evidence from the gastro-intestinal tract, genito-urinary tract and respiratory indicates that there are several health benefits to hosting a complex and diverse microbial community. Bacteria colonize the oral cavity within a few minutes after birth and form stable communities. Our knowledge of the oral microbiome has expanded exponentially with development of novel exploratory methods that allow us to examine diversity, structure, function, and topography without the need to cultivate the individual components of the biofilm. The purpose of this perspective, therefore, is to examine the strength of current evidence supporting a role for the oral microbiome in maintaining oral health. While several lines of evidence are emerging to suggest that indigenous oral microbiota may have a role in immune education and preventing pathogen expansion, much more work is needed to definitively establish whether oral bacteria do indeed contribute to sustaining oral health, and if so, the mechanisms underlying this role.

Keywords: commensal, oral, health, beneficial, ecosystem, host-bacterial interactions, inter-microbial interactions

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Homo sapiens as a Member of the Bacterial Kingdom

Bacteria predate humans on Earth by at least three billion years (Beraldi-Campesi, 2013); and have successfully survived the vicissitudes of drastic temperature changes, earthquakes, volcanic eruptions, and the advent of new species, evolving with each age and era. Along with their own evolution, these organisms have played a major role in shaping eukaryotic evolution, both as endosymbionts and as ectosymbionts (Pace, 1997). As *Homo sapiens* evolved, these organisms co-evolved with their host to such an extent that the human body is considered a super-organism consisting of functionally, metabolically, and spatially integrated bacterial and human cells (Ley et al., 2008). Modern-day man plays host to at least 10 times as many bacterial cells as human cells (Sleator, 2010). In fact, it might be more logical to view the human being as an inhabitant of the microbial world, rather than the reverse. Given this perspective, it is important to acquire a comprehensive understanding of the bacteria that inhabit us, and their collective genes (the human microbiome).

Recent large-scale public, private and crowd funded initiatives such as the Human Microbiome Project (HMP) (Human Microbiome Project Consortium, 2012), Metagenomics of the Human Intestinal Tract (MetaHIT) (Li et al., 2014), and UBiome (Costandi, 2013) have allowed us to

explore human-microbial and inter-microbial interactions to better understand the implications of hosting our microbial fellow travelers. Through these and other studies, we are beginning to learn not only how these bacteria are acquired and their colonization dynamics, but also how diverse factors, such as host genotype, host environment and host development shape these communities (Costello et al., 2009; Zaura et al., 2009; Kumar et al., 2011; Greenblum et al., 2012; Mason et al., 2013).

The Oral Microbiome as an Ecosystem

The term ecosystem was introduced by Arthur Roy Clapham to describe a community of living organisms along with their living and non-living environment, interacting as a system and linked to each other through energy transfer and nutritional flow (Blew, 1996). Based on this definition, humans may be considered a collection of microbial ecosystems (Prosser et al., 2007). The body provides several habitats for colonization—the oral cavity, nasopharynx, gastrointestinal tract, vagina, and skin—each with differing topographical, nutritional, physical, and environmental characteristics. For example, the nasopharynx, gastrointestinal tract, and vagina are all non-keratinized mucosal environments with varying degrees of oxygen tension and pH levels. In contrast, the skin provides an aerobic, keratinized epithelial surface for microbial inhabitance.

The oral cavity is a unique environment in that it is divided into several smaller habitats—biotic habitats such as the non-keratinized buccal mucosa, the keratinized mucosa of the tongue and gingiva, the subgingival sulcus, and abiotic surfaces such as the enamel, dental restorations, and dental implants. At any given time, over twenty billion organisms can be found in this environment (Loesche, 1982), representing nearly 700 different species (Aas et al., 2005). Since the oral cavity is an open ecosystem, several of these species may be allochthonous members (transient visitors), however, certain organisms colonize these surfaces (autochthonous constituents) soon after birth and form organized, cooperating communities within these niches, called biofilms (Savage, 1977). It has been shown that, in certain niches (for example the tooth surface), this colonization is a very organized event with a specific temporal and spatial sequence (reviewed by Kolenbrander et al., 2006), and can be driven by environmental and host-determined factors (Mason et al., 2013). The traditional view of these biofilms is that they are comprised of species that live in equilibrium with the host immune defenses—the so-called "commensals." However, commensalism, by definition, is a symbiotic relationship that benefits one species without harming the other. The implications of this are that the oral cavity hosts a diverse microbial community with no major benefits to the host. Since such one-sided relationships are not the norm in nature, the purpose of this perspective is to examine the currently available evidence on the health benefits of hosting a complex oral microbial ecosystem.

Evidence for Habitat Specific Colonization as a Health Benefit

A central characteristic of an ecosystem is habitat-specific colonization. For example, a wetland consists of several habitats extending from tidal creeks into low marshes and climax maritime forests, each with a specific community of flora, fauna, and microflora (Cherry, 2011). According to the physiological hypothesis, habitat specificity offers several benefits to the colonizing species, ranging from predator protection to mating to nutritional abundance (Smiley, 1978). Thus, organisms that require few host-associated benefits occupy a wide range of habitats (*generalists)*, while evolution dictates the emergence of *specialist* species that are confined to a single or narrow range of habitats. Evidence is emerging from microbial ecological systems that habitat specificity also allows a species to regulate gene expression and modify its phenotype to segregate its niche (reviewed by Young, 2006). For example, an organism determines its shape by complex algorithms that take into consideration diverse factors such as nutrient access, cell division and segregation, attachment to surfaces, passive dispersal, active motility, polar differentiation, the need to escape predators, and the advantages of cellular differentiation.

Ecologically, habitat specificity offers several benefits to the hosting species. The presence of certain algal species is important to enhance the calcifying and metabolic activities of coral-reef building anthozoans; and therefore, both species maintain their habitat specificity in all types of environments. In other marine environments, bacteria within specific habitats protect their hosts from fungal infections, detoxify host metabolites, and inhibit epibionts (White and Torres, 2009).

It is well-known that human microbial communities vary significantly by habitat. The oral microbiome is distinct from that of the gut, the ear, and the nasopharynx, even though it is geographically connected to these habitats through the esophagus, Eustachian tubes, and fauces, respectively (Frank et al., 2003; Heinemann and Reid, 2005; Flint et al., 2007; Costello et al., 2009). Within the oral microbiome, structural, spatial, functional, and compositional characteristics of supragingival and subgingival biofilms are remarkably different (Socransky and Manganiello, 1971), as are the characteristics of mucosal and tongue biofilms when compared to these tooth-related habitats (Ximenez-Fyvie et al., 2000; Socransky and Haffajee, 2005; Zaura et al., 2009). For example, *Streptococcus mitis, S. pneumoniae,* and *Granulicatella adiacens* appear to be generalists in the oral ecosystem, occupying both dental and mucosal habitats; while *Rothia dentocariosa, Actinomyces* spp., *S. sanguinis, S. gordonii*, and *A. defectiva* preferentially colonize teeth, and *Simonsiella muelleri* only colonizes the hard palate (Aas et al., 2005). Even with the same environment (supragingival or subgingival), bacterial composition varies considerably based on tooth location and site (Sreenivasan et al., 2010; Simon-Soro et al., 2013a). For example, abundances of *C. gingivalis* and *S. sanguinis* correlate with lower incisors and canines, while *Actinomyces naeslundii* 2 (also known as *A. oris*) demonstrates a positive association with upper anteriors (Haffajee et al., 2009).

While evidence demonstrates the existence of habitat-specific microbial communities in the oral cavity, the benefits conferred by this spatial segregation to community members and the implications of this phenomenon for oral health have not been as well-studied. Most of our current knowledge comes from investigations of specific species, for example, *Porphyromonas*

gingivalis (iron availability and anaerobiosis), *Fusobacterium nucleatum* (pH, anaerobiosis, etc.) and oral *Streptococci* (salivary glycans, simple carbohydrates, etc.). Investigating the effect of spatial segregation on community membership and function would be critical to elucidating the role played by distinct bacterial consortia in the etiology of site-specific diseases such as caries and periodontal disease.

Evidence for Colonization Resistance as a Health Benefit

One of the most important benefits a resident microbial community can offer to the host is resistance to invasion. In environmental ecology, invasion is defined in the process by which an exogenous species establishes itself within a resident community (Shea and Chesson, 2002). However, many human diseases are polymicrobial infections, sometimes occurring due to an overgrowth of opportunistic resident species, suggesting that "pathogens" are already present in a health-compatible environment. Hence, in human microbial ecosystems, several lines of evidence have demonstrated that the role of indigenous bacteria in controlling pathogenic colonization is by preventing pathogen expansion rather than by retarding exogenous acquisition (van der Waaij et al., 1971; Winberg et al., 1993; Drenkard and Ausubel, 2002; Wardwell et al., 2011). Disruption of resident communities with antibiotics is consistently associated with increased colonization by pathogenic species or pathologic overgrowth of certain commensals, leading to disease (Pavia et al., 1990; Pepin et al., 2005; Adams et al., 2007). This effect has been seen in the gut, vagina, and oral cavity (van der Waaij et al., 1971; Winberg et al., 1993; Ubeda et al., 2010). In certain cases, loss of colonization resistance can lead to take-over of the community not only by pathogenic bacteria, but also by higher order organisms, for example *Candida,* in both the vagina and the oral cavity (Budtz-Jörgensen, 1990; Spinillo et al., 1999). On the other hand, replenishing the resident microbiome using probiotics has been shown to reverse the effects of antibiotic-induced pathogen disease in the urinary tract, gut, and the dentition (Madden et al., 2005; Whorwell et al., 2006; Amdekar et al., 2011; Culp et al., 2011). Also, recent evidence from fecal biotherapy studies have demonstrated that restoring a native commensal population has been able to reverse pathogenic *Clostridium difficile* infection (Gough et al., 2011).

Although early evidence from non-microbial ecosystems indicated that highly diverse communities (as defined by those with more types of species) resisted exogenous invasion better than communities with fewer species (Fargione and Tilman, 2005), evidence has been emerging since then to indicate that species abundance (that is, the relative levels of each species within the community) plays a very important role, in some instances, a greater role than does species-richness (Kumar et al., 2006, 2011; Griffen et al., 2012). The first line of defense in colonization resistance is niche saturation, an ecological phenomenon where a certain number of species dominate the community, and resist colonization by pathogenic organisms (Brockhurst et al., 2007). This saturation phenomenon helps create a barrier for exogenous colonization (van der Waaij et al., 1971), prevent pathogen expansion (Gao et al., 2014), and maintains community stability, resulting in mucosal health (Abt and Pamer, 2014).

Change in species diversity is a hallmark of many bacterial dysbiotic conditions; certain diseases like bacterial vaginosis (Fredricks et al., 2005; Oakley et al., 2008), are associated with increase in diversity, while some others, for example, respiratory tract infections (influenza and bacterial pneumonia), and certain gut infections (*H. pylori* and *C. difficile)* are associated with decreased diversity. Interestingly, within the oral ecosystem, while periodontal diseases are associated with an increase in diversity (Loe et al., 1965; Listgarten, 1976; Loesche and Syed, 1978), dental caries is associated with a decrease in diversity (Simon-Soro et al., 2013b). Thus, any deviation from the stringently controlled diversity that is associated with health appears to result in disease.

In summary, literature is emerging in the gut, respiratory, urinary, and vaginal microbiomes supporting the role of colonization resistance as a health benefit. These lines of evidence include (i) pathogen colonization resulting from loss of resident microflora following antibiotic therapy, (ii) reversal of pathogen colonization by probiotic use, (iii) pathogen acquisition following changes in indigenous diversity, and (iv) reversal of pathogenic colonization following bacterial remediation (fecal transplants). Within the oral cavity, the lines of evidence have not been as robust or defined; possibly because bacteria implicated in the etiology of periodontal diseases and dental caries are already present within the health-compatible microbiome (pathobionts) (Jiao et al., 2014). Thus, the role of the oral microbiome in maintaining health may be more to prevent pathogen expansion rather than preventing exogenous acquisition. It is important to recognize the uniqueness of this ecosystem, and target research toward examining the implications of microbial homeostasis in an open, polymicrobial ecosystem.

Importance of Temporal Stability, Resistance, and Resilience in Health

In any ecosystem, three factors contribute longitudinally to health—the ability of the ecosystem to maintain its diversity, structural, and functional framework, as well as its ability to rebound from episodes of disturbance. In the pharyngeal microbiome, loss of temporal stability has been suggested the most proximal cause for the development of respiratory tract infections (Gao et al., 2014). Gao et al. have reported that patients with cystic fibrosis (CF) are the most susceptible to secondary infections, followed by chronic obstructive pulmonary disease (COPD) and asthma. Interestingly, the levels of *Bacteroidetes* were found to be lowest in CF, followed by COPD and asthma. Thus, it is hypothesized that as the protective "cover" offered by *Bacteroidetes* decreases, pathogenic *Proteobacteria* expand from their normal niche in the oropharynx and advance down the respiratory tract; their habitat-specificity being altered by the lack of competition in the "new" niche.

Oral bacteria are acquired at birth, and their colonization in the pre-dentate infant is dependent both on host genotype and on nutrition (van Steenbergen et al., 1997; Kobayashi et al., 2008). Following the development of dentition, a stable

microbiome is acquired that persists into adulthood. There is evidence that bacterial composition remains stable over long periods of time (Rasiah et al., 2005; Kumar et al., 2006), even following routine dental prophylaxis and recolonization (reviewed by Teles et al., 2013). Less is known about resilience of oral bacterial communities. We have recently demonstrated that subgingival and marginal biofilms return to nearly 90% of their original compositional structure following repeated episodes of gingivitis (Joshi et al., 2014) in never smokers, but that this resilience is lost in current smokers. The host response to this "newer" microbiome is a higher than before pro-inflammatory response, suggesting that repeated episodes of gingivitis in smokers may present a higher risk for disease than in nonsmokers.

The Role of Bacterial Cooperativity in Resistance, Stability, and Resilience of Microbial Communities

Colonization resistance and temporal stability are mediated through several inter-bacterial and host-bacterial interactions. This section will focus on what we currently know about how bacterial interactions allow species to selectively colonize, survive, and thrive in a habitat. Bacteria within human ecosystems depend upon each other for structural and metabolic cooperativity; a constraint that dictates their relative proportions within the community (Wintermute and Silver, 2010). This mutual symbiosis is one important factor in maintaining the abundances of genetically distinct species in a community and therefore, contributes significantly to microbial homeostasis. Bacterial colonization of a habitat begins through non-random species selection. This non-random event is facilitated by several inter-bacterial interactions, for example, nutritional syntrophy, coaggregation, antagonism, and communication.

Syntrophy or nutritional symbiosis [also known as cross-feeding (from Greek for eating together)] is one of the oldest mechanisms facilitating the formation of polymicrobial communities. Work from the gut has provided insight into the role of symbionts in shaping the evolution of microbial components of this microenvironment through lateral gene transfer. For example, gut dwelling *Bacteroidetes* have used this mechanism to vary their cell surface, sense their environment, and harvest nutrient resources present in the distal intestine (Xu et al., 2007).

In oral biofilms, this phenomenon is not as well-characterized, however, it has previously been shown that *Veillonella* and *Streptococcus, two* of the earliest and most abundant genera to colonize oral biofilms, share a nutritional syntrophy, in that the *Veillonellae* utilize the lactate that is produced by the *Streptococci* as a food source (Kuramitsu et al., 2007). Also, *Streptococcus sanguis* and *S. oralis* exhibit synergy in degrading mucins, thereby allowing efficient utilization of host glycopolysaccharides for nutrition (Van der Hoeven and Camp, 1991).

Coaggregation among the early colonizers is another important mechanism that controls the composition of tooth-associated biofilms. *Streptococci*, due to the presence of Antigen I/II receptors for salivary agglutinin glycoprotein, are the primary orchestrators of coaggregation events. Not only do they bind to salivary pellicle, dentin and collagen, but also, the presence of these receptors is essential for acquisition of another early colonizer *A. naeslundii* (Kolenbrander et al., 2006). Also, incorporation of the bridge species, *F. nucleatum* into the biofilm has been shown to be dependent on *A. naeslundii* (Periasamy et al., 2009). Recent evidence suggests an important role for *Candida* species in maintaining oral health, by providing metabolic, chemical, and physical support for colonization by certain bacteria (reviewed by Krom et al., 2014).

Antagonism is the collective ability of the normal microbiota to prevent colonization of exogenous and opportunistic pathogens. In the gut, for example, the presence of butyrate, a short chain fatty acid produced as a metabolic byproduct by some commensals, down regulates expression of virulence genes in *Salmonella* spp. (Gantois et al., 2006) The earliest reports of bacterial antagonism in the oral environment came from Hillman and Socransky, who demonstrated that plaque from periodontally healthy individuals was capable of inhibiting growth of certain periodontal pathogens (Hillman et al., 1985). Evidence has shown some commensal oral bacteria have antagonistic activity against periodontopathogens (van Essche et al., 2013). Specific examples of bacterial antagonism by means of producing metabolites in the oral cavity include hydrogen peroxide production by streptococcal species to inhibit growth of periodontopathongens (Hillman et al., 1985) and lactic acid production to prevent *Pseudomonas aeruginosa* incorporation into the biofilm (He et al., 2011). Evidence has shown *Streptococci* exhibit antagonistic properties toward certain *Staphylococci* in the oral cavity as well (Krzeminski and Raczynska, 1993). Some indigenous microbiota take colonization resistance a step farther by producing specific antibiotics, such as bacteriocin production in strains of *Streptococcus salivarius*, that act on specific pathogens to prevent their colonization of the community (Sanders and Sanders, 1982). This mechanism has also been studied in response to caries-causing bacteria (reviewed by Kreth et al., 2009). *S. sanguinis* and *S. gordonii* produce hydrogen peroxide, a chemical that decreases proliferation of *S. mutans* in a cell-density independent manner. *S. oligofermentans* utilizes the lactic acid produced by *S. mutans* to generate H_2O_2.

Microbes are also in direct competition for available nutrients, and in many cases the indigenous microbiota create food webs where one species end product is used by another species (Ley et al., 2006). This sequestration of nutrients by the indigenous microbiota is designed to make the colonization of non-indigenous species very difficult (Freter et al., 1983). In the gut for example, the nutrient depletion provided by the indigenous microbiota plays a role in suppressing *C. difficile* overgrowth (Wilson and Perini, 1988).

In summary, structural, metabolic, and chemical interactions between bacteria play an important role in maintaining community hemostasis by supporting the critical proportions of these species in a health-compatible microbiome. The evidence for health benefits of these interactions has been exemplified in the caries literature.

Educating the Host Immune System as a Health Benefit

Evidence is emerging to suggest that lack of a bacterial stimulus can lead to the development of atopy, a genetic predisposition to general allergic reactions. Several mechanisms have been postulated to explain this connection, with the "Hormetic Theory" being the most widely accepted (Bukowski and Lewis, 2007). The Hormetic theory suggests that exposure to a commensal bacterial flora during early years of life serves to educate the immune system, enabling it to distinguish between pathogens and host proteins. Children with low levels of the commensals *Lactobacillus* and *Bifidobacterium* demonstrate a greater predisposition to allergies (Bjorksten et al., 2001; Sepp et al., 2005). Further, administration of prenatal *Lactobacillus GG* to mothers of high-risk infants decreased the incidence of atopy (Kalliomaki et al., 2001).

Recent evidence suggests the human microbiome is also capable of directly stimulating various components of the innate and adaptive immune responses. Much of this evidence comes from studying the gut which houses a complex and diverse microbial community (Hooper and Gordon, 2001; Ley et al., 2006; Neish, 2009). The first line of immunity to foreign species is the physical barrier of the epithelial tissue. In the gut, the gut-associated lymphoid tissue (GALT), is severely underdeveloped in germ-free mice, but undergoes enlargement when exposed to antigen (Pollard and Sharon, 1970), thus suggesting a role of host-microbe interactions in the initial development of early immunity. Additional animal studies involving germ-free model systems demonstrate greater antigen transportation compared to animals with a resident biofilm (Sudo et al., 1997). Further, germ-free animals mount a severe immune response when exposed to commensal bacteria.

Contributions of Bacteria in Establishing Epithelial Barrier Function

Colonization of a germ-free model by *B. thetaiotaomicron*, a member of the indigenous gut flora, helps establish the epithelial barrier by inducing the expression of Paneth cell proteins (Hooper et al., 2003) and a decay-accelerating factor that facilitates repair (Hooper et al., 2001). Further, the release of indole, a microbial quorum-sensing molecule, has been shown to increase expression of tight junction and adherent junction molecules in the colonic epithelial tissue (Shimada et al., 2013). The increased expression of ZO-2, a tight junction protein, has also been observed following the administration of probiotics, namely *E. coli* Nissle 1917, both *in vitro* (Zyrek et al., 2007) and *in vivo* (Ukena et al., 2007).

In the oral environment, Ye et al. characterized an increase in the expression of tight junction components in oral epithelium following binding of the normal microbiota species *S. gordonii* (Ye et al., 2013) and identified that binding of these commensals through the CD24 receptor of oral epithelial is responsible for this health associated tissue phenotype (Ye et al., 2014).

Thus, currently, there is minimal evidence in the oral cavity to indicate a role for the indigenous microbiome in affecting tissue phenotype, although emerging evidence suggests that this may an important avenue of investigation.

Contributions of Bacteria to Developing TLRs

An important link between microbes and epithelial cells in innate immunity is Toll-like receptors (TLRs). Evidence suggest that the expression of TLRs on gut epithelial cells is decreased in germ-free mice when compared to mice with conventional microbiota (Lundin et al., 2008). TLRs respond to both commensals and pathogens, but evidence now suggests TLRs interaction with commensals contributes to intestinal epithelial homeostasis and protection from injury (Rakoff-Nahoum et al., 2004). The location of TLR expression in the gut epithelium has been suggested to play a role in the hosts ability to tolerate commensals and mount a more targeted inflammatory attack against pathogens (Furrie et al., 2005). Evidence also suggests that a benefit of probiotics for maintaining a healthy gut works through the TLR9 pathway, which mediates the anti-inflammatory effects observed with probiotic use (Rachmilewitz et al., 2004). The activation of TLR2 in a stress-induced inflammation model also suppressed mucosal inflammation in the gut by promoting tight junction integrity in the epithelial barrier (Cario et al., 2007). On the skin, commensal *Staphylococcal* species inhibit skin inflammation through the regulation of TLR3 by *Staphylococcal* lipoteichoic acid (LTA) activation of a TLR2-dependent pathway (Lai et al., 2009). Subsequently, the activation of TLR2 by *Staphylococcus epidermis* induces keratinocyte expression of antimicrobial peptides to enhance innate immunity toward pathogens (Lai et al., 2010).

Bacteria and Neutrophil Function

The cellular components of the innate immune system primarily include neutrophils. Evidence suggests the recruitment of neutrophils to tissue is increased by the presence of the mircobiota (Kanther et al., 2014). Similarly, work from Zenobia et al. has provided evidence that commensal bacteria in the oral cavity selectively upregulate CXCL2 expression leading to an increase in neutrophil recruitment to "prime" healthy gingival tissue (Zenobia et al., 2013). Interleukin-8 is another important chemokine in the innate immune pathway known to attract neutrophils and enhance phagocytosis. The normal microbiota has been shown to induce IL-8 (Darveau et al., 1998; Vankeerberghen et al., 2005), presumably to recruit neutrophils to a potential pathogen colonization site to help prevent overgrowth of pathogens. Not only does the commensal microbiota play a role in recruiting neutrophils, but evidence from a germ-free (Clarke et al., 2010) model suggests that a lack of commensal microbiota reduces phagocytosis and antimicrobial killing activity (Ohkubo et al., 1990, 1999). Commensal bacteria also induce low-level expression of human beta defensins, presumably to keep the innate immune system in a limited activation state (Vankeerberghen et al., 2005) and have been implicated in the regulation of gene expression of the complement system, another important arm of innate immunity (Chehoud et al., 2013).

In summary, a large body of evidence suggests that the host associated microbiome plays an important role in regulating the innate immune system, through epithelial bacterial function, bacterial recognition pathways and signals and innate immune

cell functions. However, a similar level of evidence is currently lacking in the oral microbiome.

Bacteria and Regulatory T Cell Education

Although significant evidence exists supporting the role the indigenous microbiota plays in fortifying innate immunity, evidence continues to grow linking commensal microbiota to the adaptive immunity cascade of events. The peripheral education of regulatory T cells (Treg) in the colon by antigens derived from the commensal microbiota ensures the local tolerance of this microbial community by the host (Lathrop et al., 2011). One well-identified human commensal, *Bacteroides fragilis*, directs Treg cell education using the immunomodulatory molecule, polysaccharide A (PSA) (Round and Mazmanian, 2010). PSA induces an IL-10 response in T cells that inhibits Th17 expansion preventing future mucosal damage (Round et al., 2011). Similar Treg cell induction and anti-inflammatory effects are seen with colonization of *Clostridia* species (Atarashi et al., 2011, 2013; Chiba and Seno, 2011; Nagano et al., 2012). In contrast, colonization of the gut intestinal tissue by segmented filamentous bacteria leads to an increase in mature Th17 cells and Th1 cells (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009).

Similar evidence exists in the oral cavity where members of commensal oral bacteria prime dendritic cells for Th2 and Treg differentiation (Kopitar et al., 2006). Shin et al demonstrated that *F. nucleatum*, an oral commensal, induces Th1 and Th3 immune responses, while *Treponema denticola,* a pathogenic species, induced a Th1-dominant response (Shin et al., 2013).

Bacteria and B Cell Education

Lastly, evidence indicates that commensal microbiota play a role in B cell development and maturation. In human infants, maturation of the mucosal defense system, particularly cells that secrete IgA and IgM, is dependent on the presence of a normal gut flora (Klaasen et al., 1993; Gronlund et al., 2000). This suggests that the development of immune tolerance is dependent on early and sustained exposure to a stable biofilm. Recent evidence suggests that B cell maturation may in fact be dependent on intestinal bacterial colonization as mice colonized early with *E. coli* and *Bifidobacteria* have an increased population of CD20+ B cells expressing memory marker CD27 (Lundell et al., 2012). Following development of the lymphoid tissue, the host immune system faces the challenge of finding balance between mounting a swift response toward invading pathogens, but controlling that response against commensals. One way the host controls this response in mice is by using dendritic cells to "sample" the gut lumen and keep live commensals engulfed for a few days to selectively induce IgA production (Macpherson and Uhr, 2004). This localized production of IgA helps maintain immune system homeostasis and allows the human host to distinguish between a commensal and pathogenic colonization providing the necessary defense mechanisms to control an infection.

Other Benefits

Evidence is emerging to suggest that oral bacteria may play a critical role in nitric oxide NO homeostasis (Kapil et al., 2013; Hyde et al., 2014). They do so by reducing dietary nitrate to bioactive NO, a critical symbiotic relationship since humans lack the enzymes to carry out this function. The effects of NO in maintaining cardiovascular integrity are well-established in literature. Thus, recent studies point to a cardio-protective role for oral bacteria; and may provide a critical link in the oral-systemic health connection.

Thus, although the traditional view of an indigenous microbiome is one that provides a nondestructive inflammatory stimulus to the host, thereby ensuring host-bacterial equilibrium, emerging evidence indicates that this community appears to play an active role in developing the host innate immunity and priming the adaptive immune response.

Future Steps

The role of the microbiome in maintaining health in several human ecosystems is an emerging and exciting field of study. While the health benefits of supporting a large microbial community are actively being explored in reference to the gut, genito-urinary tract, and respiratory system; similarly robust evidence is lacking in relation to the oral microbiome. Several decades of research have been focused on exploring the microbiota associated with oral diseases. While the importance of this quest cannot be downplayed, it is sometimes easy to forget that the ultimate aim of treating disease is to restore health, and the only successful method of preventing disease is by maintaining health. Hence, it is important to focus on the health benefits provided by our microbial fellow travelers and to expend some effort in cataloging and characterizing not only this community, but also the host determinants that play a role in shaping this population.

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Acquiring and maintaining a normal oral microbiome: current perspective

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The oral microbiota survives daily physical and chemical perturbations from the intake of food and personal hygiene measures, resulting in a long-term stable microbiome. Biological properties that confer stability in the microbiome are important for the prevention of dysbiosis—a microbial shift toward a disease, e.g., periodontitis or caries. Although processes that underlie oral diseases have been studied extensively, processes involved in maintaining of a normal, healthy microbiome are poorly understood. In this review we present our hypothesis on how a healthy oral microbiome is acquired and maintained. We introduce our view on the prenatal development of tolerance for the normal oral microbiome: we propose that development of fetal tolerance toward the microbiome of the mother during pregnancy is the major factor for a successful acquisition of a normal microbiome. We describe the processes that influence the establishment of such microbiome, followed by our perspective on the process of sustaining a healthy oral microbiome. We divide microbiome-maintenance factors into host-derived and microbe-derived, while focusing on the host. Finally, we highlight the need and directions for future research.

Keywords: oral microbiome, placenta, tolerance, mucosal immunity, stability, colonization resistance

INTRODUCTION

The oral microbiota needs to cope with daily physical and chemical perturbations from the intake of food and personal hygiene measures. These include fluctuations in temperature, pH, antimicrobial and dietary components, and mechanical sheer forces from brushing and mastication. Intriguingly, a long-term stable microbiome is maintained in the oral cavity, as demonstrated by Rasiah and colleagues by following an individual saliva donor over a period of 7 years (Rasiah et al., 2005). Recent data from the NIH Human Microbiome Project (HMP) revealed that the oral microbiome has the largest core of commonly shared microbes among unrelated individuals compared to other habitats such as gut or skin (Costello et al., 2009; Li et al., 2013; Zhou et al., 2013).

A key question is what governs the stability of the oral microbiome in health? Biological properties that confer stability in the microbiome are important for the prevention of dysbiosis—a microbial shift toward a disease, e.g., periodontitis or caries and sustaining general health (for review see Wade, 2013). Although processes that underlie oral diseases have been studied extensively (Bartold and Van Dyke, 2013; Bradshaw and Lynch, 2013; Nyvad et al., 2013; Belibasakis, 2014), processes behind the maintaining of a normal microbiome are poorly understood. In this review we present our hypothesis on how a healthy oral microbiome is acquired and maintained. We start by defining what constitutes a normal oral microbiome. Then we present our hypothesis on the mechanisms for acquiring a stable normal microbiome. Finally, we discuss some of the mechanisms involved in maintaining such a microbiome and highlight the directions for possible further research.

WHAT CONSTITUTES NORMAL ORAL MICROBIOME?

The human oral cavity is colonized by a wide range of microorganisms. Besides bacteria and fungi, Archaea, viruses and protozoa form a part of a normal microbiome (Wade, 2013). Current reports on a normal oral microbiome however are limited to the "bacteriome" (subsequently referred to as "microbiome") and very limited reports on the mycobiome—fungal microbiome (Ghannoum et al., 2010; Dupuy et al., 2014; Mukherjee et al., 2014). Current knowledge on the role of fungi as part of a healthy oral microbiome has been recently reviewed and is therefore not further discussed here (Krom et al., 2014). The microbiome has been studied in great detail and phylogenetic information of oral bacteria is gathered in databases dedicated to oral cavity (Palmer, 2014). The HMP assessed microbiome composition of nine intraoral sites (buccal mucosa, hard palate, keratinized gingiva, palatine tonsils, saliva, sub- and supragingival plaque, throat and tongue dorsum) from about 200 subjects and found 185–355 genera, belonging to 13–19 bacterial phyla (Zhou et al., 2013). An individual sample (i.e., from a single site of a single volunteer) contained sequences classified to 20–50 genera from 6 to 9 phyla. **Table 1** summarizes the high abundance

Table 1 | The core bacterial taxa in the oral cavity from over 200 healthy individuals participating in HMP (Li et al., 2013).

Sample type	High abundance core genera in >75% samples at >10% abundance	Other major core genera in >80% samples at >1% abundance	Minor core genera in >50% samples
Buccal mucosa	*Streptococcus (2)*	*Uncl. Pasteurellaceae (16, 19)* *Gemella (11)*	*Atopobium* *Uncl. Prevotellaceae* *Uncl. Bacilli* *Catonella*
Hard palate	*Streptococcus (2, 6)*	*Uncl. Pasteurellaceae (16)* *Veillonella (4)* *Prevotella (10)* *Uncl. Lactobacillales (13)* *Gemella (11)*	*Mogibacterium* *Catonella*
Keratinized gingiva	*Streptococcus (2)* *Uncl. Pasteurellaceae (19)*		*Uncl. Bacilli*
Palatine tonsils		*Streptococcus (2, 6)* *Veillonella (4)* *Prevotella (10)* *Fusobacterium (9)* *Uncl. Pasteurellaceae (16)*	*Mogibacterium* *Uncl. Firmicutes*
Saliva		*Prevotella (10)* *Streptococcus (2, 6)* *Veillonella (4)* *Uncl. Pasteurellaceae (16)* *Fusobacterium (9)* *Porphyromonas (7)* *Neisseria (–)*	*Uncl. Actinomycetales* *Tannerella* *Kingella*
Subgingival plaque		*Streptococcus (2)* *Fusobacterium (9)* *Capnocytophaga (–)* *Prevotella (–)* *Corynebacterium (–)* *Uncl. Pasteurellaceae (–)*	*Uncl. Firmicutes*
Supragingival plaque		*Streptococcus (2)* *Capnocytophaga (–)* *Corynebacterium (15)* *Uncl. Pasteurellaceae (–)* *Uncl. Neisseriaceae (21)* *Fusobacterium (9)*	*Uncl. Betaproteobacteria*
Throat	*Streptococcus (2, 6)*	*Veillonella (4)* *Prevotella (10)* *Uncl. Pasteurellaceae (16)* *Actinomyces (–)* *Fusobacterium (9)* *Uncl. Lachnospiraceae (–)*	*Mogibacterium* *Uncl. Firmicutes*
Tongue dorsum	*Streptococcus (2, 6)*	*Veillonella (4)* *Prevotella (10)* *Uncl. Pasteurellaceae (16)* *Actinomyces (14)* *Fusobacterium (9)* *Uncl. Lactobacillales (13)* *Neisseria (8)*	*Uncl. Actinomycetales* *Uncl. Bacilli* *Peptostreptococcus*

In the parentheses—the corresponding OTU in the genus or family. Uncl, unclassified.

core genera (defined as genera present at >10% abundance and at >75% ubiquity) and other major core genera (>1% abundance at >80% ubiquity), as well as operational taxonomic units (OTUs, 16S rRNA gene variable v3–v5 region sequences, clustered at 97% similarity) in these oral samples (Li et al., 2013). A single OTU, *Streptococcus* OTU#2, dominated nearly all oral mucosal sites of this large cohort. The reads of this OTU were obtained from Kelvin Li (personal communication) and blasted (NCBI web site, megablast against 16S ribosomal RNA sequences, default parameters) for finer taxonomic classification. The most abundant read of this OTU was identical over its entire length to 16S rRNA gene sequences of *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus peroris*.

The microbiome has evolved through hundreds of thousands of years of co-habiting into a microbe-human symbiosis with mutual benefits (Hooper and Gordon, 2001; Clemente et al., 2012). The oral microbiome in newborns has been shown to seed the gut microbiome that first resembles that of the oral cavity and diverges in 2 weeks time to gut-specific communities (Costello et al., 2013). Recently a significant association between distinct microbial community types of stool and oral samples from HMP study adult population was demonstrated (Ding and Schloss, 2014). Based on current knowledge it is apparent that the acquisition of such normal, beneficiary microbiome by newborns is an essential process. Infants are colonized rapidly after birth by bacteria present in their direct environment, through bacterial transfer from their mother but also from other sources. How does a newborn discriminate between "friend and foe" among the diverse microbes in its postnatal environment? If any randomly trespassing microbe would permanently colonize oral cavity of the newborn, there would be no core microbiome (**Table 1**) and the benefit of the long-lasting co-evolution would be lost. We suggest that development of fetal tolerance toward the microbiome of the mother during pregnancy is the major factor for a successful acquisition of a normal microbiome. We have summarized our hypothesis in the text below and in **Figure 1**.

DEVELOPMENT OF PRENATAL TOLERANCE TO MOTHER'S ORAL MICROBIOME

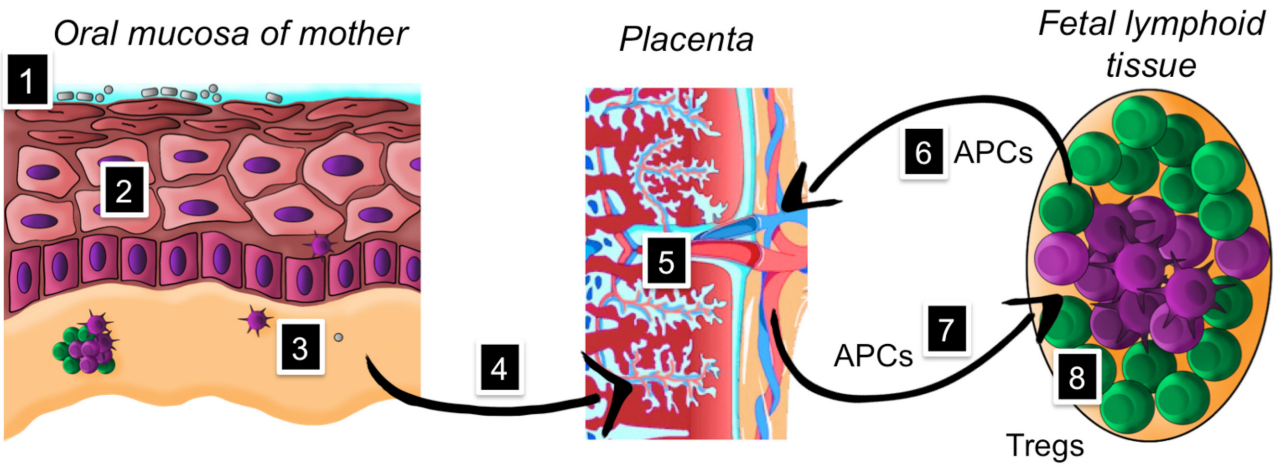
Although the first encounter of a newborn with microbiota is considered to be postnatal, there is clinical evidence for microbial presence in placenta, umbilical cord blood, amniotic fluid, and meconium in full-term pregnancies without overt infection (Bearfield et al., 2002; Jiménez et al., 2005, 2008; Stout et al., 2013; Aagaard et al., 2014). Experimental intravenous infection of pregnant mice with pooled salivary or plaque microbes resulted in colonization of placenta by selected oral microorganisms (Fardini et al., 2010). Recent comparison of sequencing results from 320 placental microbiomes with the HMP dataset showed that the placental microbiome does not resemble vaginal or gut microbiomes as previously thought, but at least at a phylum level is most similar to normal oral microbiome, especially that from tongue and tonsils (Aagaard et al., 2014). These studies may suggest that the placental microbiome has a biological function. We propose that during pregnancy the placenta becomes an antigen-collecting site for the fetal immune system to be "trained" in antigen tolerance. We suggest a hematogenous route for indigenous microbes to placenta during pregnancy. Pregnant women have increased gingival bleeding, diagnosed as pregnancy gingivitis (Niederman, 2013). We propose a new role for the increased gingival bleeding: by opening the vascular bed, oral bacteria from the mother become available in blood and thus gain access to the placenta. Jeurink and colleagues have introduced a similar mechanism for the formation of the breast milk microbiome. This involves immune cell education by the pregnancy hormone progesterone resulting in transportation of bacteria from the mother to her mammal glands (Jeurink et al., 2013). A similar process could be responsible for transporting bacteria to the placenta. Microbial cells are trapped in placental tissue to be presented to the fetal immune system. In the prenatal period, fetal antigen presenting cells (APCs) interact with the mother's microbial antigens and return to fetal peripheral lymphoid organs. The human fetus harbors large numbers of peripheral regulatory T cells (Tregs) with immunosuppressive activity (Takahata et al., 2004). Fetal Tregs can be retrieved also from umbilical cord blood offering a promising perspective for transplantation medicine, while newborns have been shown to have higher proportions of thymically derived Tregs than adults (Rabe et al., 2014). The fetal Tregs are preventing undesirable alloreactivity to maternal derivatives during the pregnancy (Takahata et al., 2004). As a result, the fetus develops prenatal tolerance to the mother's microbiome and regards it as "safe" during postnatal encounters with these bacteria. There is experimental and clinical evidence for development of fetal antigen-specific tolerance: human fetal Tregs become functionally suppressive after stimulation with maternal alloantigens and persist at least until early adulthood (Mold et al., 2008). If and to what extent early tolerance of the fetus toward the oral bacterial species is key to the colonization of the mouth and gastro-intestinal tract of the newborn and its impact in health development in the earliest phases of life needs to be investigated.

ACQUIRING THE ORAL MICROBIOME

Vertical transmission from mother to child starts at birth. The delivery mode (vaginal or Caesarian) will to a large extent determine which microorganisms—vagina or skin-derived—will be encountered first by the newborn (Dominguez-Bello et al., 2010). This affects the diversity of the oral microbiome: vaginally born infants showed higher taxonomic diversity at 3 months of age (Lif Holgerson et al., 2011). Interestingly, the birth mode may have a lasting impact as infants born with Caesarian section acquired *Streptococcus mutans* almost 1 year earlier (at 17.1 months of age) than vaginally born infants (28.8 months) (Li et al., 2005).

The method of feeding (breast-feeding or infant formula) affects the infant's microbiome as well. Breast-fed infants of 3 months of age carried oral lactobacilli with antimicrobial properties not found in formula-fed infants (Holgerson et al., 2013; Romani Vestman et al., 2013). In addition to this vertical transmission mechanism, horizontal transmission of oral microbiota, e.g., among siblings and other people sharing the same environment, contributes to oral microbiome diversity (Baca et al., 2012; Stahringer et al., 2012).

Once established, the microbiome should be sustained. Below we summarize our perspective on this process by dividing it into host-derived and microbe-derived microbiome maintenance



1 – Oral microbiome of mother; microbial cells attach to the oral epithelium
2 – Loosening of epithelial cell contacts due to pregnancy hormones; oral bacterial penetration
3 – Bacterial cells reach lamina propria and vascular tissues of the oral mucosa
4 – Transfer of bacterial cells to the placenta via blood stream
5 – Oral bacteria of mother are trapped in placental tissue
6 – Fetal Antigen Presenting Cells (APCs) acquire the alloantigen information in placenta
7 – Fetal APCs return to fetal peripheral lymphoid organs
8 – Fetal regulatory T cells (Tregs) that recognize mother's microbiome as "safe" are generated in fetal lymphoid tissue

FIGURE 1 | Hypothesis on the role of placental microbiome in the development of fetal tolerance toward oral microbiome of mother.

factors. Since microbial factors have been extensively addressed elsewhere (Kuramitsu et al., 2007; Wright et al., 2013), we focus here mainly on the role of host factors.

HOST-DERIVED MICROBIOME MAINTENANCE FACTORS

The interactions between the microbiome and the host are bidirectional. In the absence of timely and adequate stimulation by the microbiome, germ-free mice show extensive deficiencies in intestinal immune system development, with reduced lymphoid tissue and fewer lymphocytes (Macpherson and Harris, 2004). In humans, early-life exposure to microbiota is protective for immune-mediated diseases such as asthma and inflammatory bowel disease (Olszak et al., 2012). The interplay between the microbiome and innate and adaptive immunity is site specific. The skin microbiome of mice is largely unaffected by genetic manipulation of innate or adaptive immunity, while mucosal sites—oral and gut microbiomes—are shaped by innate and adaptive immune responses (Scholz et al., 2014).

The human immune system develops in a continuing dialogue with the commensal microbial populations. One communication route between microbiota and host is via the host pattern recognition receptors (PRRs), with the Toll-like receptor (TLR) family playing a key role. In the gut, ligands binding to TLR-2 can favor Treg expansion (Sutmuller et al., 2006), while TLR-9-mediated recognition of DNA from gut flora is essential for effector T-cells to overcome Treg inhibition and mount an immune response (Hall et al., 2008). Cells of the oral mucosa (keratinocytes, macrophages, dendritic cells (DCs), polymorphonuclear leukocytes and natural killer cells) express most of the TLRs (Feller et al., 2013), while altered expression patterns of TLRs have been found in oral pathology (Janardhanam et al., 2012).

Despite dense bacterial colonization, acute infections are rare in the oral mucosa, suggesting that this site is predominantly tolerogenic (Novak et al., 2008). Mucosal DCs are the arbiters of mucosal tolerance: on the one hand they are capable of mounting an effective defense against harmful pathogens, while on the other hand they inhibit immune reactions against antigens derived from commensal bacteria, preserving the health benefits acquired through thousands of years of co-evolution. DCs in the oral mucosal epithelium are of the Langerhans cell subtype and they employ complex regulatory mechanisms: deletion of T-cells via apoptosis, functional inactivation of T-cells, inhibition by co-inhibitory receptors, but also the development of antigen specific Tregs (Novak et al., 2008). Expression of LPS receptor CD14, TLR2, and TLR4 by DCs in the non-inflamed oral mucosa is crucial for the induction of tolerance. One of the mechanisms involved is the induction of Tregs characterized by interleukin (IL)-10 and transforming growth factor (TGF)-β secretion (Allam et al., 2008). In the gut, resident DCs are programmed by intestinal epithelial cells to suppress inflammation and promote immunological tolerance; in the case of a pathogen attack, "non-educated" DCs are recruited to initiate

inflammation and a protective immune response against the invader (Iliev et al., 2007). A similar mechanism could also account for the oral mucosal tolerance. Indeed, a significant infiltration of pro-inflammatory plasmacytoid DCs is found in oral inflammation (Santoro et al., 2005).

Besides the PRRs, the host may also use chemical sensing to monitor microbial activity. Well known is epithelial signaling of short chain fatty acids involving dedicated G-coupled receptors (GPR41, GPR43) on gut epithelial cells (Layden et al., 2013). Recent studies suggest a direct link between secreted bacterial products and chemosensory activation mechanisms for mucosal clearance (Lee et al., 2014). Oral mucosa, but also airway epithelial cells and airway smooth muscle cells express bitter taste receptors (T2Rs) (Prince, 2012). The T2R38 receptor is activated directly by acyl-homoserine lactone (AHL) quorum sensing molecules produced by *Pseudomonas aeruginosa* and other Gram-negative bacteria and thus provide a mechanism for chemical sensing of bacterial colonization (Lee et al., 2012). Genetic differences in the T2R38 receptor, conferring an increased ability to perceive the bitter-tasting phenyl-thiocarbamide (supertaster phenotype), have been shown to underlie differences in the ability to signal presence and subsequent clearance of *P.aeruginosa* biofilm by respiratory epithelial cells (Lee et al., 2012). Intriguingly, specific alleles of the T2R38 bitter taste receptor have also been associated with a reduced risk for caries (Wendell et al., 2010). While this association was significant for the primary dentition, no significant associations could be identified for the mixed or permanent dentition groups. In addition, decreased taste sensitivity for another bitter-tasting chemical, 6-n-propylthiouracil, was associated with increased risk for dental caries and higher mutans streptococci counts (Shetty et al., 2014). Although the ability to taste bitter components may influence dietary habits, it is tempting to suggest a mechanistic relationship between bitter taste and antimicrobial defense also in the oral cavity.

An important oral immunity factor is delivered *via* saliva and gingival crevicular fluid—the secretory immunoglobulin A (S-IgA). These antibodies limit and control microbial adhesion and colonization (Feller et al., 2013). IgA proteases that neutralize S-IgA are known virulence factors of human pathogens such as *Neisseria meningitidis* and *Streptococcus pneumoniae*. However, also commensal streptococci such as *Streptococcus mitis 1*, *Streptococcus oralis* and *Streptococcus sanguinis* are able to produce IgA proteases (Kilian et al., 1996). These commensal oral streptococci are primary colonizers and the major species colonizing infants and mucosal sites in adults (Marsh et al., 2009). Their ability to circumvent S-IgA guarantees survival in the oral cavity and underlines their long-lasting symbiotic co-evolution with the human host.

Salivary glycoproteins contain glycans that may act as decoys to prevent pathogens from adhering to epithelial cells, thereby influencing a healthy microbial homeostasis. This was shown *in vitro* on inhibition of adhesion of the fungus *Candida albicans* to epithelial cells (Everest-Dass et al., 2012). Other salivary proteins that influence the oral microbiome include lactoferrin, agglutinins, lysozyme, peroxidase, statherin, histatins, defensins, and mucins (for extensive review see Dodds et al., 2005). For instance, histatin 5 has candicidal activity and its concentration is reduced in saliva of elderly and HIV patients (Khan et al., 2013)—two populations prone to candidiasis. Salivary flow rate as well as composition thus play key role in maintaining a healthy oral microbiome.

The impact of the immune system on oral health is most obvious once it becomes dysfunctional, as in hematopoietic stem cell transplant patients who have received immunosuppressive therapy. In these patients, the mucosal barrier is often damaged leading to severe mucositis with life-threatening viral and fungal infections (Petti et al., 2013), as well as oral infections by non-oral species (Soga et al., 2011; Diaz et al., 2013).

MICROBE-DERIVED MICROBIOME MAINTENANCE FACTORS

Co-evolution of the microbiome with the host has resulted in host-associated microbial communities that are equipped with mechanisms that allow them to prevent colonization and establishment of foreign microbes, so called "colonization resistance" (He et al., 2013). An extensive review on interspecies interactions within oral communities has been provided by Kuramitsu and colleagues. The authors distinguished five types of interaction between oral bacteria: competition for nutrients, synergy, antagonism, neutralization of virulence factors and interference in signaling mechanisms (Kuramitsu et al., 2007). Integrity of the microbial community is maintained by specific inter-microbial adhesion, cell signaling through cell-to-cell contact, metabolic interactions and quorum sensing (Wright et al., 2013). Recently a social structure in the murine oral community was reported where a concerted action by "sensor," "mediator" and "killer" bacteria in the community formed a pathway that prevented colonization by the non-oral species *Escherichia coli* (He et al., 2013).

Besides bacterial inter-species communication, also interkingdom communication plays a role in oral microbial ecosystem (Morales and Hogan, 2010; Jarosz et al., 2011). Bacteria produce a range of signaling molecules that affect *C. albicans* biofilm formation or morphogenesis, while *C. albicans* metabolites are known to influence bacterial growth (Wright et al., 2013). Such interactions, as well as interkingdom adhesion events, are also likely with the other members of the oral mycobiome (Krom et al., 2014).

FUTURE RESEARCH DIRECTIONS

A stable ecosystem is the result of complex interactions between all members of the system (Jenkinson, 2011). The entire oral microbiome of an individual, in addition to bacteria also fungi, viruses, Archaea and protozoa, has so far not been approached as an entity. The current knowledge is based on studying individual community components without the complexity of their mutual interactions. The first article on assessment of both, fungal and bacterial profiles in the same oral samples has just been published (Mukherjee et al., 2014), although focusing on diseased individuals, infected with HIV.

Microbiology of oral health has been neglected for decades. With the advent of health-related microbiome research grants such as HMP, an important turning point has been set, with a paradigm shift from disease as a starting point toward health as main interest. Since our current knowledge on the mechanisms

of oral microbiome stability is only revealing the tip of the iceberg any initiatives similar to NIH HMP need to be encouraged. The Dutch Top Institute of Food and Nutrition (TIFN) (www.tifn.nl) has established public-private partnership that aims to identify the biological processes in the oral ecosystem responsible for maintaining oral health and to develop *in vitro* and *in vivo* technologies for the development of novel preventative strategies and to evaluate their efficacy. The project is based on the hypothesis that oral health reflects the ability of the oral ecosystem to adapt to and counteract perturbing stresses, where the oral ecosystem is defined as the oral microbiota, the saliva and host (mucosal) immunity.

The introduction of novel sequencing technologies has led to a significant advance in our knowledge of the oral microbiome in its broadest sense, and has revealed a stable commensal population, suggesting symbiotic beneficial relationship. However, the underlying principles of the beneficial interactions between host and the oral microbiota urgently require attention. Intriguing questions relate to the acquisition of the oral microbiota in early life, with a possible involvement of pre-natal tolerance development toward the oro-pharyngeal microbiota of the mother. Why do placentas in healthy pregnancies harbor microbiota at all and why does this microbiome resemble the oral communities? Is the fetus already seeded with maternal microbes *in utero*, as has been proposed recently (Aagaard et al., 2014)? How are these microbiota transported to placenta? Is this transportation selective? Do fetal APCs acquire antigens from placental tissues? If our views on fetal tolerance training by the placental microbiome are confirmed, how is the immune-modulation of the fetus guided by the placental microbiome? Are then vital bacteria involved in recognition by fetal immune system, or just their DNA fragments?

Once the microbiome is established, sustaining it at health becomes an issue. Colonization resistance mechanisms are just becoming apparent and more research needs to be performed using complex model systems and longitudinal clinical studies. Extremely exciting is the overlap between taste and chemical sensing of bacteria in the airways with possible similar mechanisms operating in the mouth. In addition to modulating taste, does the oral microbiota dictate the feeling of hunger or craving for specific dietary habits? Such interactions have been suggested recently where ingestion of bacterial LPS was shown to inhibit taste responses to sucrose in mice (Zhu et al., 2014). Substantial research has been done in the past few years, while one thing is certain—much more research needs to be done in the near future, leading to exciting discoveries directed to maintenance of oral and overall health in an ever-changing population.

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Comparison of bacterial culture and 16S rRNA community profiling by clonal analysis and pyrosequencing for the characterization of the dentine caries-associated microbiome

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Culture-independent analyses have greatly expanded knowledge regarding the composition of complex bacterial communities including those associated with oral diseases. A consistent finding from such studies, however, has been the under-reporting of members of the phylum *Actinobacteria*. In this study, five pairs of broad range primers targeting 16S rRNA genes were used in clonal analysis of 6 samples collected from tooth lesions involving dentine in subjects with active caries. Samples were also subjected to cultural analysis and pyrosequencing by means of the 454 platform. A diverse bacterial community of 229 species-level taxa was revealed by culture and clonal analysis, dominated by representatives of the genera *Prevotella*, *Lactobacillus*, *Selenomonas*, and *Streptococcus*. The five most abundant species were: *Lactobacillus gasseri*, *Prevotella denticola*, *Alloprevotella tannerae*, *S. mutans* and *Streptococcus* sp. HOT 070, which together made up 31.6 % of the sequences. Two samples were dominated by lactobacilli, while the remaining samples had low numbers of lactobacilli but significantly higher numbers of *Prevotella* species. The different primer pairs produced broadly similar data but proportions of the phylum *Bacteroidetes* were significantly higher when primer 1387R was used. All of the primer sets underestimated the proportion of *Actinobacteria* compared to culture. Pyrosequencing analysis of the samples was performed to a depth of sequencing of 4293 sequences per sample which were identified to 264 species-level taxa, and resulted in significantly higher coverage estimates than the clonal analysis. Pyrosequencing, however, also underestimated the relative abundance of *Actinobacteria* compared to culture.

Keywords: caries, oral microbiome, caries-infected dentine

INTRODUCTION

Dental caries, or tooth decay, is the dissolution of tooth structure by acids formed by bacteria as a result of the fermentation of dietary carbohydrate, particularly sucrose. *Streptococcus mutans* was one of the first species associated with dental decay leading to carious lesions in teeth (Clarke, 1924; Loesche et al., 1975). *S. mutans*-free caries lesions have been observed, however, (Marsh et al., 1989) and it has been recognized that the plaque biofilm as a whole, rather than individual species, is responsible for acid production and lesion formation, particularly in the early stages (Marsh, 2003; Takahashi and Nyvad, 2011). Consequently, it is important to comprehensively determine the composition of the bacterial community associated with dental caries to better understand the bacterial factors and host and environmental interactions that are responsible for the initiation and progression of dental decay. This will make it possible to develop novel preventative and/or therapeutic strategies for this disease.

Traditionally, microbiologists have used culture media to grow and characterize bacterial species, but it has been realized that not all species can be readily grown under laboratory conditions. Consequently, in recent years molecular methods targeting the 16S rRNA gene to characterize complex microbial communities have been established and many sequences representing novel species have been detected. There are around 700 bacterial species found in the human mouth, around 65% of which have been cultured (Paster et al., 2001, 2006; Dewhirst et al., 2010).

The culture-independent methods themselves have biases and deficiencies; for example it has been found that the proportions of *Actinobacteria* were underestimated using molecular analysis when a direct comparison to culture was available (Munson et al., 2002, 2004; de Lillo et al., 2006). Furthermore, recent studies by Tanner et al. (2011) and Kanasi et al. (2010) observed greater diversity of species detected in early childhood caries (ECC) using culture compared to clonal analysis.

The caries-associated microbiota has yet to be completely characterized; many recent studies in have reported the detection of novel species, genera or even higher taxonomic orders (Munson et al., 2004; Nadkarni et al., 2004; Chhour et al., 2005; Kanasi et al., 2010; Tanner et al., 2011). Tanner et al. (2011) reported *Scardovia wiggsiae* to be significantly associated with severe ECC children in the presence and absence of *S. mutans* detection and showed for the first time a strong association of *S. wiggsiae* together with *S. mutans* in ECC. These findings clearly demonstrate that continued efforts to characterize the microbiota of caries and distinguish mechanisms of disease progression are needed.

The aim of this study was firstly, to design novel primers for 16S rRNA-based community profiling of the microbiota associated with carious dentine, and, secondly, to compare the results obtained with cultural and pyrosequencing analyses of the same samples.

MATERIALS AND METHODS

SUBJECTS AND SAMPLE COLLECTION

Ethical approval for the study was granted by the Lewisham Local Research Ethics Committee South London REC Office (4) (Reference 08/H0810/61). Six subjects, four male and two female, aged 22–35 years (mean age 26.6 years), who were medically healthy participated in the study with their informed consent, provided in writing. Subjects were included if they had a carious lesion that had spread into the middle or inner third of dentine, that was checked radiographically with cavitation. Local anesthesia was administered where necessary, and the carious teeth isolated with rubber dam to minimize saliva contamination during the excavation procedure. Following removal of carious enamel to the enamel-dentine junction with a sterile, water-cooled diamond bur in an air-turbine handpiece, the dentine lesion was hand excavated with a sterile, spoon excavator (Ash G5; Claudius Ash Ltd., Potters Bar, UK). After the superficial layer of debris had been removed and discarded, the sample, consisting of soft necrotic dentine, was collected using a fresh, sterile spoon excavator at a level that represented the infected dentine lesion. Samples were placed in 1 ml of reduced transport medium (1% w/v tryptone, 0.5% w/v yeast extract, 0.1% w/v L-cysteine, 0.1% w/v D+glucose, 2% v/v horse serum in distilled water and adjusted to pH 7.5, RTM). Samples were then vortex-mixed for 1 min and then divided.

BACTERIAL CULTURE

Ten-fold serial dilutions of 100 µl of the sample suspensions were prepared in RTM within an anaerobic workstation. One hundred µl of appropriate dilutions were used to inoculate pre-reduced Fastidious Anaerobe Agar (LabM, Bury, UK) +5% horse blood (FAA) plates, in triplicate, which were incubated anaerobically for 10 d at 37°C. Plates with between 30 and 300 colonies were counted and 96 colonies were selected randomly and sub-cultured on FAA plates, with a *Propionibacterium acnes* feeder streak. Isolates were incubated anaerobically for a further 4–5 days, after which the purity of all isolates was visually checked using a plate microscope. Mixed cultures were subcultured to achieve purity and pure cultures were stored at −70°C in Brain Heart Infusion (BHI) +10% glycerol. Cells were harvested from FAA plates of the isolates, and suspended in 1 ml PBS (Oxoid). DNA was extracted by means of the GenElute™ bacterial genomic kit (Sigma Aldrich), following the modification for Gram-positive bacteria. 16S rRNA genes were amplified by PCR with primer pair 27F CM/1492R. Reactions were prepared containing 4 µl 5× Phusion buffer GC, 0.4 µl 10 mM dNTPs, 0.2 µl Phusion HF polymerase (0.4 U, Finnzymes), 0.5 µl of each primer (10 µM), 1 µl of template and 13.4 µl sterile water. Initial denaturation was at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 30 s and extension at 72°C for 45 s.

PCR PRIMER DESIGN

The Human Oral Microbiome Database (Chen et al., 2010) reference dataset was aligned and manually inspected for regions of homology suitable for PCR primer design. Novel primer 39F (**Table 1**) was selected for use in this study. Primer 61F was modified from primer 63F described by Marchesi et al. (1998) and was truncated at the 3′ end to remove the mis-match with many oral streptococcal species (**Table 1**).

COMMUNITY PROFILING BY CLONAL ANALYSIS

The remaining 900 µl of the original samples was centrifuged for 10 min at 13,000 g, the supernatant discarded and the pellet subsequently used for DNA extraction using the GenElute™ bacterial genomic kit. 16S rRNA genes of the extracted DNA from each patient sample were amplified with the following primer combinations: 27F YM/1492R (library 1), 27F CM/1492R (library 2), 39F/1387R (library 3), 39F/1492R (library 4) and 61F/1387R (library 5) (**Table 1**). Five replicate amplification reactions were set up for each sample and combined. Initial denaturation was at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 90 s. Ten µl of the five replicate *Taq* polymerase PCR products of each primer set were pooled and cloned into the TA cloning vector pCR4-TOPO (Invitrogen) following the manufacturer's instructions. Transformants were detected on LB agar supplemented with 50 µg/ml kanamycin. Ninety-six clone colonies were chosen at random and the insert amplified by PCR with vector-specific primers M13 FWD and REV.

Sequencing

PCR products from clone insert amplification or from isolates amplified with Phusion polymerase were purified using the ExoSAP-IT (Exonuclease I/Shrimp Alkaline Phosphatase) clean up kit (Affymetrix, High Wycombe, UK). Five µl of PCR

Table 1 | Primers used in the study.

Primer	Sequence (5′-3′)	
27F CM	AGAGTTTGATCMTGGCTCAG	Lane, 1991
27F YM	AGAGTTTGATYMTGGCTCAG	Frank et al., 2008
39F	ATCMTGGCTCAGRWYGAACGC	This study
61F	CAGGCCTAACACATGCAAG	This study
519R	GWATTACCGCGGCKGCTG	Lane, 1991
1387R	GGGCGGWGTGTACAAGGC	Marchesi et al., 1998
1492R	TACGGYTACCTTGTTACGACTT	Lane, 1991

product were mixed with 1 μl ExoSAP-IT and 1 μl water and incubated in a thermal cycler for 15 min at 37°C followed by incubation at 80°C for 15 min. For each sample, 96 isolates and 96 clones from each library were partially sequenced using primer 519R. Reactions were set up with 0.5 μl BigDye (Applied Biosystems, Life Technologies), 1.75 μl 5 × sequencing buffer (Applied Biosystems), 0.3 μl primer 519R (10 μM), 5.45 μl deionised, autoclaved water and 2 μl cleaned up PCR product as template. Thirty cycles were run consisting of 10 s at 96°C, 5 s at 50°C and 2 min at 60°C. Sequencing reaction products were purified by ethanol precipitation and then dissolved in 10 μl 0.1 × TE and sequenced by means of an AB3730xl DNA analyser (Applied Biosystems). Sequences were identified by BLASTn interrogation of the Human Oral Microbiome Database (HOMD), by means of the HOMD BLAST on-line tool (www.homd.org). The following parameters were used with BLASTn: cost to open a gap = 5; cost to extend a gap = 5; penalty for a mismatch in the blast portion of run = −5; reward for a match in the blast portion of run = 4. A word length of 11 was used. Sequences were compared with database sequences at a sequence identity level of 98.5%, and greater than 90% shared coverage for positive identification. Sequences with multiple database hits above 98.5% were reported with all possible identification options.

PYROSEQUENCING

For amplicon library construction, 16S rRNA genes of the DNA extracted from the samples from the initial patient sample were amplified using six barcoded forward primers which consisted of the 27FYM template-specific primer sequence, a 12-base unique Golay barcode and the Lib-L Adapator A. The reverse primer consisted of template-specific reverse primer 519R sequence with Lib-L Adapator B. Three replicate amplification reactions were set up for each sample. Reactions were prepared containing 12.5 μl Extensor PCR mastermix (High fidelity Taq polymerase, Thermo Scientific), 2 μl of template, 0.5 μl of each primer (10 μM) and 9.5 μl sterile water. Initial denaturation was at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 45 s, annealing at 53°C for 45 s, extension at 72°C for 90 s and a final extension at 72°C for 15 s. PCR amplicons were pooled and then purified using the QIAquick PCR purification kit (Qiagen, Crawley, UK) following the manufacturer's instructions. The size and purity of the amplicons were assessed using the Agilent 2100 Bioanalyzer and the Agilent DNA 1000 kit (Agilent Technologies, Inc., Wokingham, UK), and quantified using the Quant-iT-Picogreen fluorescent nucleic acid stain (Invitrogen). The amplicons were then pooled at an equimolar concentration of 1×10^9 molecules/μl. The pooled samples were amplified clonally by emulsion-PCR using the GS emPCR Lib-L Kit. The GS PicoTiterPlate Kit was then used to sequence individual clonally amplified molecules on a Roche 454 GS-FLX Titanium sequencer. The data were analyzed using the mothur pipeline (Schloss et al., 2009). Trim.flows was used to remove sequences with more than two mismatches to the primer sequences or more than one mismatch to the barcode and sequences with fewer than 320 or greater than 720 flows. Sequences were denoised by means of shhh.flows after which trim.seqs was used to remove the primer and barcode sequences, and sequences shorter than 350 bp. The sequences were then aligned to the Silva reference file by means of align.seqs, after which non-aligned sequences and columns without bases were removed. Pre.cluster was used to combine sequences that were within 1 bp per 100 bp of total sequence length of a more abundant sequence with that sequence. Chimerae were detected using chimera.uchime and removed using remove.seqs. The classify.seqs command was used to classify sequences using the HOMD version 10 reference sequence and taxonomy databases. The dist.seqs program calculated uncorrected pairwise distances between aligned sequences and the cluster command was used to assign sequences to OTUs. Following this, a table was created indicating the number of times an OTU was present in each sample using the make.shared command. Because the groups for the different patients contained varying amounts of sequences, all samples were normalized to the size of the smallest sample group (4293 sequences) by means of sub.sample. The classify.otu command was used to obtain a consensus taxonomy for each OTU. The collect.single command was used to calculate the Chao1 richness and the Inverse Simpson diversity index. A table containing the number of sequences, sample coverage, number of observed OTUs and the Inverse Simpson diversity estimate was compiled using the summary.single command.

The pyrosequencing data was deposited in the NCBI SRA database as accession SRP047474.

STATISTICAL ANALYSIS

The relative abundance of taxa in samples and by different analysis methods was compared by means of a two tailed *Z*-Test calculator for paired comparisons, with a significance threshold of 0.05.

RESULTS

CULTURE AND 16S rRNA GENE SEQUENCING AND CLONING ANALYSIS

The mean bacterial count estimated from culture on the anaerobically FAA agar was 6.5×10^7, and ranged from 5.4×10^4 to 2.0×10^8. The sequences from 2700 cloned 16S rRNA genes and 540 isolates were analyzed together. 229 taxa were found at species level, representing 8 phyla: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, TM7, *Spirochaetes* and *Synergistetes*. 216 taxa were detected using molecular analysis, of which 143 were only found using this method. 86 taxa were isolated in culture, 12 of which were not detected using molecular analysis. Sequences representing 16 novel taxa were identified and were sequenced to near full length and the sequences deposited in the NCBI nucleotide database (**Supplementary Table 1**).

The distribution of taxa found in the dentine caries lesions detected by culture and clonal analysis is shown in **Supplementary Table 2**. A highly taxon-rich community was seen, including numerous representatives of the genera *Prevotella*, *Lactobacillus*, *Selenomonas*, and *Streptococcus*. The five most abundant species overall were *Lactobacillus gasseri*, *Prevotella denticola*, *Alloprevotella tannerae*, *S. mutans* and *Streptococcus* sp. HOT 070, who together represented 31.6% of the total sequences. There were differences in the composition of the microbiota in samples from different subjects; e.g., lactobacilli made up 93.2 and 57.6% of the microbiota in samples A and E, respectively

but only 0.2 and 1.5% in B and D, while none were detected in patients C and F ($p < 0.05$). Few other *Firmicutes* were observed in subject A, whilst the sample from subject E showed a significantly higher proportion of *S. mutans* and *Veillonella dispar/parvula* ($p < 0.05$). Samples from subjects B, C, D, and F had a wide range of other *Firmicutes*.

Subject C had significantly higher levels of *Atopobium rimae* and *Atopobium* OT416 compared to all other subjects ($p < 0.05$) and 88 of the detected 92 *Pseudoramibacter alactolyticus* clones were detected in this sample ($p < 0.05$). Compared to the levels of lactobacilli seen, significantly fewer *Prevotella* species were detected in samples A and E ($p < 0.05$). *Prevotella* spp. comprised between 21.1 and 40.2% of the microbiota in samples B, C, D, and F, which was significantly higher than that found in samples A and E. Of the 57 *Olsenella profusa* sequences detected, 19 and 37 were detected in isolate libraries of samples D and F, respectively, which was significantly higher than was found in the molecular libraries of these patients as well as in the remaining subjects ($p < 0.05$). Spirochetes and TM7 were both detected in patient B and D, but the relative occurrence was reversed; i.e., patient B had significantly more TM7 than *Spirochaetes* ($p < 0.05$), while patient D had significantly more of the latter and only few TM7 ($p < 0.05$).

All clone libraries significantly under-reported *Actinobacteria* numbers compared to culture analysis ($p < 0.05$). The detection of *Bacteroidetes* seemed to be influenced mostly by the choice of reverse primer as libraries 3 and 5 that used primer 1387R had a significantly higher rate of detection of *Bacteroidetes* ($p < 0.05$) than the libraries/culture analysis using primer 1492R. The highest detection of *Firmicutes* was seen in the libraries where 27F CM was used. The proportion of the microbiota represented by the genus *Streptococcus* was significantly higher in libraries 1, 2, 3, and 4 compared to culture ($p < 0.05$, **Figure 1**). Streptococcal proportions in library 5, were not significantly different to those revealed by culture, but there was a significant difference compared to the other molecular libraries ($p < 0.05$).

Figure 2 shows the proportions of selected taxa among the isolates and clones. The taxa shown were chosen because the total number of sequences detected for each taxon was greater than 1% of the total and at least one library showed an at least 50% higher incidence compared to the other libraries or detection rates in two libraries was at most 50% of the detection rate of the other libraries. For example, libraries prepared with reverse primer 1387R (3 and 5) detected between 50 and 75% less *Pseudoramibacter alactolyticus* compared to reverse primer 1492R

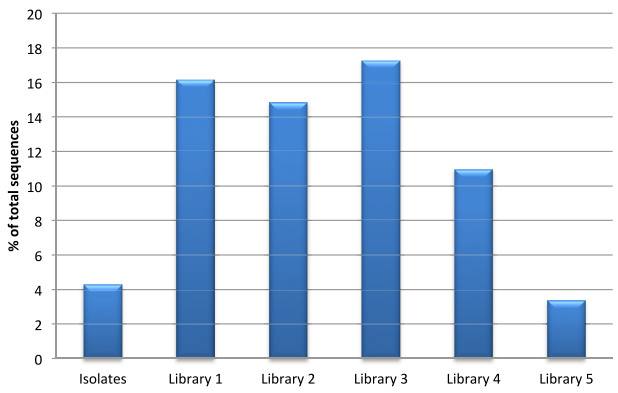

FIGURE 1 | Detection of the genus *Streptococcus* by culture and clonal analysis.

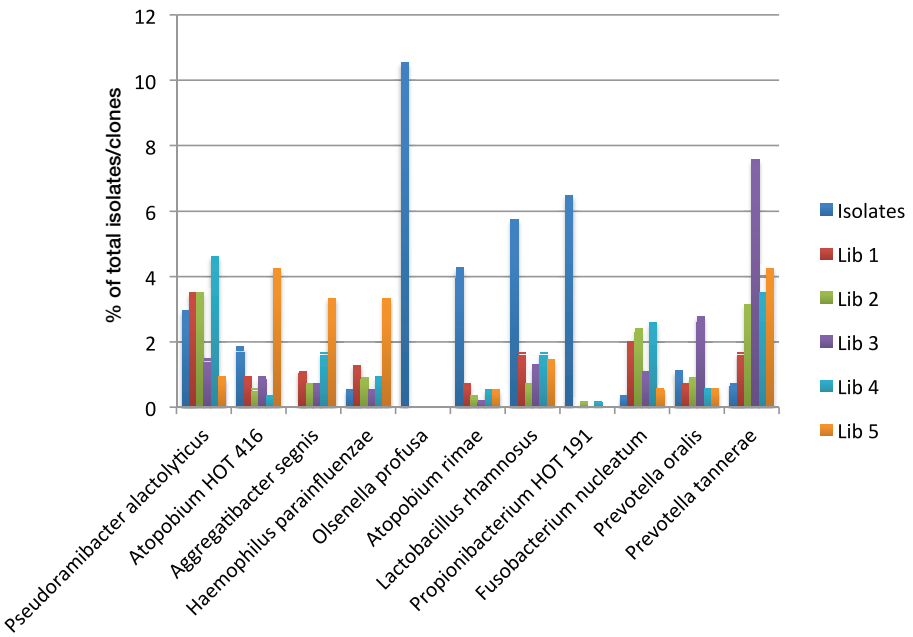

FIGURE 2 | Detection of selected oral taxa by culture and clonal analysis.

($p < 0.05$). Detection of *Atopobium* OT 416, *Aggregatibacter segnis* and *Haemophilus parainfluenzae* was significantly increased with primer pair 61F/1387R (library 5) to more than double of that detected with culture analysis or any other primer pair combination used ($p < 0.05$). *Olsenella profusa* was only found using culture methods ($p < 0.05$). Detection of *Atopobium rimae*, *Lactobacillus rhamnosus*, and *Propionibacterium* OT 191 showed a similar trend in that detection using culture was on average 11.0, 5.5, and 34.1 times higher than that using molecular methods, respectively ($p < 0.05$). Detection of *Fusobacteria* was significantly reduced when culture methods or reverse primer 1387R (libraries 3 and 5) were used; detection of *Prevotella oralis* and *Prevotella tannerae* was significantly increased using primer pair 39F/1387R (library 3) ($p < 0.05$).

PYROSEQUENCING

35,191 sequences were obtained by pyrosequencing. After filtering for quality and removal of chimerae, 29,835 sequences were analyzed. The number of sequences per sample ranged from 4293 to 6488 and thus the sample libraries were sub-sampled to select 4293 sequences from each sample.

Table 2 shows a comparison of the OTU-based analysis of the clonal data obtained with library 2 (primer set 27FYM/1492R) and pyrosequencing. The number of sequences obtained from pyrosequencing was almost 50-fold higher, whilst the number of observed OTUs (S_{obs}) was between 4.05 and 7.44 times greater in 454 sequencing. Chao1 estimates of total species richness were 2 and 14 times higher for pyrosequencing. Good's coverage estimates were 97% or higher for pyrosequencing but generally substantially lower for the clonal analysis, ranging from 67 to 97%.

The distribution of phyla in the samples seen in the pyrosequencing analysis was broadly similar to that revealed by cloning and Sanger sequencing. The detection of *Actinobacteria* was significantly lower than that seen by culture ($p < 0.05$). Eighty seven genera were detected by pyrosequencing, 65 of which had been found by Sanger sequencing, whilst 25 were only detected by pyrosequencing. Four genera were found only with Sanger sequencing. Of the 25 genera found only by pyrosequencing most (17 of 25) were represented by five or fewer sequences.

Table 2 | Comparison of coverage and taxon richness estimates by clonal analysis and pyrosequencing.

Analysis sample	Clonal analysis—Library 2 ($n = 90$)			Pyrosequencing ($n = 4293$)		
	Sobs	Good's coverage	Chao1	Sobs	Good's coverage	Chao1
A	9	0.97	10	67	0.99	142.6
B	51	0.67	73.56	331	0.97	486.22
C	25	0.88	34.17	176	0.98	282.64
D	42	0.73	69.6	287	0.97	441.89
E	19	0.84	64.5	77	0.99	121
F	39	0.73	94.2	187	0.98	263.24

The identification of the sequences to species level using the mothur command classify.seqs in conjunction with the HOMD reference dataset is shown in **Supplementary Table 3**. A bootstrap cut-off value of 80% was used. Unclassified sequences were those that did not match any database sequence or where two closely related species could not be differentiated. 264 species-level taxa were detected by pyrosequencing, including numerous representatives of the genera *Prevotella*, *Lactobacillus*, *Streptococcus*, *P. alactolyticus*, and *Fusobacteria*. The five most abundant species-level taxa were *Lactobacillus gasseri*, a group of unclassified streptococci, *S. mutans*, *P. alactolyticus*, and *P. denticola*; together representing 40.7% of all detected sequences. Three of the five most abundant species were the same as for Sanger sequencing: *L. gasseri*, *S. mutans*, and *P. denticola*, but their proportions were significantly different ($p < 0.05$).

Differences in the composition of the microbiota in samples from different subjects were observed as seen in the Sanger sequencing analysis and the same proportional trends were observed for the pyrosequencing data. For example, both sequencing methods showed that samples A and E had significantly higher proportion of lactobacilli than the other patients, with few other *Firmicutes* observed in patient A, whereas sample E showed a high proportion of *S. mutans* and *Veillonella parvula* ($p < 0.05$). Similarly, pyrosequencing confirmed the results of the Sanger analysis in that *Prevotella* species were detected at low levels in patient samples A and E (0–0.32% of sequences), while making up a substantial proportion of the microbiota in samples B (14.2%), C (43.5%), D (37.1%) and F (11.9%).

DISCUSSION

One of the primary aims of this study was to improve the detection of high G+C species in the molecular analyses. This was not achieved and the results were similar to those from previous studies (Munson et al., 2002, 2004; de Lillo et al., 2006), in that the proportions of *Actinobacteria* in the molecular analyses were reduced compared to culture, although primer pair 61F/1387R was superior to the other primer pairs tested. The different primers in their various combinations used for the molecular analysis of the carious samples resulted in different, but minor biases in the detection of various species. Although the primers used to target 16S rRNA genes in molecular ecology studies are often referred to as "universal" primers, it would be more appropriate for them to be termed broad range primers, since there is sequence variation in even the conserved regions of the gene (Wang et al., 2009; Palatinszky et al., 2011). Furthermore, primers have been designed using alignments of species known to date, which frequently contain sequencing errors (Taylor et al., 2007). Single mismatches in the first 3–4 nucleotides from the 3′ end of a PVR primer can greatly reduce and even hinder extension (Bru et al., 2008; Wu et al., 2009). Bru et al. (2008) reported differences in the detection rates using forward and reverse primers with a single mismatch, which could explain the observed differences in detection for the various primers. They found forward primers with a mismatch located more than four bases away from the 3′ end underestimated gene copy numbers, while no effect was seen on the reverse primers. They conceded that the severity of this bias is determined by numerous factors, such as the

primer length, the nature, and position of the mismatches and the annealing temperature of the primers. Overall, it appears that the choice of primers cannot explain the bias against members of the phylum *Actinobacteria*.

A potential source of bias is the cloning used in the clonal analysis. It has been found that libraries from an environmental sample constructed using the TA-cloning of multi-template PCR products showed significant differences compared to a length heterogeneity PCR which did not employ cloning (Palatinszky et al., 2011). However, Taylor et al. (2007) found no biases due to TA cloning when testing phylogenetic bias in fungal environmental clone library construction comparing TA and blunt-ended cloning. The abundance of OTUs was found to be correlated and phylogenetic tests showed no significant differences between the two libraries (Taylor et al., 2007). Some OTUs differed in abundance between libraries, however, indicating a potential phylogenetic bias during cloning. Palatinszky et al. (2011) suggested that clone libraries with low diversity may be more prone to phylogenetic biases and that drawing conclusions from diverse communities such as used by Taylor et al. (2007) could lead to underestimation of the extent of the bias.

When comparing the distribution of taxa of all libraries combined for each patient it became apparent that when a patient has a high incidence of lactobacilli (patients A and E), few *Prevotella* species (A = 1, E = 8 sequences identified as *Prevotella*) and no *Pseudoramibacter* were observed and vice versa (patient F = 8 sequences identified as lactobacilli). Furthermore, where the microbiota of two samples was dominated by lactobacilli the overall number of taxa was lower compared to the other samples (22 and 41 taxa vs. 113, 60, 117, and 88 taxa). Chhour et al. (2005) reported similar findings when looking at the microbial diversity in advanced caries lesions of 10 patients. They, too, reported *Lactobacillaceae* and *Prevotellaceae* to make up the majority of all identified sequences and that lesions could be grouped into *Prevotella*-dominated or *Lactobacillus*-dominated samples. They argued that colonization, or exclusion thereof, could depend on fermentation by-products, but that lactate did not appear to be the major fermentation by-product, since they observed lactate-dependent *Veillonella* spp. infrequently and no further species capable of metabolizing lactate. Similar results were found here, particularly that lactate-utilizing phylotypes, such as *Selenomonas* spp. and *Pseudoramibacter alactolyticus*, were detected in lesions high in *Prevotella* (Chhour et al., 2005). The conclusion from these observations are that the incidence of the dominant species depends on factors early in the colonization of the dentine matrix and successive inclusion or exclusion of subsequent colonizers is likely to be determined by metabolic by-products of the initial colonizers (Byun et al., 2004; Nadkarni et al., 2004; Chhour et al., 2005).

Another approach to advance the detection of novel species could be to use the universal primers to detect new groups of bacteria, as was achieved in this study, and to subsequently design family/genus-specific primers to broaden the knowledge of that particular group. It was shown in several other studies that greater diversity could be observed using genus specific primers (Vartoukian et al., 2009; Lin et al., 2011; Xie et al., 2011).

As previously mentioned, this study, employing five primer sets (some of them novel), resulted in similar findings to other studies looking at the microbiota of dentine caries. Munson et al. (2004) analysing the microbiota of five carious dentine samples found 95 taxa when applying two primer sets and culture analysis. They found three taxa to be detectable in all five samples and found *O. profusa* and *P. acidifaciens* to be predominant in culture analysis, but very few clones could be identified as these two species. In this study, a total of 228 taxa were found of which only a single taxon, *S. mitis/pneumonia*e, was found in all six patient samples. Similar to the above mentioned study, *O. profusa*, *O. uli*, and *P. acnes* were predominant in culture analysis, but none or only a few were detected in the molecular analysis. These findings are in contrast to those by Chhour et al. (2005) who, analysing ten carious dentine samples using primer pair 331F/797R, found *O. profusa* and *P. acidifaciens* to make up to 14.4 % and 30 % of the detected bacterial load, respectively. Studies by Tanner et al. (2011) and Kanasi et al. (2010) looked at culture and molecular analysis of the same plaque samples taken from children with and without early childhood caries (ECC). The culture-based study strongly associated *S. mutans* with disease, but also found a diverse microbiota as well as a novel potential pathogen associated with ECC, *Scardovia wiggsiae* (Tanner et al., 2011). Kanasi et al. (2010), analysing the same sample pool using molecular techniques reported 139 taxa (and 35 provisional taxa) and found *S. mutans* and *Bifidobacteriaceae* to be significantly associated with ECC. However, this was only discovered using specific PCR primers, indicating that caries sites are highly diverse and that, while important in disease, the organisms may be present only in low proportions. Another explanation could of course be that the universal primers are not specific enough to allow for full detection of those species. In this study, *S. mutans* was detected in five of the six patient samples using culture and molecular techniques, albeit in varying frequencies. These results suggest that the combination of some patients having low numbers of this species and the choice of primers greatly influences rate of detection. Overall, had only primer pair 27F YM/1492R been used 62 taxa that were detected in this study would have been overlooked. Even using primer pair 27F CM/1492R, that allowed detection of the most taxa not found with any other primer combination, would result in 51 taxa not being detected.

Similarly, if no culture analysis had been performed, none of the 57 *O. profusa* isolates would have been detected and thereby this taxon would have been completely missed in the clonal analysis, although it was detected by pyrosequencing. It seems less likely that novel species will be detected using established culture methods. Tanner et al. (2011), however, by using careful sample handling and multiple culture media, isolated 45 previously uncultivated taxa and 45 potentially novel species-level taxa not in the HOMD database.

It was clear that the use of pyrosequencing and its inherent increased depth of analysis did increase coverage compared to clonal analysis, especially in taxon-rich samples, such as B and D. Pyrosequencing also helped enhance knowledge of the microbiota in dentine caries, in that a number of genera were found, which were not seen with Sanger sequencing. Most of those sequences were detected in low abundances and may represent part of

the rare biosphere (Pedros-Alio, 2012). This can be explained by the high number of sequences generated by pyrosequencing, whereby the chance of detecting a rare species is increased. Indeed, two sequences belonging to the phylum SR1 and one sequence belonging to the phylum *Chloroflexi* were detected that have previously been described as rare (Keijser et al., 2008). Diaz et al. (2012) argued that OTUs representing singletons should be eliminated, since it was found that in communities with known numbers of OTUs pyrosequencing and following analysis generated more OTUs than expected. However, findings from this study show that OTUs from the rare biosphere would thus be missed and steps to eliminate or include OTUs in the analysis have to be given careful consideration (Reeder and Knight, 2009; Schloss and Westcott, 2011).

In previous studies, detection of *Veillonella* spp. was reported in most patients at all stages ranging from intact to deep dentine cavities, but no significance could be drawn in relation to any stage of caries lesion progression (Aas et al., 2008; Gross et al., 2010; Lima et al., 2011), showing that the significance of the detection of certain species and genera is still not clear. When Belda-Ferre et al. (2012) made comparisons at species level between health and caries status, however, *Veillonella parvula* was found in caries-active and caries-free patients. Only the metagenomic analysis showed that different strains were present in health and disease, since the *Veillonella* found in caries-active individuals contained genes that *Veillonella* in caries-free subjects did not, which could indicate that different genes are involved in pathogenesis (Belda-Ferre et al., 2012). Metagenomic analyses which take into account genetic variation among strains within a species and metatranscriptomic methods which determine which genes are actively transcribed clearly have advantages over 16S rRNA-based analyses in determining the function of the oral microbiome in health and disease.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00164/abstract

Supplementary Table 1 | Accession number of 16S rRNA gene sequences for novel taxa identified in the study.

Supplementary Table 2 | Caries-associated bacterial biota determined by culture and clonal analysis. Library 1 – primers 27F CM/1492R; Library 2 – 27F YM/1492R; Library 3 – 39F/1387R; Library 4 - 39F/1492R; Library 5 – 61F/1387R.

Supplementary Table 3 | Caries-associated bacterial biota determined by pyrosequencing.

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Challenges in the culture-independent analysis of oral and respiratory samples from intubated patients

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The spread of microorganisms in hospitals is an important public health threat, and yet few studies have assessed how human microbial communities (microbiota) evolve in the hospital setting. Studies conducted so far have mainly focused on a limited number of bacterial species, mostly pathogenic ones and primarily during outbreaks. We explored the bacterial community diversity of the microbiota from oral and respiratory samples of intubated patients hospitalized in the intensive care unit and we discuss the technical challenges that may arise while using culture-independent approaches to study these types of samples.

Keywords: endotracheal aspirate, supraglottic secretions, microbiota, 16S rDNA profiling, bacterial communities

SUBJECTS AND SAMPLE TYPES

Supraglottic secretions (SGS) and endotracheal aspirates (ETA) were collected on a daily basis from five subjects over a 4-day period following intubation (Supplementary Material). For each subject, we also included a sample on day 5–11 depending on availability. None of the patients developed ventilator-associated pneumonia (VAP), so their oral/respiratory bacterial communities likely represent "healthy" microbiota of mechanically ventilated patients.

SEQUENCE DATA PROCESSING

After pyrosequencing of 16S rDNA V1-3 amplicon libraries from the reverse primer (Supplementary Material), a total of 383,302 sequence reads had an exact match to the barcode sequence. Removal of sequence reads based on (1) the match to the 16S rDNA sequence of the reverse primer, (2) length, (3) quality score, (4) the presence of homopolymer runs and (5) ambiguous bases, resulted in 375,612 (98%), 375,210 (99.8%), 266,338 (69.5%), 266,338 (69.5%), and 264,358 (69%) sequences, respectively. The BLASTN-based OTU picking, performed as described previously (Lazarevic et al., 2013a) using the Greengenes taxonomy (McDonald et al., 2012), further reduced the dataset to 217,531 sequences (56.8%) of which 209,477 derived from the 50 clinical (25 SGS and 25 ETA) samples and 8054 sequence reads corresponded to 8 negative controls (reagents). After removal of possibly contaminant 16S rDNA sequences (see below) the sample dataset was represented by 194,322 sequences. The number of sequences per sample varied between 9 and 7665 (average 3886, median 4500). The average number of sequences per individual were 24,712 (median 22,763) and 14,153 (median 15,082) for SGS and ETA, respectively.

MICROBIOTA PROFILES

The phyla Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, Tenericutes and Actinobacteria were highly prevalent (42–50 positive samples) and corresponded on average to >97% of the 16S rDNA sequences in both SGS and ETA samples datasets. The less abundant phyla Spirochaetes, TM7 and Synergistetes were also identified in both samples types (24–30 positive samples). Other phyla, SR1, Cyanobacteria, Thermi and WPS-2 were found each in less than 5 samples at low proportion (<0.2%). A total of 115 genera were identified in the dataset. At the genus level, SGS and ETA microbiota showed high similarity in terms of the prevalence (Pearson $R = 0.884$) and average relative abundance (Pearson $R = 0.854$). Genera *Streptococcus*, *Neisseria*, and *Prevotella* had the highest proportion in both

samples types and represented together 56 and 57% of sequence reads in SGS and ETA, respectively. In contrast, *Mycobacterium*, the fourth most abundant genus in SGS (6.7%) and ETA (3.1%) has been identified in saliva at very low levels (Lazarevic et al., 2011). Interestingly, in a study which included intubated patients, the genus *Mycoplasma* was found in bronchoalveolar lavages but only in individuals who developed VAP or community associated pneumonia (Bousbia et al., 2012). The other genus from the phylum Tenericutes, *Ureaplasma*, was frequently identified as dominant organism in tracheal aspirates from mechanically ventilated preterm infants (Mourani et al., 2011).

We compared the SGS and ETA microbiota with those from other body sites available from published studies. Both SGS and ETA bacterial communities determined in our study clustered together with salivary (Zaura et al., 2009; Lazarevic et al., 2010, 2013b; Segata et al., 2012; Ling et al., 2013) and throat (back wall of oropharynx) microbiota (Segata et al., 2012), and were clearly distinct from the skin microbiota (Ling et al., 2013) (unpublished, MG-RAST ID 6526), nasopharyngeal microbiota (Bogaert et al., 2011; Ling et al., 2013) and gut microbiota (Claesson et al., 2009; Segata et al., 2012; Krych et al., 2013; Ling et al., 2013) (**Figure 1**). This significantly supports the validity of our experimental approach and shows that differences between anatomical sites outweighed the methodological differences related to DNA extraction, PCR amplification and bioinformatics analysis. Our result is consistent with the recent metagenomic studies indicating that the lower respiratory tract microbiota (including trachea) originate mainly from the oral and upper respiratory tract (including oropharynx) in healthy subjects and in disease (Charlson et al., 2011; Cabrera-Rubio et al., 2012; Segata et al., 2012). Similarly, culture-based studies showed that bacterial communities of the pharyngeal and tracheal secretions are similar (Pirracchio et al., 2009).

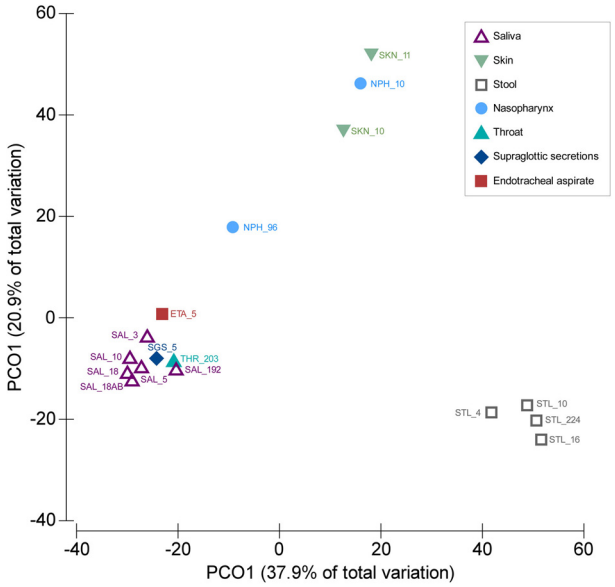

FIGURE 1 | Similarities between SGS, ETA, and bacterial communities from other body parts. PCoA was based on Bray–Curtis similarity matrix constructed using square-root transformed average relative abundance of genera. The number following underscore corresponds to the number of subjects analyzed. Saliva samples: SAL_192 (Segata et al., 2012), SAL_10 (Ling et al., 2013), SAL_18 (from children, before antibiotic treatment) (Lazarevic et al., 2013b), SAL_18AB (from children, at the end of the antibiotic treatment) (Lazarevic et al., 2013b), SAL_5 (three time points for each subject) (Lazarevic et al., 2010), SAL_3 (Zaura et al., 2009). Throat swab: THR_203 (Segata et al., 2012); Supraglottic secretions: SGS_5 (five time points for each subject, this study); Endotracheal aspirate: ETA_5 (five time points for each subject, this study). Skin samples: SKN_10 (Ling et al., 2013), SKN_11 (MG-RAST ID 6526); Nasopharyngeal swabs: NPH_96 (Bogaert et al., 2011), NPH_10 (Ling et al., 2013). Stool samples: STL_224 (Segata et al., 2012), STL_10 (Ling et al., 2013), STL_16 (five time points for each subject) (Krych et al., 2013), STL_4 (data designated V4-0.5) (Claesson et al., 2009). The abundance of genera were taken directly from the tables provided in corresponding publications, except for the SKN_11 (unpublished) for which the MG-RAST data were processed using the bioinformatics pipeline as described in this paper.

TECHNICAL CHALLENGES

Since many bacteria are not readily cultivable, the studies of bacterial communities using culture-independent methods provide a benefit over the traditional approaches in which bacterial identification requires growth under laboratory conditions. However, culture-free molecular methods introduce biases related to: DNA extraction procedure, PCR amplification, sequencing platform used, and bio-informatic analysis (Lazarevic et al., 2013a; Lozupone et al., 2013). Below we discuss some of the challenges in the culture-independent analysis of SGS and ETA related to the physical and microbiological nature of these samples.

VISCOSITY OF SAMPLES

Because of the high viscosity observed in about 5% of ETA samples, we added dithiothreitol (DTT) in the lysis buffer for DNA extraction. By dissolving mucus, DTT treatment liquefies samples (Olsson et al., 1993) and allows for further and optimized sample processing in a semi-automated workflow. DTT and other thiol-reducing agents used to reduce *in vitro* viscosity of the mucin (Sheffner, 1963) have the potential to inhibit the subsequent PCR amplification (Deneer and Knight, 1994). We performed DNA extraction with or without DTT addition for six ETA samples and we found that 16S rDNA amplicon yields were higher when the extraction procedure included DTT-treatment (not presented). Therefore, DTT may be systematically added to viscous samples provided that it is efficiently washed away before the PCR step, as evidenced in these conditions.

CO-EXTRACTION OF BACTERIAL AND HUMAN DNA

Real-time PCR revealed that the bacterial DNA concentration in extracts varied from 0.1 to 4723 pg/μL with median values of 96.2 and 2.3 pg/μL for SGS and ETA, respectively. The yield of human DNA was generally much higher (median 10.1 ng/uL) and showed less variation in concentration (**Figure 2A**). Grice et al. (2008) showed that a mixture of human and bacterial DNA in up to a 100,000:1 mass ratio (100:1 cell ratio) did not significantly alter 16S rDNA amplification. However, the presence of human DNA is more critical when a whole genome shotgun sequencing approach is to be used to study microbial communities because many reads will derive from host DNA. To circumvent this potential limitation, a method for selective enrichment of microbial

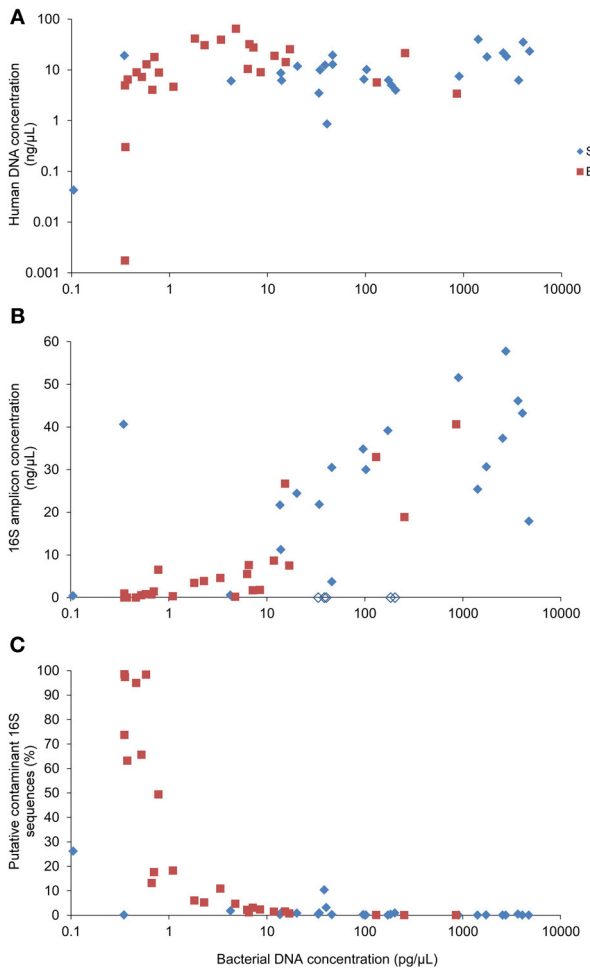

FIGURE 2 | Correlation between bacterial DNA yield and (A) human DNA yield, (B) concentration of the 16S rDNA amplicons or (C) percentage of putative contaminant 16S rDNA sequences. Bacterial and human DNA concentration in purified extracts was determined by qPCR. The yield of the 16S rDNA amplicon concentration was determined using Bioanalyzer. Empty lozenges (in **B**) correspond to SGS samples from the subject #5.

DNA from contaminating human host DNA has been developed (Feehery et al., 2013), but further progress is needed in this area. Alternatively, host DNA sequences may be recognized and removed by bio-informatic analysis (Schmieder and Edwards, 2011).

PCR INHIBITION

We observed that all of the five SGS samples from one patient (#5) presumably contained PCR inhibitors. **Figure 2B** shows that non-diluted SGS samples from this subject did not produce measurable amounts of PCR products. A 100-fold sample dilution was required to obtain a visible band of the 16S rDNA V1-3 amplicon upon electrophoresis. However, sample dilution may not be optimal in cases where the bacterial concentration is low, as it may reduce already low input DNA. The qPCR analysis of the samples from patient #5 performed after serial dilution did not reveal the inhibitory effect (not presented). This is in line with evidence that PCR inhibition depends on amplification conditions and the DNA polymerase being used (Al-Soud and Rådström, 1998). Therefore, to reduce PCR inhibition, it may be helpful to use genetically engineered DNA polymerases highly tolerant to inhibition (Kermekchiev et al., 2009).

CONTAMINANT DNA

Reagents used for DNA extraction and PCR may contain bacterial DNA which is overruled by DNA from high-density samples. However, sequence reads derived from samples with low DNA concentration may largely originate from exogenous DNA contamination.

We included in the pyrosequencing run the PCR amplification products obtained using eight negative controls. Any operational taxonomic unit (OTU) that had greater average relative abundance in negative controls than in clinical samples was considered as contaminant. The proportion of putative contaminant 16S rDNA sequences was inversely correlated with bacterial DNA concentration in DNA extracts (Spearman $r = -0.850$) (**Figure 2C**). Most contaminating OTUs (93/127) were assigned to Proteobacteria, already identified before as common reagent contaminants (Tanner et al., 1998; Biesbroek et al., 2012; Willner et al., 2012). The sequence reads assigned to the putative contaminating OTUs represented 7.2% of the reads in the sample dataset. Processing of the sequence datasets using a minimum identity threshold of 99% and the reference OTU database pre-clustered at 99% resulted in only slightly higher proportion (8%) of putative contaminating sequences. However, distinction between putative contaminants and "true" sequences will remain an important variable in metagenomic approaches.

LOW DNA YIELD

It remains unclear whether prophylactic chlorhexidine oral rinse, given to all patients in our study, decreases total bacterial load in the trachea as it has been the case with saliva (Veksler et al., 1991). 16S rDNA amplicon libraries deriving from the samples with very low bacterial load resulted in a low number of sequence reads. DNA extraction using larger sample volume (if available) and/or concentration of bacteria by centrifugation may provide a solution. Performing additional PCR cycles in order to increase the amplicon yield has been shown to introduce amplification biases in salivary samples (Lazarevic et al., 2012). Another strategy to cope with low DNA concentration is the use of multiple displacement amplification (MDA) prior to 16S rDNA amplification (Pragman et al., 2012), but MDA may also introduce a representational bias (Marine et al., 2014).

OUTLOOK

In this pilot study, involving a small number of intubated patients, we pointed to some common issues that may arise when analysing their oropharyngeal and respiratory-tract microbiota. We provided a preliminary characterization of the microbiota associated with these specific sample types that have been only

weakly (ETA) or not at all (SGS) studied so far using culture-independent methods. The analysis of larger cohorts of intubated patients with a longer follow-up period may allow to (1) answer whether the oropharyngeal and respiratory microbiota from different patients converge to one or several distinct states during hospitalization and to (2) link microbiome structure to the development of VAP which occurs in up to 30% of patients receiving mechanical ventilation (Morrow et al., 2010).

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SUPPLEMENTARY MATERIAL

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Historical and contemporary hypotheses on the development of oral diseases: are we there yet?

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Dental plaque is an oral biofilm that much like the rest of our microbiome has a role in health and disease. Specifically, it is the cause of very common oral diseases such as caries, gingivitis, and periodontitis. The ideas about oral disease development have evolved over time. In the nineteenth century, scientists could not identify bacteria related to disease due to the lack of technology. This led to the "Non-Specific Plaque Hypothesis" or the idea that the accumulation of dental plaque was responsible for oral disease without discriminating between the levels of virulence of bacteria. In the twentieth century this idea evolved with the techniques to analyze the changes from health to disease. The first common hypothesis was the "Specific Plaque Hypothesis" (1976) proposing that only a few species of the total microflora are actively involved in disease. Secondly, the "Non-Specific Plaque Hypothesis" was updated (1986) and the idea that the overall activity of the total microflora could lead to disease, was enriched by taking into account difference in virulence among bacteria. Then, a hypothesis was considered that combines key concepts of the earlier two hypotheses: the "Ecological Plaque Hypothesis" (1994), which proposes that disease is the result of an imbalance in the microflora by ecological stress resulting in an enrichment of certain disease-related micro-organisms. Finally, the recent "Keystone-Pathogen Hypothesis" (2012) proposes that certain low-abundance microbial pathogens can cause inflammatory disease by interfering with the host immune system and remodeling the microbiota. In this comprehensive review, we describe how these different hypotheses, and the ideas around them, arose and test their current applicability to the understanding of the development of oral disease. Finally, we conclude that an all-encompassing ecological hypothesis explaining the shifts from health to disease is still lacking.

Keywords: dental plaque, ecological plaque hypothesis, keystone pathogen hypothesis, specific plaque hypothesis, non-specific plaque hypothesis

INTRODUCTION

The human body contains 10 times more bacterial cells than human cells (Turnbaugh et al., 2007), with hundreds of times more bacterial than human genes (Yang et al., 2009). This microbiome has a significant influence on the physical and mental well-being of the host (Wikoff et al., 2009; Archambaud et al., 2013) and interactions between the microbiome and the host dictate health and disease.

The mouth is a nutrition-rich, humid environment of around 35/36°C with a resting-pH between 6.75 and 7.25 (Marsh, 2003; De Almeida et al., 2008). These conditions are optimal for growth of many bacteria that can form biofilms—a structured, often polymicrobial community—on oral surfaces (Jenkinson and Lappin-Scott, 2001). Oral biofilms, called dental plaque, were first observed by Antoni van Leeuwenkoek in the seventeenth century (van Leeuwenhoek, 1684) and are associated with all of the most common oral diseases: caries, and periodontal disease.

Poor oral health has been linked to many systemic diseases, including cardiovascular disease, diabetes, adverse pregnancy outcomes (Seymour et al., 2007), rheumatoid arthritis (Mercado et al., 2001), gastrointestinal disease (Watabe et al., 1998), oral cancer (Tezal et al., 2009), and pre-eclampsia (Kumar et al., 2014). Furthermore, the two most common oral diseases, caries and periodontal disease, are highly abundant among the population of industrialized countries, having a major impact on the populations' well-being and health care providers (Petersen and Lennon, 2004). To effectively treat and prevent these oral diseases, it is important to understand how healthy plaque develops into pathological plaque.

The ideas about how changes in dental plaque relate to a shift from oral health to disease have changed over time. In this review we discuss the main hypotheses of oral disease development that were proposed between the nineteenth and the twenty first century: the Traditional and Updated Non-Specific Plaque Hypothesis (NSPH), the Specific Plaque Hypothesis (SPH), the

Ecological Plaque Hypothesis (EPH) and the Keystone Pathogen Hypothesis (KPH) (**Table 1**). We then test their current applicability to the understanding of the development of oral disease.

TRADITIONAL NON-SPECIFIC PLAQUE HYPOTHESIS (T-NSPH)

The NSPH are part of a controversy that took place for over a century (Miller, 1890; Loesche, 1976; Theilade, 1986). At the end of the nineteenth century the most common idea about dental infections was that they were caused by the non-specific overgrowth of all bacteria in dental plaque (Black, 1884, 1899; Miller, 1890; Loesche, 1986). This idea is referred to as the "Non-specific plaque hypothesis" (NSPH) (Loesche, 1976) and was based on work of researchers such as Black (1884) and Miller (1890). Applying the NSPH it was postulated that it was the quantity of plaque that determined the pathogenicity without discriminating between the levels of virulence of bacteria. Believing this, the host would have a threshold capacity to detoxify bacterial products (e.g., saliva neutralizing acid) and disease would only develop if this threshold was surpassed and the virulence factors could no longer be neutralized (Theilade, 1986). The conclusion was that if any plaque has an equal potential to cause disease, the best way of disease prevention would be non-specific mechanical removal of as much plaque as possible by e.g., tooth brushing or tooth picking. The improvement of techniques to isolate and identify bacteria in the mid-20th century led to the abandoning of the NSPH. Nonetheless, mechanical plaque removal remained the most efficient way of preventing disease.

THE SPECIFIC PLAQUE HYPOTHESIS (SPH)

In the 1970s, culture-based techniques and microscopy allowed discrimination of specific bacterial species and opened the hunt for disease-related micro-organisms. It was noticed that the antibiotic kanamycin was particularly effective against cariogenic species such as oral streptococci and reduced caries formation (Loesche and Nafe, 1973; Loesche et al., 1977). This suggested that removing cariogenic bacteria from the oral cavity using antibiotics could prevent caries. In 1976, Walter J. Loesche announced the "Specific Plaque Hypothesis" (SPH), postulating that dental caries was an infection with specific bacteria in the dental plaque of which the most relevant were "mutans streptococci" (main species: *Streptococcus mutans* and *Streptococcus sobrinus*) and lactobacilli (Loesche, 1976).

This hypothesis proposed that use of antibiotics against specific bacterial species could cure and prevent caries (Loesche and Nafe, 1973; Loesche, 1976, 1986; Loesche et al., 1977). However, results from clinical studies, then and today, are not very promising. For instance, even though the use of kanamycin resulted in an overall reduction of caries, at some surfaces the caries rate increased. This indicates that kanamycin failed to penetrate certain niches allowing cariogenic species to have a selective advantage and accumulate there (Loesche et al., 1977; Banas, 2009). Furthermore antibiotics reduced the abundance of cariogenic bacteria but failed to eliminate them thus as soon as the treatment was stopped, abundance increased (Loesche and Nafe, 1973; Loesche et al., 1977), while a long period of treatment leads to antibiotic resistance (Kornman and Karl, 1982). These suggested "specific-pathogens" are part of the indigenous microflora and unlike foreign pathogens cannot be eliminated from the oral cavity (van Palenstein Helderman, 1984).

The development of the anaerobic hood in the 1970s for the first time allowed cultivation of the strict anaerobic species. This extended the SPH to periodontal diseases which were proposed to be inflammations caused by specific periopathogens and antibiotic treatment would be effective (Loesche, 1986). However, in line with the use of antibiotics in caries treatment, recent clinical studies evaluating the effectiveness of antibiotics as adjunct in periodontal therapy have not booked significant success either. A Cochrane review stated that the use of the antibiotic chlorhexidine after scaling and root planing in patients with chronic periodontitis had only a modest positive effect, and concluded that the extensive use of chlorhexidine may be questioned (Eberhard et al., 2008).

In the decade after the SPH was introduced, potential periopathogens included: protozoa, spirochetes, streptococci, and actinomyces. In addition, Gram-negative, anaerobic rods including black-pigmented *Bacteriodes* such as *Bacteriodes melaninogenicus* (renamed to *Prevotela melaninogenica*) and others from the genus *Wolinella* (re-classified as *Campylobacter*) and facultative anaerobic, Gram-negative rods of the genera

Table 1 | Comparison of the different hypotheses.

Hypothesis	References	Bacteria involved in disease	Relates to	
			Ecological changes	Host specific factors*
Traditional NSPH	Miller, 1890	All	–	–
SPH	Loesche, 1976	Specific bacteria	–	–
Updated NSPH	Theilade, 1986	All, difference in virulence	+	–
EPH	Marsh, 1994	All, enrichment of specific pathogenic bacteria	+++	–
KPH	Hajishengallis et al., 2012	Specific bacteria, dependent on (some of) remaining microbiota	++	+

**Factors that could differ amongst hosts, e.g., innate immune system (levels of cytokine and TLR expression), response to certain bacteria, GCF properties (iron concentration), saliva properties (buffer capacity) and enamel repair. – not or only briefly mentioned, + mentioned, ++ mentioned and described, +++ described in detail.*

Capnocytophaga, *Eikenella* and *Actinobacillus* (van Palenstein Helderman, 1981; Socransky et al., 1982; Slots and Genco, 1984 and reviewed by Theilade, 1986) were identified as periopathogens. However, these findings were limited due to the large number of uncultivable species (∼50%) (Siqueira and Rôças, 2013) and the bias toward easily cultivable species (Handelsman, 2004). The finding of different species related to periodontal disease led to the idea that oral disease could be initiated by a number of specific pathogens (Socransky, 1977; Theilade, 1986). This idea was further investigated over the next decades and led to the famous Socransky-complexes which include bacterial clusters based on their association with periodontal disease (Socransky et al., 1998).

UPDATED NON-SPECIFIC PLAQUE HYPOTHESIS (U-NSPH)

Else Theilade also noticed that the "specific-pathogens" from the SPH were indigenous bacteria and sometimes common bacteria in health, which led to an updated NSPH in 1986 focusing on periodontal disease (Theilade, 1986). At this time most researchers seemed to agree that gingivitis was a non-specific inflammatory reaction to a complex indigenous microbiota. However, the updated NSPH took into consideration that some indigenous subgingival bacteria can be more virulent than others and that plaque composition changes from health to disease. Nevertheless, it stated that all bacteria in plaque contribute to the virulence of the microflora by having a role in either colonization, evasion of the defense mechanism, and/or provocation of inflammation and tissue destruction (Theilade, 1986). Theilade's statement that "any microbial colonization of sufficient quantity in the gingival crevice causes at least gingivitis" was supported by the fact that a non-pathogenic plaque (i.e., not causing gingivitis in the absence of oral hygiene) had never been observed. Additionally, it was considered that some people have gingivitis for a lifetime without tissue and bone destruction, while others encounter rapid progression into periodontitis. Unlike the classic NSPH, the updated NSPH could explain this by taking into account that differences in the plaque microbial composition could lead to differences in pathogenic potential.

ECOLOGICAL PLAQUE HYPOTHESIS (EPH)

In 1994 Philip D. Marsh proposed a hypothesis that combined key concepts of the earlier hypotheses. In his "Ecological Plaque Hypothesis" (EPH), disease is the result of an imbalance in the total microflora due to ecological stress, resulting in an enrichment of some "oral pathogens" or disease-related microorganisms (Marsh, 1994). This idea was not entirely new since Theilade, in the review proposing the U-NSPH concluded that "increased virulence of plaque (leading to disease) is due to a plaque ecology unfavorable to the host and favorable for overgrowth by some of the indigenous bacteria having a pathogenic potential" (Theilade, 1986). Importantly, Marsh expanded this theory and related the changes in microbial composition to changes in ecological factors such as the presence of nutrients and essential cofactors, pH and redox potential (Marsh, 1994, 2003). For example, frequent exposure to a low pH, for instance as the result of sugar fermentation, leads to a relative increase of acid-tolerant species. The thought arose that disease could be prevented by interfering with processes that break down homeostasis and change composition. For example, non-fermentable sweeteners could be used to replace sugar and thus prevent acidification.

Marsh provided and collected convincing evidence to support his hypothesis, and it is still generally accepted that the composition of dental plaque depends on the environment. Thus, the classical "everything is everywhere, but, the environment selects" (Baas Becking, 1934) was successfully applied to dentistry (Marsh, 2003; De Wit and Bouvier, 2006). Marsh also considered the reverse: the bacteria in dental plaque affect the environment. For instance, early colonizers of supragingival dental surfaces, are usually facultative anaerobic bacteria that use the oxygen, producing carbon dioxide and hydrogen (Alexander, 1971; Marsh, 2003). This lowers the redox potential giving strict anaerobes a chance to settle and multiply in the biofilm. Bacterial growth is dictated by the environment, which in turn is influenced by bacterial metabolism, leading to mutual dependencies in health but also a chain of events that lead to diseases.

The importance of the host-dependent environment in selection of bacterial species that colonize should not be neglected. A simple but convincing example is a study indicating that, even though there is a continuous passage of bacteria from saliva to the gut, only 29 out of over 500 taxa found in the mouth were recovered in fecal samples (Moore and Moore, 1994). However, like the other hypotheses, the traditional EPH does not address the role of genetic factors of the host that significantly contribute to the composition of dental plaque and to susceptibility to disease (Mason et al., 2013).

KEYSTONE PATHOGEN HYPOTHESIS (KPH)

The concept of keystone species is derived from basic ecological studies. Certain species have an effect on their environment that is *disproportional* relative to their overall abundance (Paine, 1969; Power et al., 1996; Darveau et al., 2012). George Hajishengallis and colleagues applied this concept to (oral) microbiology by proposing "The Keystone-Pathogen Hypothesis" (KPH) (Hajishengallis et al., 2012). The KPH indicates that certain low-abundance microbial pathogens can cause inflammatory disease by increasing the quantity of the normal microbiota and by changing its composition (Hajishengallis et al., 2012). For instance, *Porphyromonas gingivalis* is shown to be able to manipulate the native immune system of the host (reviewed by Darveau, 2010). By doing so it was hypothesized that it does not only facilitate its own survival and multiplication, but of the entire microbial community. In contrast to dominant species that can influence inflammation by their abundant presence, keystone pathogens can trigger inflammation when they are present in low numbers (Hajishengallis et al., 2012). When disease develops and advanced stages are reached, the keystone pathogen are detected in higher numbers (Socransky et al., 1998). Importantly, even though their absolute number increases, keystone pathogens can decrease in levels compared to the total bacterial load which increases as plaque accumulates in periodontitis (Abusleme et al., 2013).

The KPH was developed by observing the properties of the "red complex" (Socransky et al., 1998) bacterium *P. gingivalis*.

Studies in mouse models showed that very low presence of *P. gingivalis* (<0.01% of the total bacterial count in plaque) could alter the plaque composition, leading to periodontitis (Hajishengallis et al., 2011). In germ-free mice, *P. gingivalis* was able to colonize by itself, but was not able to trigger disease without the presence of other bacterial species. This indicates that (some of) the commensal microbiota is essential in the disease process. Evidence of *P. gingivalis* acting as a keystone pathogen was also obtained in rabbit models (Hasturk et al., 2007) and non-human primates (Page et al., 2007).

The role of the host-immune system is critical in the KPH. At health, periodontal tissue contains a wall of neutrophils, between the plaque and the epithelial surface, residing just outside the epithelial cells. Expression of mediators such as interleukin 8 (IL-8), intercellular adhesion molecule (ICAM) and E-selectin is required to form this neutrophil wall (Moughal et al., 1992; Nylander et al., 1993; Gemmell et al., 1994; Tonetti, 1997). E-selectin is required for neutrophil migration from the highly vascularized gingival tissue, IL-8 is a key neutrophil chemoattractant produced by epithelial cells, and ICAM facilitates adhesion of neutrophils to the tissue allowing formation of this wall (Springer, 1994; Darveau, 2009). Furthermore, the epithelium expresses low levels of a wide range of toll-like receptors (TLR's), including TLR1-TLR9 that mediate the response to a broad range of microorganisms (Sugawara et al., 2006; Mahanonda and Pichyangkul, 2007; Beklen et al., 2008). The array of different TLRs in combination with the multitude of bacterial species lead to a large variety of cytokines that are expressed at health (Kumar et al., 2011; Matthews et al., 2013). Studies in germ-free mice show that there are low levels of innate host mediators, such as IL-1B, present in the periodontal tissue (Dixon et al., 2004). This indicates that a basic level of cytokine expression is genetically programmed without bacterial challenge. The composition and amount of bacteria in plaque modifies cytokine expression further (Dixon et al., 2004; Kumar et al., 2011; Matthews et al., 2013).

Evidence was found of three major KPH mechanisms of *P. gingivalis* that could impair the above mentioned host defenses: (1) Toll-like receptor (TLR) response manipulation, (2) interleukin 8 (IL-8) subversion and (3) the corruption of the complement system (reviewed by Darveau, 2009, 2010; Hajishengallis and Lambris, 2011; Darveau et al., 2012).

In vitro, the TLR response is manipulated by *P. gingivalis* with the help of two types of lipopolysaccharides (LPS) with different lipid A structures—PgLPS1690 (type I) and PgLPS1435/1449 (type II). Type I is a TLR4 agonist thus activating the immune system, while Type II is a TLR4 antagonist inhibiting the immune response to *P. gingivalis* (Coats et al., 2005, 2007). The concentration of iron determines which type of LPS is expressed (Hanioka et al., 1990, 1991; Olczak et al., 2005). In the oral cavity, the main source of iron is hemin, found in the gingival crevicular fluid (GCF). During inflammatory process, GCF increases indicating that *P. gingivalis* type II LPS expression increases which reduces the TLR4 response. It was proposed that this could facilitate survival and multiplication of the entire microbial community (reviewed by Darveau, 2010).

Porphyromonas gingivalis can block production of IL-8 *in vitro*, which is produced by gingival epithelial cells in response to other bacteria, by secreting a serine phosphatase that inhibits the synthesis of IL-8 (Darveau et al., 1998; Hasegawa et al., 2008). This process is called "local chemokine paralysis" and delays the recruitment of neutrophils preventing proper neutrophil wall formation, of which was proposed that it could facilitate initial microbial colonization of the periodontium (Madianos et al., 1997; Darveau et al., 1998). Other "red complex" bacteria such as *T. denticola*, are also able to manipulate the IL-8 response of the host however the mechanism(s) involved is not understood (Ji et al., 2007).

The third and best *in vivo* documented keystone pathogen mechanism is the interference with the complement system (Hajishengallis et al., 2011; Abe et al., 2012). The complement system is a major component of the innate immune response involved in recognizing and destroying microorganisms (Walport, 2001) with complex roles in homeostasis and disease (Ricklin et al., 2010). To be a successful pathogen in humans (and any other mammal) a microorganism needs to be able to avoid complement-mediated detection and killing. Again, the best-studied example in the oral cavity is *P. gingivalis* that produces membrane bound and soluble arginine-specific cysteine proteinases called "gingipains" (Imamura, 2003). Gingipains can cleave complement factors C3 and C5 into active fragments C5a (cell activator) and C3b (phagocytosis enhancer). These fragments can be further degraded by gingipains resulting in loss of their function (Wingrove et al., 1992). However, this takes up to 1 h when adding purified compounds together *in vitro*. More relevant is that in the presence of gingipains the levels of the inflammatory mediator C5a increase within seconds (reviewed by Hajishengallis et al., 2012). This leads to an increased activation of the C5a receptor (C5aR) on leukocytes. C5aR is involved in crosstalk with TLR2, which is activated in parallel by *P. gingivalis* (and other bacterial) surface ligands. While this crosstalk leads to increased inflammation, it impairs the killing capacity for leukocytes (Wang et al., 2010; Liang et al., 2011). In mouse models this mechanism has a major role in accelerating periodontitis development and bone loss (Liang et al., 2011). A *P. gingivalis* strain that lacks gingipains failed to change the oral microbiota and induce bone loss (Liang et al., 2011). Additionally, periodontitis did not develop in mice lacking one of the two involved receptors—C5aR or TLR2 (Hajishengallis et al., 2011; Liang et al., 2011). This provides clear evidence that in mice the dysbiosis caused by *P. gingivalis* is mainly due to complement subversion.

In conclusion, it was proposed that currently known and unknown keystone pathogens use a combination of these and presently unknown mechanisms to manipulate the innate defense system leading to destructive periodontitis (Darveau, 2010).

APPLICABILITY OF THE PLAQUE HYPOTHESES TO THE DEVELOPMENT OF ORAL DISEASES

CARIES AND ECOLOGICAL PLAQUE HYPOTHESIS

Dental caries is a multifactorial disease, greatly influenced by the diet of the host (Touger-Decker and van Loveren, 2003). Therefore, this process fits EPH well (**Figure 1**): Subjects that frequently consume a considerable amount of fermentable carbohydrates, select for bacteria that ferment these carbohydrates and produce acids (Bradshaw and Marsh, 1998; Marsh, 2003).

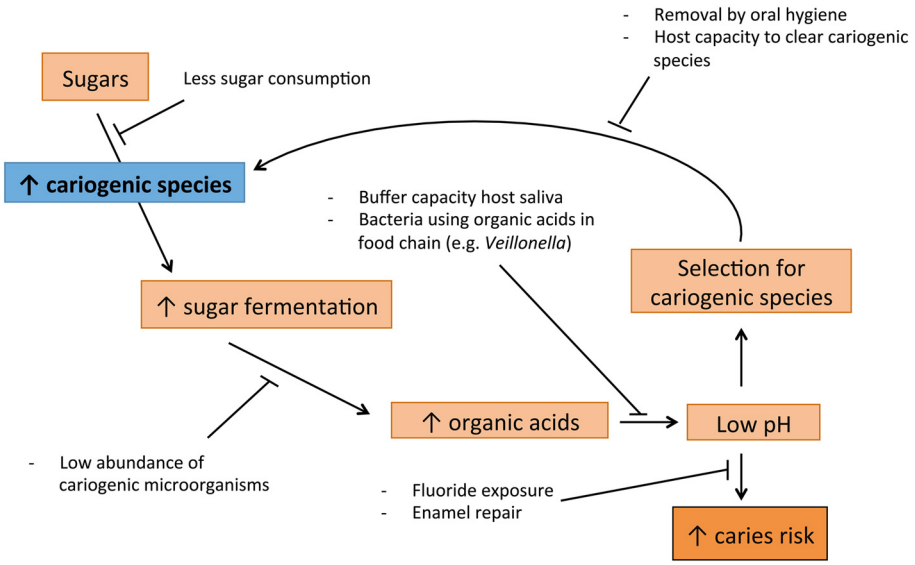

FIGURE 1 | Suggested cycle of disease leading to caries, supported by Ecological Plaque hypothesis (EPH).

This leads to more sugar fermentation and thus acid production, increasing the cariogenic bacteria even more (Bradshaw and Marsh, 1998; Welin et al., 2003). Acidogenic (acid-producing) and aciduric (acid-tolerating) bacteria such as the classic *S. mutans*, *S. sobrinus* and *Lactobacillus* spp., and the later discovered *Bifidobacterium* spp., lower the pH to levels at which enamel is demineralized, which can result in caries (Loesche, 1986; Becker et al., 2002; Beighton et al., 2010). The EPH is supported also by the caries-protective role of the host-related factors such as salivary properties and fluoride exposure (Marsh and Martin, 2009).

Acid stress is a well-known biological stress factor (ergo a selective pressure) and therefore, fermentable carbohydrates are the type of nutrition that has the highest impact on the ecology of the mouth (Touger-Decker and van Loveren, 2003). Studies have shown that it is the acidic pH caused by the sugar fermentation, and not the availability of sugar itself, that leads to the disturbance of microbial homeostasis associated with caries (Bradshaw and Marsh, 1998). The aciduric bacteria are able to proliferate at acidic pH. For instance, *S. mutans* up-regulates a number of specific proteins when exposed to an acidic pH, which enhance the chances of survival under these conditions (Welin et al., 2003). In contrast, some bacteria associated with oral health are sensitive to acidic pH and are thus outcompeted in individuals that regularly consume fermentable sugars (Marsh, 2003).

S. mutans has carried the role of the main caries pathogen for decades (Banas, 2009) which, as discussed above, resulted in the SPH. However, demineralization can take place without the presence of this specific species, as non-mutans streptococci (e.g., *S. oralis* and *S. mitis biovar*), can metabolize sugars in comparable way to *S. mutans* (De Soet et al., 2000). It has also been shown that phenotypic heterogeneity among different *S. mutans* strains determines the rate of the carbohydrate fermentation and thus their cariogenic potential (Burne et al., 2012).

PERIODONTAL DISEASES AND THE FUSION OF NON-SPECIFIC, ECOLOGICAL AND KEYSTONE PATHOGEN HYPOTHESES

Unlike caries, periodontal diseases—gingivitis and periodontitis—do not fit to a single hypothesis. The intimate interaction of bacteria with the host leading to inflammatory reaction adds to the complexity of these diseases (**Figure 2**).

The U-NSPH can partly explain the development of gingivitis. If plaque is allowed to accumulate without intervention by oral hygiene methods, gingivitis is established without exceptions, generally after a period of 2–3 weeks (Loe et al., 1965; Theilade et al., 1966). The finding that many members of the TLR family, including TLR1-TLR9, are present in the gingival tissue (Sugawara et al., 2006; Mahanonda and Pichyangkul, 2007; Beklen et al., 2008) has brought an old thought into reconsideration: "any microbial colonization of sufficient quantity in the gingival crevice causes at least gingivitis" (Theilade, 1986). With such a broad variety of TLRs most commensal species should be able to trigger inflammation. A broad range of bacterial products can be recognized including DNA, flagella and fimbriae, peptidoglycan and lipoteichoic acids, and LPS. Another point supporting the idea that all bacteria have a role in the gingival inflammation is that Gram-negative rods and spirochetes are not able to form plaque without Gram-positive species (Slots and Gibbons, 1978). Thus, many, if not all, species are probably directly or indirectly involved in triggering the early stages of gingivitis.

The ecological changes as indicated by the EPH are also relevant in gingivitis development. Health-associated species with an insignificant role in gingival inflammation could significantly contribute to a change in growth conditions favoring pro-inflammatory bacteria. For instance, facultative anaerobic *Rothia* spp. have recently been associated with oral health (Griffen et al., 2012; Abusleme et al., 2013; Kistler et al., 2013) and reduce the oxygen levels in the direct environment. This in turn allows

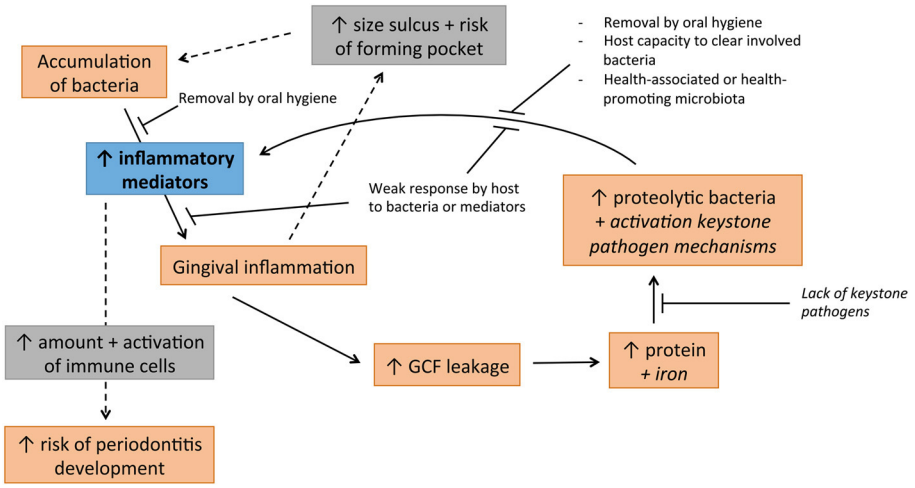

FIGURE 2 | Cycle of disease involved in development of periodontal diseases, jointly supported by non-specific, ecological and keystone pathogen hypotheses. Italised text indicates hypothetical involvement.

proliferation of strict anaerobes, which include proteolytic Gram-negative bacteria that contribute to triggering the inflammation. The same holds for facultative anaerobic *Streptococcus* spp. that dominate the plaque in relative levels in health but decrease in disease (Matthews et al., 2013). As gingivitis develops, inflammation, gingival bleeding on probing (BoP) and the volume of GCF also increases (Kistler et al., 2013; Matthews et al., 2013). Accumulation of commensal microbiota results in an increase in GCF that in turn changes the environment because GCF contains high levels of proteins that are a novel source of nutrients (Marsh, 2003). Furthermore, GCF contains iron that triggers keystone pathogen mechanisms in *P. gingivalis* (and perhaps other bacteria) decreasing TLR4 activity *in vitro*, which could enhance survival for the whole community. This could lead to a protein-rich environment with a decreased innate response in which some bacteria can evade the immune system and are able to accumulate. Increased bacterial accumulation triggers more inflammation, which leads to a vicious circle where the host is producing more GCF, more protein and more iron (**Figure 2**).

Periodontitis results from complex interactions of micro-organisms and the immune system (Sanz et al., 2011). Due to increased plaque amount and increased abundance of more virulent and keystone pathogen bacteria, the concentration of inflammatory mediators increases (reviewed by Graves, 2008; Darveau, 2010). An increase in the concentration of pro-inflammatory cytokines in periodontal tissue can directly affect bone loss (Nagasawa et al., 2007).

It should be taken into account that there are differences between susceptibility for oral disease among people even if they share the same lifestyle. This could be due to the different proportions of bacterial species at health, determined by genetic factors. In recent a pyrosequencing study, subgingival plaque composition of 192 people belonging to four ethnic affiliations: non-Hispanic blacks, non-Hispanic-whites, Chinese, and Latinos, was determined. Subgingival plaque composition differed significantly between these four ethnicities, a difference that, according to the authors, was not dependent on diet and cultural differences (Mason et al., 2013). Although African Americans and Caucasians shared similar environmental factors over several generations they had different plaque compositions. Even though the authors did not provide any data to prove the consistence of environmental factors over several generations, they were able to use their results to predict ethnicity based on the dental plaque composition of random subjects. This indicates that genetic factors could have an influence in determining the core microbiome (nature over nurture), just like the ethnic background determines susceptibility for diseases such as cholera (Levine et al., 1979), pneumonia (Salnikova et al., 2013) and cystic fibrosis (Kilpatrick, 2002). These results suggest that—after controlling for socioeconomic, dietary, and other environmental factors—susceptibility for periodontal disease and caries varies among ethnicities (Cruz et al., 2009; Mason et al., 2013). Proteins involved in innate immune responses to bacterial virulence factors, e.g., TLR4 and heat shock proteins, also vary among ethnic groups (Nguyen et al., 2004; Miller and Cappuccio, 2007). This indicates that genetic host-factors have an important role in health and the shift to disease. For example, people with high expression of complement factor C5 might be more susceptible to disease progression by *P. gingivalis*-produced gingipains (*vide supra*). The role of the host in periodontal disease development should not be neglected. In transgenic mice overexpressing IL-1α, periodontitis developed even in the absence of a significant bacterial challenge (Dayan et al., 2004). In another study, it was found that aging-associated periodontitis was accompanied by lower expression of Del-1, an endogenous inhibitor of neutrophil adhesion (Eskan et al., 2012). Young Del-1-deficient mice had excessive neutrophil infiltration and developed spontaneous periodontitis. This was prevented by local administration of Del-1 which inhibited neutrophil accumulation and bone loss. It is therefore not unimaginable that periodontal disease develops in individuals with a defective immune homeostasis. For instance, individuals with defective IL-1α or Del-1 regulation could develop periodontal disease with bacterial

profiles that would maintain health in other individuals. This is supported by a recent study in 385 individuals, which concluded that a single nucleotide polymorphism (SNP) in *IL-1α* was associated with periodontitis (Laine et al., 2013).

"Red complex" bacteria are in general strongly associated with periodontitis. However, there are examples of studies in which the "red complex" bacteria are below the detection threshold at diseased sites. At the same time, "red complex" bacteria can be frequently detected, be it in low numbers, in healthy sites (Socransky et al., 1998; Bik et al., 2010). Periodontitis is always been preceded by gingivitis. However, there are people with gingivitis for a lifetime who do not develop periodontitis, while others encounter rapid progression into periodontitis after only a short gingivitis episode. Apart from the genetic host factors discussed above, these findings could also be related to microbial phenotypic heterogeneity and plasticity as suggested previously (Burne et al., 2012). The role of both the host genetic background and the microbial phenotypic heterogeneity, is illustrated in a recent study by Haubek et al. (2008). *Aggregatibacter actinomycetemcomitans* is a Gram-negative rod that expresses a leucotoxin that specifically lyses human neutrophils. Compared to other strains, *A. actinomycetemcomitans* JP2 has several genetic differences (Haubek et al., 2008). As a consequence of a deletion of 530 base pairs in the promoter of the gene encoding leucotoxin, the JP2 clone produces significantly higher levels of leucotoxin. In a study in Moroccan adolescents, individuals that carried the JP2 clone had a higher risk of developing periodontitis (relative risk 18.0 vs. 3.0) (Haubek et al., 2008). Population genetic analysis suggested that, despite the global presence of the JP2 clone, it is strongly associated with the West African ethnicity thus indicating a significant host tropism effect (Haubek et al., 2008).

ALL THESE HYPOTHESES, BUT ARE WE THERE YET?

The key to oral health has been described as having a diverse microbiome with two main characteristics (Zarco et al., 2012). Firstly, it should practice *commensalism within itself*, meaning that bacteria in the microbiome benefit from others without affecting them (Mikx and van Der Hoeven, 1975). Secondly, the microbiome should practice *mutualism with its host*, meaning that there is a relationship in which both partners benefit. On the one hand, the host provides nutrients and a protective environment for the microbiome. On the other hand, the microbiome contributes to the host physiology and defenses against pathogens (Zarco et al., 2012). A healthy microbiome is maintained by bacterial homeostasis which is achieved by a balance of inter-microbial as well as host-microbial interactions, which can be synergistic and antagonistic (Marsh, 1994, 2003). In this respect it should be noted that although other inhabitants of the oral cavity, including archaea, protozoa, viruses and fungi, might have significant roles in health and disease (Krom et al., 2014), most common studies on the oral microbiota are limited to bacteria. The role of these "co-inhabitants" is not well explored and thus also lacking in the above-listed hypotheses.

For the caries process, the best-fitting EPH does not consider host genetic components at all. This is striking in light of the most-unethical experiment in the history of cariology, performed in the 1950's in Sweden, known as the Vipeholm study. Mentally disabled, institutionalized individuals received high frequency carbohydrate snacks for a period of 2 years and caries incidence was scored. Depending on snack type and frequency, high levels of caries developed with the exception of about 20% of the individuals, who did not develop dental caries even at these highly cariogenic conditions (Vieira, 2012). A genetic sensitivity to caries was further supported by the observation that the parents and siblings of these individuals showed lower caries prevalence than the rest of the population (Böök and Grahnén, 1953). Since then, more and more evidence has been delivered that supports genetic susceptibility to caries (Werneck et al., 2010; Wang et al., 2013) and should be implemented in explaining the disease development.

With the KPH, the periodontal diseases, especially periodontitis, heavily depend on a single periodontal bacterium—*P. gingivalis*. This is probably due the relative ease of cultivation and genetic modification compared to the other species (Darveau et al., 2012) combined with the, by definition, low relative abundance of keystone pathogens. Other species might be equally or even more active in the process that leads from periodontal health to disease and should be investigated. Besides, it should be noted that the "keystone pathogenesis" itself has yet to be demonstrated in humans.

The large number of microbiome studies that are appearing provide a wealth of information at the taxonomic (OUT) or species level. However, in light of the known phenotypic plasticity and heterogeneity it is important to study strain differences. Although differences in bacterial phenotypes are reflected in the discussed hypotheses, mechanisms that govern them are not. The differences at strain-level rather than species level, among patients with a lifetime of gingivitis and patients that develop periodontitis rapidly should be investigated in more detail. Also, micro-evolution within the oral cavity, in which bacterial traits can be exchanged, deleted and changed, from birth to death of an individual is not covered. The host-to-host, species-to-species and strain-to-strain differences all play a role in the functioning of the oral microbiome and the way it handles environmental changes related to the ease in which it triggers disease. So far it is not known if there are fixed patterns in the shift from health to disease among people with different genetic backgrounds. The virulence of certain species or strains could differ enormously among different ethnic groups and individuals.

The recently described polymicrobial synergy and dysbiosis (PSD) model for periodontitis highlights the importance of other bacteria in keystone pathogenesis and the thought that other than the classical "red complex" species could have similar keystone roles in periodontitis (Hajishengallis and Lamont, 2012). It states that in periodontitis polymicrobial synergy can lead to dysbiosis. In this model, there are more ways to skin a cat, since different members or different gene combinations can result in a disease-provoking microbiota. In another very recent review, the importance of bacteria acting upstream and downstream of *P. gingivalis* pathogenesis is further described (Hajishengallis and Lamont, 2014). Our idea that all bacteria could have a role in periodontal disease development is supported by the PSD model which states that "traditional concepts of pathogen and commensal have become obsolete" (Hajishengallis and Lamont, 2014).

For example, *S. gordonii* (commensal) can act as an accessory pathogen by increasing the virulence of *P. gingivalis*. In another very recent review applying the PSD model, the conclusion is that the transition to periodontitis requires a dysbiotic microbiota and a susceptible host (Hajishengallis, 2014). We agree that this might be true. In a Sri Lankan population with no oral hygiene habits nor dental care at all, the majority of the population (89%) experienced periodontal breakdown (Löe et al., 1986). However, 11% of this group did not have any periodontal breakdown beyond gingivitis. It is highly probable that the long-term accumulated plaque in these "periodontitis-resistant" individuals contained many late colonizing bacteria associated with periodontitis development (Kolenbrander et al., 2010). However, further research should be performed to confirm this and to conclude if it were host factors (innate immune system), bacterial factors (metagenome activity) or both functioning in a way that kept these individuals periodontitis free under conditions that would cause tissue break-down in the average person. In our opinion, the PSD-model is currently the most extensive, however it is modeled only for periodontitis.

All presently available hypotheses fall short of combining actual microbial and host behavior that lead to maintenance of health or the shift to disease. An all-encompassing hypothesis is needed, but this is only possible when sufficient knowledge is obtained between the complex relationships of the oral microbiome and the hosts' innate immune system. The advancement of technology for sequencing allows detailed analysis of the metagenome (all potentially expressed host and microbial functions) and meta-transcriptome (all actually expressed host and microbial functions). Combined with increased computational power and more advanced bioinformatics technology, future studies will provide a more holistic view of the oral ecology and lead to unraveling of mechanisms that govern change from health to disease.

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