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INVITED MEDICAL REVIEW

A practical guide to the oral microbiome and its relation to health and disease

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The oral microbiome is incredibly complex with the average adult harboring about 50–100 billion bacteria in the oral cavity, which represent about 200 predominant bacterial species. Collectively, there are approximately 700 predominant taxa of which less than one-third still have not yet been grown *in vitro*. Compared to other body sites, the oral microbiome is unique and readily accessible. There is extensive literature available describing the oral microbiome and discussing the roles that bacteria may play in oral health and disease. However, the purpose of this review is not to rehash these detailed studies but rather to educate the reader with understanding the essence of the oral microbiome, namely that there are abundant bacteria in numbers and types, that there are molecular methods to rapidly determine bacterial associations, that there is site specificity for colonization of the host, that there are specific associations with oral health and disease, that oral bacteria may serve as biomarkers for non-oral diseases, and that oral microbial profiles may have potential use to assess disease risk.

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Introduction

Although there have been wildly diverse estimates of the total number of oral bacterial taxa, it is generally accepted that, collectively speaking, there are 687 predominant species in the oral cavity (www.homd.org) (Dewhirst *et al*, 2010). These estimations are based on years of traditional identification of bacteria from cultural and phenotypic characterization studies, but mostly from identification of bacteria from culture-independent molecular studies using 16S rRNA gene comparative analyses (Paster *et al*, 2001, 2006; Aas *et al*, 2005, 2008; Dewhirst *et al*, 2010). As of May 2016, 31% of oral bacterial taxa have not been grown *in vitro* (www.homd.org). These not-yet-cultivated taxa are typically referred to as phylotypes, or colloquially as 'uncultivables'. About 400–500 oral taxa have been detected in the subgingival crevice alone (Paster *et al*, 2001; Aas *et al*, 2005). The remaining taxa are distributed on the many oral habitats including different areas on the tongue, tooth surface, buccal mucosa, tonsils, soft and hard palate, and lip vestibule (Aas *et al*, 2005; Human Microbiome Project, 2012a; Human Microbiome Project, 2012b; Segata *et al*, 2012). The salivary microbiome would essentially be comprised of a mixture of microbes sloughed off from all sites. Although there is considerable overlap of species detected in all oral sites, such as certain species of *Streptococcus*, *Gemella*, *Granulicatella*, *Neisseria*, and *Prevotella*, there is often site specificity. For example, species of *Rothia* typically colonize the tongue or tooth surfaces, *Simonsiella* colonizes only the hard palate, *Streptococcus salivarius* mainly colonizes the tongue, and treponemes are typically restricted to the subgingival crevice (Kazor *et al*, 2003; Mager *et al*, 2003; Aas *et al*, 2005; Segata *et al*, 2012).

It is well known that specific bacterial taxa that colonize the oral cavity are associated with oral health and oral diseases or afflictions, such as dental caries, periodontal diseases, endodontic lesions, dry socket, halitosis, and odontogenic infections (Socransky *et al*, 1998; Paster *et al*, 2001, 2006; Mager *et al*, 2003; Aas *et al*, 2005, 2008; Socransky and Haffajee, 2005; Dewhirst *et al*, 2010; Johansson *et al*, 2016). Furthermore, oral bacteria may be linked or serve as biomarkers for certain systemic diseases, such as pancreatic cancer (Farrell *et al*, 2012), diabetes type II (Demmer *et al*, 2015), pediatric Crohn's disease (Docktor *et al*, 2012), heart disease (Leishman *et al*, 2010), and low weight, preterm birth (Shira Davenport, 2010). However, it is yet to be established if there is a causal relationship between the oral microbiome and these systemic disorders.

The Human Microbiome Project

Our ability to study the human microbiome has been greatly improved by the advances made in sequencing technologies

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and recent developments in bioinformatics. These advances have led to a plethora of genomic and metagenomic studies investigating the role of microbes in several different ecosystems (Gilbert and Dupont, 2011). Established in 2008, the Human Microbiome Project (HMP) aimed to determine the microbiomes from 242 healthy human subjects from sites including the oral cavity (seven sites), nasal cavity, skin, gastrointestinal tract, and urogenital tract. The data obtained from sequencing was used for taxonomic assignment and is also available through the HMP Data Analysis and Coordination Center data browser. This enables the advance of research relating to the human microbiome by acting as a community resource that is widely accessible. The establishment of such an effort led to the development of a variety of new protocols including methods for laboratory and sequence processing, and analysis of 16S rDNA and whole-genome shotgun sequences and profiles of the microbiome (Human Microbiome Project, 2012a). Results from the HMP analyses indicated that repertoire and abundance of microbiota found on individuals varies greatly depending on multiple factors, with ethnic/racial background having one of the strongest associations to microbes with clinical metadata (Human Microbiome Project, 2012b). Such studies provided insights into what constitutes the normal microbiota of each organ or mucosa in the body, enabling a better understanding of how they impact human health. As of April 2016, over 1300 reference strains isolated from the human body were sequenced and the data publicly available for researchers.

The Human Oral Microbiome Database

The purpose of the Human Oral Microbiome Database (HOMD; www.homd.org) is to provide the scientific community with comprehensive information on the approximately 700 predominant bacterial species that inhabit the human oral cavity (Dewhirst *et al*, 2010); www.homd.org. This curated 16S rDNA database provides a provisional naming scheme for currently unnamed species or phylotypes. The HOMD also links sequence data of 687 oral taxa with phenotypic, phylogenetic, clinical, and bibliographic information. A phylogenetic tree of 118 of the most predominant and other key taxa, that were mostly identified from 16S rRNA cloning studies, is shown in Figure 1. Note that there are many well-known oral species, for example, including species *Prevotella*, *Porphyromonas*, *Treponema*, *Tannerella*, *Fusobacterium*, and *Streptococcus*, as well as perhaps lessor known species, for example, phylotypes of members of the phyla SR1, GN02, and TM7, *Fretibacterium*, *Solobacterium*, and *Abiotrophia*. Many are associated with oral health and disease and will be discussed in more detail below. Phylogenetic trees of members of each bacterial phylum or family can be downloaded from the HOMD website. As part of HOMD, HMP, and other sequencing projects, genome sequences are available for approximately 400 oral bacterial taxa, which represent 58% of the known oral species. BLAST tools are available to rapidly determine oral bacterial identification from 16S rDNA sequences. Easy-to-use tools for viewing all publically available oral bacterial genomes are also offered on the HOMD site.

CORE, another phylogenetically curated 16S rDNA database of the oral microbiome, is also available and can be used to identify the bacterial taxa from large next-generation sequence (NGS) 16S rDNA datasets (Griffen *et al*, 2011).

Bacterial–bacterial and host–bacterial interactions

It has long been known that oral bacteria exhibit specificity for their respective colonization sites and to each other, directed by adhesin-receptor binding (Kolenbrander, 2000). Thus, adhesins on bacterial cells bind to receptors on epithelial cells or to other bacteria, including pili, autotransporters, and extracellular matrix-binding proteins (Nobbs *et al*, 2011). Some receptors are derived from salivary components, such as proline- or serine-rich proteins, that undergo conformational change when they are adsorbed onto surfaces such as the tooth surface. Consequently, bacteria do not simply bind or randomly pile on to oral surfaces or other bacteria – there is a specific interaction with a strong affinity. Such specificity can be readily seen *in situ* using Combinatorial Labeling and Spectral Imaging FISH (CLASI-FISH), which enables simultaneous identification of tens to possible hundreds of bacterial species (Valm *et al*, 2011; Mark Welch *et al*, 2016). Figure 2 illustrates a specific spatial organization of bacterial taxa within dental plaque and that the bacteria do not randomly aggregate. Based on these CLASI-FISH data, the authors were able to propose a model for plaque microbiome development integrating known metabolic, adherence, and environmental information. Thus, we can deduce functional traits of the specific members of the consortium; for example, anaerobic species are at the center with facultatively anaerobic or aerobic species being at the edge.

Tools to define oral microbiome

Culture techniques

Historically, bacterial taxa were identified using culture-dependent methodologies such as microscopy, biochemical and other phenotypic tests, growth conditions, sugar utilization, and antibiotic sensitivity. As 31% of the known oral taxa still cannot be grown *in vitro*, bacterial culture is still important in microbiology (Vartoukian *et al*, 2010). However, for diagnostic purposes, except for antibiotic sensitivity, culture-dependent methods are labor-intensive, costly, and not as comprehensive as the molecular DNA-based technologies, which circumvent the need for culture.

Gel-based technologies

High-throughput analysis of microbial communities has been possible due to several culture-independent methodologies. Early on, community-fingerprinting techniques such as denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis separated DNA of the same size, which, in turn, could be sequenced for identification purposes (Nishigaki *et al*, 2000; Anderson and Cairney, 2004; Deng *et al*, 2008). Restriction fragment length polymorphism was used to digest homologous

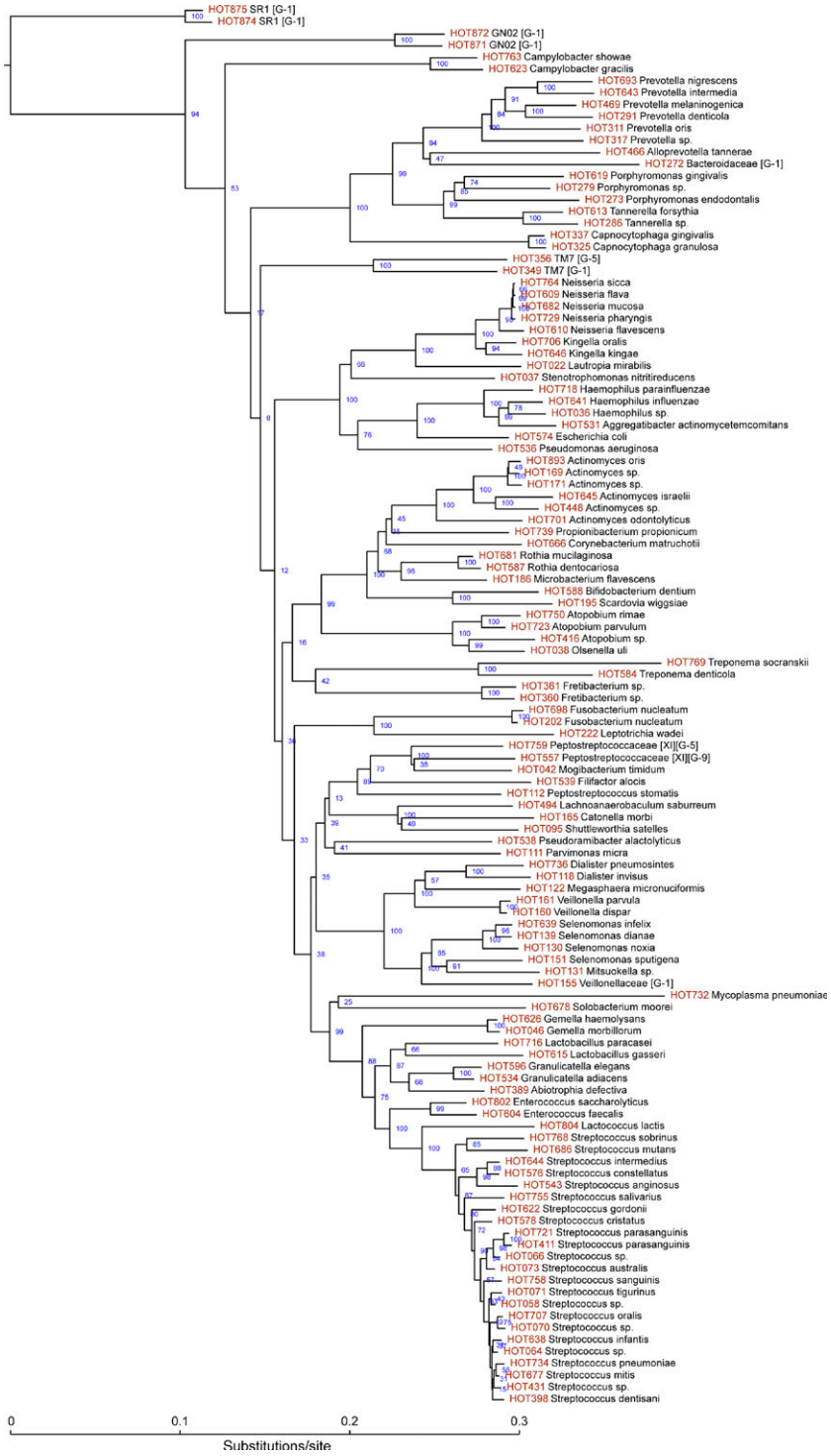

Figure 1 16S rRNA Phylogenetic tree of 118 human oral microbial species. The aligned sequences (HOT designations, printed in red, means Human Oral Taxon) were downloaded from HOMD (www.homd.org), and sequences of selected taxa were subjected to QuickTree software v1.1 (Howe *et al*, 2002) using the Kimura DNA substitution model for pairwise distances. The bootstrap values are printed in blue based on 100 iterations. For those species without a specific epithet (e.g., species name), they are either not-yet-cultured phylotypes or cultured unnamed species

DNA sequences, and variation in the resulting fragment length was then used as a tool for genome mapping (Deng *et al*, 2008). This enabled a macro-level analysis where large shifts or variations in the population of a particular microbial community could be identified. These community-profiling methods were based on PCR where specific primers were used to amplify regions of interest and then subjected to analysis.

DNA microarrays

One of the first methods to rapidly assess specific bacterial associations in oral health and disease was DNA–DNA checkerboard hybridization on a solid membrane (Socransky *et al*, 1994). In the original method, 30 whole-genomic probes were hybridized to 45 DNA samples bound to a membrane for up to 1350 simultaneous hybridizations. Another version of checkerboard hybridization was a

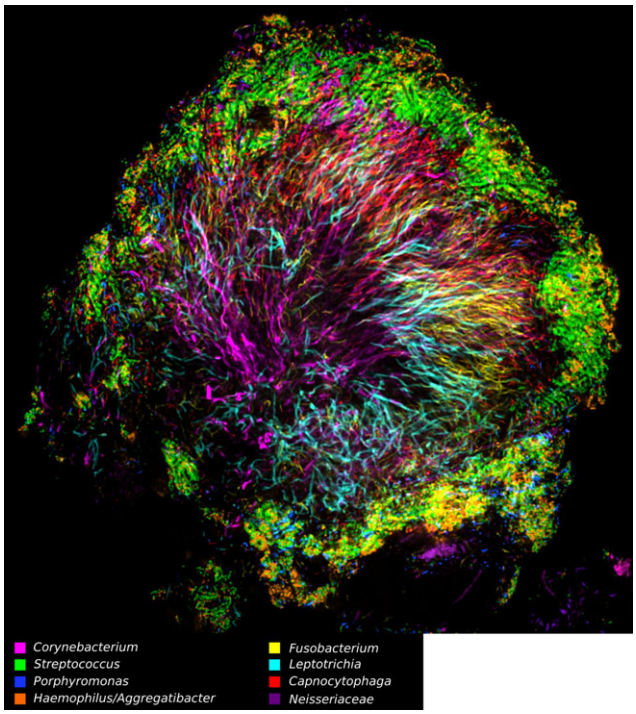

Figure 2 A microbial consortium in human dental plaque. Spatial organization of eight specific bacterial taxa can be clearly seen. Different colors represent different bacterial genera. For example, note that *Corynebacterium* (purple) and *Leptotrichia* (light blue) are centrally localized with *Streptococcus* (green) and *Haemophilus* (orange) on the periphery. Courtesy of Jessica Mark Welch, Marine Biological Laboratory, and Gary Borisy, The Forsyth Institute

reverse-capture protocol that utilized labeled 16S rDNA PCR products that were hybridized to 16S rDNA taxa-specific oligonucleotide probes bound to the membrane (Socransky *et al*, 1994; Paster *et al*, 1998).

DNA microarrays used signals from hybridization of DNA fragments to hundreds or thousands of complementary probes arrayed on a glass slide for expression profiling (Schena *et al*, 1995). This was modified to identify microbial populations (Zhou, 2003; Bodrossy and Sessitsch, 2004), such as the PhyloChip to screen for 16S rDNA (Wilson *et al*, 2002) and GeoChip for functional analysis (He *et al*, 2007). The Human Oral Microbe Identification Microarray (HOMIM), another reverse-capture protocol, was developed using 379 species-level probes to identify approximately 290 oral bacterial species and has been used in several disease-related and oral microbiome characterization studies (Colombo *et al*, 2009, 2012; Duran-Pinedo *et al*, 2011; Lif Holgerson *et al*, 2011; Olson *et al*, 2011; Torlakovic *et al*, 2012; Belstrom *et al*, 2015). An array platform with broad range, higher taxa probes can help to identify/estimate a community population composition at a family or phylum level even if species-level specificity is absent (Hamady and Knight, 2009).

16S rRNA gene sequencing

The 16S rRNA has been used as an evolutionary clock (Woese, 1987) for the identification and classification of pure cultures of bacteria as well as for estimation of bacterial diversity in environmental samples (Rajendhran and Gunasekaran, 2011). Comparative analyses of 16S rDNA sequences have been the primary basis of defining the microbiome from all environments, including the oral cavity. With pure bacterial cultures, PCR amplicons (approximately 1500 bp) of 16S rRNA genes were simply sequenced using Sanger sequencing (Sanger *et al*, 1977). Phylogenetic identity of bacterial taxa, whether they are cultivable or not-yet-cultivated, in a mixed population, for example, plaque, was determined using what has been referred to as the 16S rRNA approach (Paster *et al*, 2006). Briefly, DNA isolated from any given environment is amplified using universally-conserved PCR primers for 16S rRNA genes. The resultant amplicons were cloned into *Escherichia coli*, and the 16S rDNA inserts were sequenced to determine species identity (Hugenholtz and Pace, 1996; Paster *et al*, 2001). Typically, a >98.5% identity defines a species/phylotype. Consequently, 16S rDNA sequences from an isolate or cloned insert with <98.5% similarity to previously defined phylotypes would be considered representatives of new species (Dewhirst *et al*, 2010).

Next-generation sequencing platforms

In the last decade, next-generation sequencing methods have revolutionized the study of microbial diversity, which allow for large-scale sequencing projects to be completed in a few days or sometimes hours. The main technologies for next-generation sequencing are as follows.

- 454 pyrosequencing. This method clonally amplified fragmented DNA on beads within an emulsion (Margulies *et al*, 2005). The sequencer was able to generate over 250-bp-long reads and about 400 000 reads per run.
- Applied Biosystems (Life Technologies)
  - Sequencing by Oligo Ligation Detection (SOLiD). This technique was similar to 454 pyrosequencing in that fragmented DNA was amplified on agarose beads. However, this technique utilized the incorporation of a ligase and universal oligonucleotides, which resulted in millions of reads.
  - Personal Genome Machine, or Ion Torrent. Newer technology with a similar emulsion PCR for amplification technique but with an underlying semiconductor technology (Nakano *et al*, 2003).
- Illumina
  - Early instruments utilized a sequencing-by-synthesis platform where DNA fragments were clonally amplified on a flow cell and binding of complementary fluorescently labeled dNTPs is detected. Millions of 35-bp reads could be produced in one run, and, depending on the instrument, multiplexing of sample across lanes was possible.
  - In the past few years, Illumina has emerged as the market leader with a suite of instruments such as HiSeq, HiSeq X, NextSeq 500, and MiSeq with varying abilities for sequencing length and number of reads. The MiSeq can generate up to

2×300bps reads, and HiSeq X can produce approximately 600 Gb of data. The MiSeq is generally used for 16S rDNA profiling.

- Pacific Biosciences (PacBio), single-molecule real-time technology. This instrument is sensitive enough to detect a single fluorescently-labeled nucleotide (Levene *et al*, 2003; Korlach *et al*, 2008; Lundquist *et al*, 2008) and is purportedly able to generate approximately 10 000-bp reads. The PacBio platform is often used to determine whole-genomic sequences, without the need for a reference genome.
- Oxford Nanopore, MinION technology. One of the most recent technologies, released in May 2015, enables sequencing of single DNA molecules (Mikheyev and Tin, 2014; Quick *et al*, 2014). As with the PacBio, MinION would allow for *de novo* sequencing of whole genomes.

All NGS analysis requires extensive bioinformatic capabilities and involved data quality control, filtering for good quality reads, aligning and mapping to good reference genomes, removing chimeras, normalizations across samples, and populations for meaningful interpretations.

16S rDNA profiling. The 16S rRNA approach was refined further using NGS methodologies to rapidly sequence hypervariable regions of the 16S rRNA genes (Caporaso *et al*, 2011). This involves amplification of DNA samples using universally-conserved PCR primers of 16S rDNA and sequencing of the amplified regions to produce millions of reads enabling multiplexing of several samples in one run. The length of sequencing reads varies depending upon the primers used, but many studies utilize about 500-base pair reads for a typical sequencing run, which allows for microbial community identification (Liu *et al*, 2007, 2008; Wang *et al*, 2007; Mougeot *et al*, 2016). At the present time, using an Illumina platform (described below), 500- to 600-base pair reads is the size limit for sequencing. This technique does rely on PCR amplification, and care should be taken on which region of the 16S rRNA gene is used in analysis for accurate classification of the population (Yang *et al*, 2016), but nevertheless it is a valuable tool to identify species in a population.

A common bioinformatics tool for analysis has been Quantitative Insights Into Microbial Ecology (QIIME), which picks operational taxonomic units and assigns taxonomic identities based on comparisons to sequences from a reference database (Caporaso *et al*, 2010). Typically, especially with limited base pair reads, these analyses identify taxa at the genus level with some species-level identification.

Human Oral Microbe Identification using Next-Generation Sequencing (HOMINGS), http://homings.forsyth.org, is the new HOMIM, which utilizes standard NGS methodologies (Caporaso *et al*, 2010), and is capable of species-level identification for most of the prevalent oral bacterial taxa. This is achieved by an *in silico* search for specific 'probe' sequences, called ProbeSeq for HOMINGS, that targets approximately 600 species. ProbeSeq is an iterative process in which for those sequences that are not identified, the search is repeated with 129 genus-level probes that will identify those species at the genus level (Gomes *et al*, 2015). The advantages of HOMINGS are that it is computationally efficient, rapid, and reproducible and can identify the majority of the oral microbiome at the species level. There is good correlation between HOMINGS and HOMIM (Mougeot *et al*, 2016). HOMINGS has been used in several recent studies demonstrating bacterial associations with endodontic lesions (Gomes *et al*, 2015), salivary microbiomes in caries and periodontitis (Belstrom *et al*, 2016b), temporal differences in salivary microbiomes (Belstrom *et al*, 2016a), and in biofilm models in response to sucrose-induced dysbiosis (Rudney *et al*, 2015).

Most recently, a multistage algorithm for 16S rDNA NGS reads was developed for species-level identification. Although this method requires more computing power, it is able to maximize the percentage of reads classified at the species level (Al-Hebshi *et al*, 2015). In that study, this technique was used to determine the oral microbiome in subjects with oral cancer.

Whole-genome shotgun metagenome sequencing. The entire DNA (genome) of a single microbial culture or a complex microbial population can now be sequenced to great depth allowing us to generate reference genomes (*de novo* assembly) as a resource for future studies or identify the composition of microbial community, respectively (mapping back to a reference genome). These culture- and PCR-independent techniques allow parallel sequencing and identification of several organisms. Long read lengths enable a more accurate assembly of the genomes present in the population; however, the huge volume of data generated still poses a bioinformatic challenge (Grice and Segre, 2012). Fortunately, at least for the oral microbiome, there are complete genomes for about 400 bacterial species that facilitate assembly. At great depth of short read sequencing, metagenomic analysis also allows quantification of copy number and allelic variants of genes within the microbial population (Vincent *et al*, 2016).

Microbial metatranscriptomic sequencing. Both 16S rDNA and metagenome sequencing allow us to determine 'who is there'; however, metatranscriptomic analysis would tell 'what they are doing'. The metatranscriptome represents the RNA encoded by the microbial population; this functional analysis is performed by enriching for the mRNA, converting it into cDNA and sequencing the fragments. The reads are mapped back to reference genomes for gene expressions profiling within the microbial communities. Clinical samples can be sequenced to identify changes in gene expression between disease and normal states to identify key pathways upregulated in disease and expression patterns of potential pathogenic factors and microbial diversity (Wade, 2011; Benitez-Paez *et al*, 2014; Duran-Pinedo *et al*, 2014; Yost *et al*, 2015).

Bacterial associations in health and disease

Unlike many human diseases, oral bacterial diseases, such as caries and periodontitis, are not caused by a single

species, but by a consortium of species that are likely living harmlessly in very low numbers (often below the limit of detection) in the oral cavity. In essence, oral bacterial diseases are opportunistic infections and thus disease occurs under the proper circumstances and conditions, for example, diet, host immune response, complicating systemic or genetic disorders, pH, poor oral hygiene, lifestyle, and even bad luck.

In using the molecular techniques described above, bacterial associations have been determined in their relationships to health status. Such studies help to determine the role of specific species in oral health and disease, including extraoral sites in systemic diseases. However, note that these associations do not necessarily identify actual etiological agents, hence often the designation 'putative' pathogens or biomarkers of disease. Also of note is that, in all cases, bacterial associations are usually more complex than previously believed.

Oral health

The oral microbiome changes during the life of an individual from bacterial acquisition at birth (Berkowitz and Jones, 1985; Asikainen and Chen, 1999) to bacterial colonization of the elderly (Preza *et al*, 2008). Species of *Streptococcus* are usually the first pioneering microorganisms to colonize the oral cavity with *S. salivarius* found mostly on the tongue dorsum and in saliva, *Streptococcus mitis* on the buccal mucosa, and *Streptococcus sanguinis* on the teeth (Socransky and Manganiello, 1971; Gibbons and Houte, 1975; Smith *et al*, 1993). The growth and metabolism of these pioneer species change local environmental conditions such as local redox potential, pH, coaggregation, and availability of nutrients, thereby enabling more fastidious organisms to colonize after them (Marsh, 2000). Over time, other microbial communities take over including *Prevotella melaninogenica*, *Fusobacterium nucleatum*, *Veillonella*, *Neisseria*, and non-pigmented *Prevotella* (Kononen *et al*, 1992). With the development of teeth, an increase in the presence of genera such as *Leptotrichia* and *Campylobacter* is observed and along with colonization by additional species such as *Prevotella denticola* and members of the *Fusobacterium* and *Selenomonas* genera (Kononen *et al*, 1994). The eruption of teeth creates a new habitat, the gingival crevice, which is nourished by the gingival crevicular fluid (GCF). Along with saliva, GCF is critical for the maintenance of the integrity of the gingival crevices and contains antimicrobial peptides, immunoglobulins, and a range of other active proteins that enable it to influence the ecology of the oral cavity. Moreover, it also contains nutrients that support the resident microflora (Marsh, 2000). This continual succession of microbes is eventually replaced with a stable homeostasis of microbial communities that is referred to as the climax community whereby different bacteria interact to establish an ecosystem where each community contributes in some form (Marsh, 2000). The 'keystone pathogen' hypothesis suggests that specific low-abundance pathogens can influence periodontal disease by altering the 'healthy' microflora to a disease state (Hajishengallis *et al*, 2012). However, you still have to know health before you know disease.

Depending upon the oral site and individuals, many health-associated species have been identified. Using the 16S rRNA approach, Aas *et al* (2005) analyzed sites from five clinically healthy subjects. Sites included tongue dorsum, lateral sides of tongue, buccal mucosa, hard and soft palate, palate, supragingival and subgingival plaque, maxillary anterior vestibule, and tonsils. Species typically associated with periodontitis and caries were not detected. Using NGS, Zaura *et al* (2009) performed a similar analysis of multiple oral sites. In both of these studies, species and phylotypes of *Streptococcus*, *Granulicatella*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Rothia*, *Actinomyces*, *Prevotella*, *Capnocytophaga*, *Porphyromonas*, and *Fusobacterium* were common. They concluded that most oral taxa found in unrelated healthy individuals was similar and supported the concept of a healthy core microbiome. From the HMP 16S rRNA gene data of 200 subjects, Segata *et al* (2012) found similar taxa, but there was significant subject-to-subject variation.

Periodontitis

Putative pathogens have long been implicated in periodontal disease including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, and species of *Treponema* and *Prevotella*. However, using checkerboard hybridization, Socransky *et al* (1998) determined that oral diseases were better defined as based on a combination of species, or complexes, rather than a single specific etiologic agent. The authors defined five complexes of which the 'red complex' was the most pathogenic one. This complex contained *P. gingivalis*, *T. forsythia*, and *Treponema denticola* and depended on earlier colonization of the pocket by the orange complex (Socransky *et al*, 1998; Socransky and Haffajee, 2005).

The literature is quite extensive in regard to those species that are associated with periodontal disease (Socransky and Haffajee, 2005; Teles *et al*, 2013). Several studies suggested that there may be additional red complex species (Paster *et al*, 2001; Kumar *et al*, 2003) that are associated with chronic periodontitis. Recently, there was systematic review (Perez-Chaparro *et al*, 2014) of 1450 bacterial association studies of subgingival plaque, of which 41 studies qualified for analysis. Consequently, based on these analyses, they concluded that there were 17 additional disease-associated species or phylotypes and these are listed in Table 1 along with their human oral taxon (HOT) designations (www.homd.org).

Refractory periodontal disease

Some subjects who have destructive periodontal disease do not respond to conventional therapy and continue to lose periodontal attachment. This has often been termed as refractory periodontal disease (Adams, 1992). It has been suggested that subjects with refractory disease may be mildly immunocompromised making them more susceptible to periodontal disease. Early studies of refractory periodontal disease demonstrated a lack of typical periodontal pathogens (Magnusson *et al*, 1991). In contrast, using newer molecular techniques such as HOMIM, Colombo *et al* (2009) demonstrated that refractory periodontitis

Table 1 Newly identified putative periodontal pathogens (from Perez-Chaparro *et al*, 2014)

Bacterial taxa	
Anaeroglobus geminatus HOT 121	Cultivable
Archaea spp.	Cultivable
Bacteroidales [*G-2*] sp. oral taxon 274	Unnamed
Desulfobulbus sp. oral taxon 041	Phylotype
Eubacterium [*XI*] [*G-5*] *saphenum* HOT 759	Cultivable
Filifactor alocis HOT 539	Cultivable
Fretibacterium fastidiosum HOT 363	Cultivable
Fretibacterium sp. oral taxon 360	Phylotype
Fretibacterium sp. oral taxon 362	Phylotype
Mogibacterium timidum HOT 042	Cultivable
Peptostreptococcus stomatis HOT 112	Cultivable
Porphyromonas endodontalis HOT 273	Cultivable
Selenomonas sputigena HOT 151	Cultivable
TM7 [*G-5*] sp. oral taxon 356	Phylotype
Treponema lecithinolyticum HOT 653	Cultivable
Treponema medium HOT 667	Cultivable
Treponema vincentii HOT 029	Cultivable

differed from treatable periodontitis by having a higher frequency of putative periodontal pathogens as listed above and in Table 1. However, they also found additional species that are not commonly detected in treatable periodontal disease, including *Peptoniphilus alactolyticus*, *Brevundimonas diminuta*, *Shuttleworthia satelles*, *Dialister invisus*, *Granulicatella adiacens*, *Veillonella atypica*, and *Mycoplasma salivarium*. A more recent study implicated *Actinetobacter baumannii*, an important nosocomial pathogen that is notoriously antibiotic resistant, as a risk factor for refractory periodontitis (Richards *et al*, 2015).

Aggressive periodontitis

This periodontal disease was previously referred to as localized juvenile periodontitis or generalized juvenile periodontitis. As the name implies, this is an aggressive form of periodontitis that typically affects only incisors and first molars in adolescents or young adults. There is usually a lack of gingival inflammation, even with deep probing depths (Albandar, 2014). *Aggregatibacter (Actinobacillus) actinomycetemcomitans* has long been considered the etiologic agent of aggressive periodontitis; however, more recent evidence has shown that the subgingival plaque microbiome of aggressive periodontitis resembles that of chronic periodontitis (Kononen and Muller, 2014). Shaddox *et al* (2012) showed that *A. actinomycetemcomitans*, *Filifactor alocis*, *Tannerella* spp. *Solobacterium moorei*, *Parvimonas micra*, and *Capnocytophaga* spp were most abundant in aggressive periodontitis. A recent study showed that a consortium of *A. actinomycetemcomitans*, *Streptococcus parasanguinis*, and *F. alocis* may serve as a biomarker of disease, that is, predict bone loss before it occurs (Fine *et al*, 2013).

Caries

One of the most prevalent human bacterial infections is dental caries, which leads to tooth decay and potentially tooth loss. *Streptococcus mutans* has long been considered as the etiological agent of caries inasmuch as it not only produces lactic acid, but also thrives in the low-pH environment. However, 10–20% of subjects with caries do not have detectable levels of *S. mutans*, so clearly other acid-producing bacterial taxa must be involved. Molecular methods such as the 16S rRNA approach or microarrays (Aas *et al*, 2008) have demonstrated that in carious lesions with *S. mutans*, additional species belonging to the genera *Atopobium*, *Propionibacterium*, and *Lactobacillus* were present at significantly higher levels. In those subjects with no detectable levels of *S. mutans*, *Lactobacillus* spp., *Bifidobacterium dentium*, and low-pH non-*S. mutans* streptococci were predominant. Based on these results, it was suggested that bacterial species other than *S. mutans*, for example, species of the genera *Veillonella*, *Lactobacillus*, *Scardovia*, and *Propionibacterium*, low-pH non-*S. mutans* streptococci, *Actinomyces* spp., and *Atopobium* spp., may play an important role in caries progression. NGS analyses of the microbiome of populations with a low and high prevalence of caries found that adolescents in Romania, who had limited access to care, were colonized with *S. mutans* and *Streptococcus sobrinus*. In contrast, those adolescents in Sweden, who had very good care, were colonized only infrequently with *S. mutans* and *S. sobrinus*, but were colonized more with species of *Actinomyces*, *Selenomonas*, *Prevotella*, and *Capnocytophaga* (Johansson *et al*, 2016).

The oral microbiomes do differ between primary and secondary dentitions as well as in root surface caries. In the primary dentition (Becker *et al*, 2002), *S. mutans* was typically detected at high levels. Other disease-associated species included *Actinomyces gerencseriae*, *Scardovia wiggsiae*, *Veillonella*, *S. salivarius*, *Streptococcus constellatus*, *S. parasanguinis*, and *Lactobacillus fermentum* were found more in the secondary dentition. Root surface caries differ from primary and secondary caries in that root surfaces do not have enamel. Preza *et al* (2008) demonstrated that *S. mutans* was also associated with root surface caries, but that the predominant taxa included *Actinomyces* spp., *Lactobacillus*, *Enterococcus faecalis*, *Mitsuokella* sp. HOT131, *Atopobium* and *Olsenella* spp., *Prevotella multisaccharivorax*, *P. alactolyticus*, and *Propionibacterium acidifaciens.* Of note, detectable levels of *E. faecalis* and *P. alactolyticus* are typically found only in endodontic lesions and not in dental plaque.

Odontogenic infections

These pus-laden infections typically originate within a tooth or surrounding structures resulting in swellings of the head, face, and neck (Flynn *et al*, 2012). Using the 16S rRNA approach, several predominant species that had been previously associated with odontogenic infections were detected (Flynn *et al*, 2012). These species included *Fusobacterium* spp, *P. micra*, *Porphyromonas endodontalis*, and *Prevotella oris*. However, they also detected newly associated species including *Dialister pneumosintes*, *D. invisus*, and *Eubacterium brachy*, as well as several phylotypes. An interesting finding in this study was that species of *Streptococcus* were not detected.

Endodontic lesions

In primary infections, predominant taxa detected include species of *Peptostreptococcus*, *P. micra, F. alocis*, and *P. alactolytcus* and species of *Dialister*, *F. nucleatum*,

T. denticola, *P. endodontalis*, *P. gingivalis*, *T. forsythia*, *Prevotella baroniae*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Bacteroidaceae* [G-1] HOT272 (Siqueira and Rocas, 2009). *Enterococcus faecalis* was detected, but as lower levels. However, in retreatment cases, the predominant taxa include *Enterococcus* species such as *E. faecalis*, *P. micra*, *F. alocis*, *P. alactolytcus*, *S. constellatus* and *Streptococcus anginosus*, and *Propionibacterium propionicum*. The microbiomes of endodontic-periodontal lesions possessed similar profiles including *E. faecalis*, *P. micra*, *Mogibacterium timidum*, *F. alocis*, and *Fretibacterium fastidiosum* (Gomes *et al*, 2015).

Oral bacteria as biomarkers for non-oral diseases

Oral bacteria have been linked to a number of systemic diseases including bacterial endocarditis (Berbari *et al*, 1997), ischemic stroke (Joshipura *et al*, 2003), cardiovascular disease (Beck and Offenbacher, 2005; Teles and Wang, 2011), pancreatic cancer (Farrell *et al*, 2012), pediatric Crohn's disease (Docktor *et al*, 2012), and pneumonia (Awano *et al*, 2008). Periodontal disease has been shown to predispose individuals to cardiovascular disease through its ability to induce chronic inflammation (Syrjanen, 1990; Valtonen, 1991). Similarly, the presence of several anaerobic oral bacterial species has been shown to predispose to bacterial pneumonia including *Actinobacillus actinomycetemcomitans* and *S. constellatus* (Shinzato and Saito, 1994; Venkataramani *et al*, 1994). In Alzheimer's disease, inflammation, a key feature of the disease (Olsen and Singhrao, 2015), could be caused in part by peripheral infections, such as periodontal disease. Periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* and *P. intermedia* are capable of eliciting systemic inflammation, which results in the release of pro-inflammatory cytokines that traverse the blood–brain barrier.

An intriguing suggestion has been that oral bacteria may play a role in nitric oxide (NO) homeostasis, which is important in renal and cardiovascular health (Hezel and Weitzberg, 2015). Dietary nitrates can be reduced to nitrites by oral bacteria, and nitrite, absorbed in the blood, is further reduced to NO by a variety of mechanisms. NO then acts on vascular smooth muscle to stimulate vasodilation.

Important considerations and conclusions

Researchers are now routinely identifying bacterial composition, and high-throughput sequencing of the microbiome will likely progress into functional studies encompassing genomics, transcriptomics, and metabolomics of both host and pathogens. Such analyses could provide insights into activity of the microbes, their relationship to hosts, and possible causative mechanisms. The second phase of the NIH Human Microbiome Project will study the host–microbiome relationship in longitudinal studies (Integrative, 2014). These data will guide researchers to develop new therapies that target key mechanisms. With such huge datasets, we will likely identify 'community signatures' of certain diseases. It can be envisioned that chair side or bedside, point-of-care diagnostics could be developed that target key bacterial taxa. Consequently, these potential biomarkers of disease, the proverbial 'canary of the coal mine in human disease' could be used to warn dentists or physicians of disease yet-to-come or to assess risk of disease. Thus, for the dentist, oral medicine specialist, or periodontist, these warning 'danger' microbial profiles would allow for early treatment to combat disease in the preclinical stages. The oral microbiome could be used further to monitor health status after treatment, that is, "Is the treatment working to establish a more healthy microbial profile?" Regardless of the specific application, microbial analysis is in an exciting phase of research with huge prospects for the clinic.

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Author contributions

K. Krishnan previously set up and ran the sequencing core at the Forsyth Institute and provided expertise in descriptions of sequencing platforms. T. Chen constructed the phylogenetic tree from sequence data from the HOMD database. He also provided bioinformatic expertise. K. Krishnan and B. Paster wrote the review and B. Paster finalized the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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