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CHAPTER

Taxonomy of Oral Bacteria 7

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GLOSSARY

ARB	software package comprising tools for sequence database handling and data analysis
CORE	a 16S database of the core human oral microbiome
FROGS	find, rapidly, OTUs with galaxy solution. A pipeline to analyse large sets of amplicon sequences and produce abundance tables of OTUs and their taxonomic affiliation
Greengenes	a chimera-checked 16S rRNA gene database and workbench
HMP	human microbiome project: a catalogue of reference genomes from the human microbiome
HOMD	the human oral microbiome database
MEGAN	mEtaGenome analyzer. Used to perform taxonomic and functional analysis of large metagenomic datasets
MG-RAST	rapid annotation using subsystems technology for metagenomes. A public resource for the automatic phylogenetic and functional analyses of metagenomes
mothur	open-source, platform-independent, community-supported software for describing and comparing microbial communities
NGS	next-generation sequencing. Non-Sanger-based, high-throughput DNA sequencing
QIIME	quantitative insights into microbial ecology, an open-source software pipeline for analysis of high-throughput community sequencing data
RDP	ribosomal database project. A database of quality-controlled bacterial and archaeal small subunit rRNA alignments and analysis tools
SILVA	a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB
SMURF	short mUltiple regions framework. A method to combine sequencing results from different PCR-amplified regions
VAMPs	the visualisation and analysis of microbial population structures. Enables analysis of microbial community diversity using marker gene sequence data

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1 INTRODUCTION

The various structures that make up the oral cavity: the tongue, cheeks, palate, gingiva and teeth allow us to talk, chew, taste, whistle and smile. These structures provide the oral cavity with a diverse range of microenvironments, each inhabited by a community of microorganisms, the majority of which are bacteria and their phages. Community membership is influenced by myriad factors including available nutrients, pH, redox potential, substratum for adherence, receptors for adhesion and microbial interactions both agonistic and antagonistic.

The Human Oral Microbiome Database currently lists 688 prokaryotic taxa as having been found in the oral cavity (Chen et al., 2010), a figure that is still thought to underestimate the true microbial diversity in this part of the human gastrointestinal tract (Siqueira & Rocas, 2017) and its multiple unique environments (Simon-Soro et al., 2013). The oral cavity of a healthy individual is likely to provide a home to about 30–70 predominant prokaryotic species (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005); friends, foes and some we do not yet know well enough to determine.

Since 1683, when Antony Van Leeuwenhoek described 'living animalcules' upon viewing dental plaque using a hand-crafted microscope of his own design (Porter, 1976), the quest to characterise the microbiota inhabiting the oral cavity has continued unabated. From morphologic descriptions of dental plaque in the classic microscopy studies of Listgarten and colleagues (Listgarten, 1976; Listgarten, Mayo, & Tremblay, 1975) to the comprehensive culture studies of W.E. Moore and L.V. Moore and into the genomics era of the 21st century our appreciation of the true diversity of prokaryotes in the oral cavity continues to evolve.

Studying the diversity of and relationships between prokaryotes in the oral cavity requires taxonomy: a circinate process that involves the classification of organisms based on similarities and differences, naming them according to a defined code (Parker, Tindall, & Garrity, 2015) and characterisation which allows an organism to be classified and named, or reclassified and renamed (Brenner, Staley, & Krieg, 2005). As such, prokaryotic taxonomy is not fact (Garrity, 2016) but is in a constant state of flux and is influenced in large part by the evidence provided by different laboratory techniques and methods of characterisation used at any one time. Which raises the question of why bother? In the oral cavity, classification of microorganisms allows the microbial communities at different sites, and associated with states of health or disease, to be defined. This may then enable the monitoring of health and disease with predictive models to inform targeted prevention. The aim of this chapter is to review the methods used to characterise the bacteria calling the oral cavity home, and point to the clinical significance of classifying oral prokaryotes not only at a species level, but at the whole community level.

1.1 THE ORAL CAVITY: NOT ONE, BUT MANY ENVIRONMENTS

With its combination of mucosal surfaces, and nonshedding hard tooth surfaces, the oral cavity is not one single environment, but a collection of unique microenvironments (Fig. 1). Different sites in the oral cavity such as the tongue, buccal mucosa,

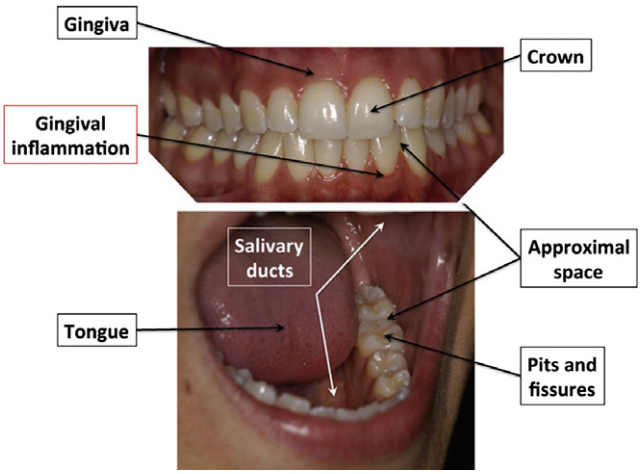

FIG. 1

The oral cavity and its associated environments.

Figure reproduced from Costalonga, M., & Herzberg, M. C. (2014). The oral microbiome and the immunobiology of periodontal disease and caries. Immunology Letters, 162*(2 Pt. A), 22–38. https://doi.org/10.1016/j.imlet.2014.08.017.*

teeth and gingival crevice have been found to exhibit differences in the bacterial composition of their associated microbial communities (Eren, Borisy, Huse, & Mark Welch, 2014; Hall et al., 2017; Mager, Ximenez-Fyvie, Haffajee, & Socransky, 2003; Simon-Soro et al., 2013; Xu et al., 2015; Zhou et al., 2013). Further variability exists within these sites. For example, the location of the tooth surface in the oral cavity may affect the bacterial composition of the complex polymicrobial biofilm (plaque) forming at a site (Haffajee et al., 2009), and even different surfaces of the same tooth (Simon-Soro et al., 2013). This variation between microbial communities at different oral sites is likely to be the combined result of multiple factors including the surface available for colonisation (Mager et al., 2003), salivary flow and composition (Proctor et al., 2018) and the presence of other species (Eren et al., 2014). Oral microbial community profiles can also be affected by myriad environmental perturbations including dietary sugar intake, pH, oral hygiene practices, plaque mass and gingival inflammation (Haffajee et al., 2009; Haffajee, Teles, & Socransky, 2006; Kianoush et al., 2014; Uzel et al., 2011).

The tissues of the oral cavity are at risk of several diseases resulting from bacteria residing in dental plaque biofilms. The two most common of these diseases, dental caries and periodontal diseases, are considered polymicrobial, opportunistic diseases which can result in damage to the hard tooth structure, or supporting bone and soft tissues, respectively. Unlike diseases caused by exogenous pathogens, dental caries and periodontal diseases are caused by bacteria (pathobionts) that are part of our normal microbiota, the associated microorganisms being able to reside in the human mouth at low levels without causing disease. Under certain conditions, however,

these bacteria can proliferate and cause destruction of soft and/or hard tissue either directly or indirectly via the host immune response (Marsh, Head, & Devine, 2015).

While evidence of both dental caries and periodontal diseases exists in ancient populations (Eshed, Gopher, & Hershkovitz, 2006; Lopez-Valverde, Lopez-Cristia, & de Diego, 2012), there has been a marked increase in the prevalence of these diseases in modern populations (Muller & Hussein, 2017; Raitapuro-Murray, Molleson, & Hughes, 2014). The oral cavity has been home to pathobionts associated with oral disease since at least prehistoric times (Warinner et al., 2014; Weyrich et al., 2017), signifying an enduring association between these microorganisms and the oral cavity. However, not only has there been an increase in the prevalence of pathobionts such as *Streptococcus mutans* and *Porphyromonas gingivalis* in modern times (Adler et al., 2013) but also an increase in the pathogenic potential of the oral microbiome. This is illustrated by the rapid evolution of sugar transport, metabolism and acid tolerance in *S. mutans* in response to dietary changes associated with the move from hunter-gatherer to agricultural lifestyles and the accompanying increased consumption of grains and fermentable carbohydrates (Cornejo et al., 2013). Cooccurrence of additional risk factors for oral disease such as smoking may also be related to the increased prevalence of periodontal diseases in modern populations (Hujoel, del Aguila, DeRouen, & Bergstrom, 2003). While it is possible to coexist with our oral microbiome and remain in a state of oral health, the ubiquitous nature of oral diseases in modern times raises the question of which is the atypical environment? The modern diseased oral cavity? Or are oral diseases now so prevalent that the modern disease-free oral cavity should be considered the atypical environment? Both dental caries and periodontal diseases are largely preventable with sufficient control of oral microbial biofilms and appropriate diet, and it can be argued that while our modern lifestyle leaves us more susceptible to some oral diseases than our ancestors, the disease-free oral cavity is an appropriate and achievable aim of modern dental care (Axelsson, 2014; Axelsson, Nystrom, & Lindhe, 2004; Moynihan & Kelly, 2014).

1.2 THE DIVERSITY OF THE ORAL MICROBIOME

A single 10 s kiss can transfer 80 million bacterial cells from one mouth to another (Kort et al., 2014), but what is the vast and diverse collection of bacteria humans carry in their oral cavity composed of? Dewhirst and colleagues characterised the most prevalent and abundant bacterial taxa in the human oral cavity as being in the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, *Fusobacteria* and *Saccharibacteria* (TM7) using an impressively comprehensive analysis of oral clones and isolates (Table 1) (Chen et al., 2010; Dewhirst et al., 2010). The Human Microbiome Project determined the bacterial community composition of 18 sites in and on the human body of over 200 healthy individuals, including 9 sites in the oral cavity (Huttenhower et al., 2012). Across all oral samples, taxa from between 185 and 322 genera were found to inhabit various oral sites which included buccal mucosa (cheek), keratinised gingiva (gums), hard palate, saliva,

Table 1 Bacterial Phyla, Genera and Species Representatives Commonly Found in the Oral Cavity

Phyla	Genera	Species-Level Representatives
Firmicutes	*Dialister*	*D. invisus*, *D. pneumosintes*
	Enterococcus	*E. faecalis*
	Filifactor	*F. alocis*
	Gemella	*G. morbillorum*
	Lachnoanaerobaculum	*L. saburreum*
	Lactobacillus	*L. salivarius*, *L. paracasei*
	Mogibacterium	*M. timidum*, *M. pumilum*
	Mycoplasma	*M. faucium*, *M. orale*
	Oribacterium	*O. asaccharolyticum*, *O. parvum*
	Peptoniphilus	*P. lacrimalis*
	Selenomonas	*S. artemidis*, *S. noxia*
	Streptococcus	*S. mitis*, *S. sanguinis*, *S. oralis*, *S. mutans*
	Veillonella	*V. atypica*, *V. parvula*
	Veillonellaceae[G-1]	
Bacteroidetes	*Alloprevotella*	*A. rava, A. tannerae*
	Bacteroidetes[G-1]	
	Bergeyella	
	Capnocytophaga	*C. gingivalis*, *C. ochracea*
	Porphyromonas	*P. catoniae, P. gingivalis,*
	Prevotella	*P. dentalis*, *P. intermedia, P. maculosa*
	Tannerella	*T. forsythia*
Proteobacteria	*Aggregatibacter*	*A. actinomycetemcomitans*, *A. aphrophilus*
	Campylobacter	*C. concisus*, *C. gracilis*, *C. showae*
	Haemophilus	*H. aegyptius*, *H. haemolyticus*
	Kingella	*K. denitrificans*, *K. oralis*
	Neisseria	*N. mucosa*, *N. oralis*
	Pseudomonas	
Actinobacteria	*Actinomyces*	*A. israelii*, *A. johnsonii*, *A. naeslundii*, *A. odontolyticus*
	Bifidobacterium	*B. dentium*, *B. longum*
	Corynebacterium	*C. durum*, *C. matruchotii*
	Olsenella	*O. profusa*, *O. uli*
	Propionibacterium	*P. avidum*, *P. propionicum*
	Rothia	*R. dentocariosa*, *R. mucilaginosa*
	Scardovia	*S. wiggsiae*
Spirochaetes	*Treponema*	*T. denticola*, *T. lecithinolyticum*, *T. parvum*, *T. socranskii*
Fusobacteria	*Fusobacterium*	*F. nucleatum*, *F. periodonticum*
	Leptotrichia	*L. buccalis*, *L. hofstadii*
Saccharibacteria (TM7)	Saccharibacteria (TM7) [G-1]–[G-8]	
Synergistetes	*Fretibacterium*	*F. fastidiosum*

Compiled from the Human Oral Microbiome Database Chen, T., Yu, W. H., Izard, J., Baranova, O. V., Lakshmanan, A., & Dewhirst, F. E. (2010). The Human Oral Microbiome Database: A web accessible resource for investigating oral microbe taxonomic and genomic information. Database (Oxford), 2010, *baq013. https://doi.org/10.1093/database/baq013.*

tongue dorsum and both supragingival (above the gingival margin) and subgingival (below the gingival margin) plaque. Individuals were found to harbour between 41 and 72 genera at a site and saliva and subgingival dental plaque exhibited the greatest diversity of microbiota (Zhou et al., 2013). At a genus level, similarity exists across sites, with *Streptococcus* and *Pasteurellaceae unclassified* identified as core genera, having been found at an abundance of >1%, in >80% of samples from all nine oral sites and *Fusobacteria*, *Prevotella* and *Veillonella* present in >80% of samples from at least five of the nine oral sites sampled (Li, Bihan, & Methe, 2013).

While some species such as *Streptococcus mitis* and *Gemella haemolysans* appear ubiquitous across all oral sites (Aas et al., 2005; Hall et al., 2017), others show a site preference, such as *Simonsiella muelleri* with its specificity to the hard palate (Aas et al., 2005; Eren et al., 2014). Comparison of communities across sites indicates similarity in community membership, with site preference demonstrated by an increased abundance at a site or sites. For example, while *Rothia aeria*, *Lautropia mirabilis*, *Streptococcus sanguinis* and *Streptococcus salivarius* are found across all oral sites, their abundance indicates site preference, where *R. aeria*, *L. mirabilis* and *S. sanguinis* show a preference for supragingival and subgingival plaque, *S. salivarius* shows a preference for soft tissues being present in low abundance in supragingival and subgingival plaque (Eren et al., 2014).

1.3 THE TWO MAJOR ORAL POLYMICROBIAL DISEASES AND THEIR AETIOLOGIES

1.3.1 Dental caries

Dental caries is a disease of the hard tissues of the teeth whereby acids produced by the bacterial metabolism of dietary sugars results in a reduced pH in the plaque fluid surrounding the tooth which disrupts the chemical equilibrium with the tooth and results in dissolution of calcium and phosphate ions from the tooth (Pitts et al., 2017). *S. mutans* has long been associated with dental caries due to its abundance in supragingival dental plaque over carious lesions (Becker et al., 2002; Loesche, Rowan, Straffon, & Loos, 1975); and its ability to rapidly convert a wide range of dietary sugars to acid (Ajdic et al., 2002; de Soet, Nyvad, & Kilian, 2000), its ability to adapt to and survive in low pH environments (Dashper & Reynolds, 1992) and the production of extracellular polysaccharides (Bowen & Koo, 2011). The importance of *S. mutans* as the major microbial aetiologic factor associated with dental caries is considered doubtful with findings from both bacterial culture and molecular studies unveiling a far more complex bacterial community associated with dental caries, including *Lactobacillus gasseri* (Aas et al., 2008; Schulze-Schweifing, Banerjee, & Wade, 2014), *Lactobacillus fermentum*, *S. salivarius*, *Streptococcus parasanguinis* and *Veillonella* sp. (Aas et al., 2008; Becker et al., 2002) and *Actinomyces gerencseriae* (Becker et al., 2002; Tanner et al., 2011). While most of these species can produce lactic acid from the fermentation of dietary sugars and directly contribute to the dissolution of tooth mineral, *Veillonella* sp. do not produce, but consume lactate. *Veillonella* sp. have been found to be predictive of future

dental caries and have been proposed as an indicator of total acid production in the biofilm, regardless of the microbial source (Gross et al., 2012). Not only is dental caries a polymicrobial disease, the microbial aetiology may be tissue dependent with different microbial communities associated with disease in dentin and enamel (Simon-Soro, Guillen-Navarro, & Mira, 2014). These observations serve to highlight the importance of what the bacteria in these communities are doing, rather than who is present as will be discussed in the sections on metagenomics and metatranscriptomics.

1.3.2 Chronic periodontitis

Periodontitis is a multifactorial, polymicrobial infection characterised by irreversible destruction of the supporting tissues of the teeth. If left untreated, it can progress to the point at which teeth exfoliate (Kinane, Stathopoulou, & Papapanou, 2017). Based on considerable human and animal studies it has been proposed that in a susceptible host under particular environmental conditions, *P. gingivalis* is able to dysregulate protective host responses to shape and stabilise a dysbiotic, disease-provoking microbial community, and subsequently work with a variety of other pathobionts to sustain an uncontrolled inflammatory state resulting in irreversible periodontal bone loss (Hajishengallis, 2014). *Treponema denticola* and *Tannerella forsythia* are frequently found with the aforementioned *P. gingivalis*, with all three species associated with periodontal pocket depth (Gatto, Montevecchi, Paolucci, Landini, & Checchi, 2014; Pradhan-Palikhe et al., 2013; Socransky, Haffajee, Cugini, Smith, & Kent, 1998), and *P. gingivalis* and *T. denticola* also associated with disease progression (Byrne et al., 2009). However, 16S rRNA gene sequencing of subgingival dental plaque samples in cross-sectional studies has implicated a wider range of species to be associated with disease including *Filifactor alocis* (Camelo-Castillo, Novoa, et al., 2015; Chen et al., 2015; Griffen et al., 2012), *Peptostreptococcus stomatis* (Griffen et al., 2012), *Treponema maltophilum* (Hong et al., 2015), *Desulfobulbus propionicus* (Camelo-Castillo, Mira, et al., 2015; Camelo-Castillo, Novoa, et al., 2015), *Fretibacterium* sp. (Park et al., 2015) and *Rothia* sp. (Park et al., 2015). However, the role of these species in disease initiation and progression, if any, has yet to be elucidated.

2 METHODS FOR CHARACTERISING ORAL BACTERIA

In 1876, Alexander Graham Bell invented the telephone and around the same time, German physician Robert Koch was attempting to culture bacteria on pieces of boiled potato (Simonson, Robinson, Pranger, Cohen, & Morton, 1992). Just as technology has advanced to allow video pictures to be sent around the world in seconds to and from wireless telephones that fit in the palm of a hand, laboratory techniques have advanced such that tiny fragments of DNA can be used to detect and identify microorganisms in a matter of hours without the need for bacterial culturing and phenotypic testing. We will now examine how the evolution

of laboratory methods for identifying microorganisms has aided the classification of the bacteria we carry around in our mouths.

2.1 MICROSCOPY

The diversity of species now known to inhabit the oral cavity was hinted at in the light and electron microscopy studies of temporal dental plaque development from the 1960s and 1970s. These beautiful images captured the diversity of morphotypes present in developing dental plaque with the diversity of undisturbed oral biofilms unsurprisingly increasing over time (Listgarten, 1976; Listgarten et al., 1975). Certain morphotypes showed a location preference such as the lack of filamentous forms at the tooth surface (Listgarten, 1976; Listgarten et al., 1975) and general morphological changes in plaque communities were found to be associated with clinical evidence of health or disease. Gram-positive cocci dominated in health, with Gram-negative cells found infrequently and an absence of flagellated or spirochaetal cells (Listgarten, 1976), which were then increased with gingival inflammation (Loe, Theilade, & Jensen, 1965), and periodontal disease (Armitage, Dickinson, Jenderseck, Levine, & Chambers, 1982). Intimate relationships between different bacterial species in dental plaque were also observed (Jones, 1972; Listgarten et al., 1975). However, without the recent addition of molecular 'helpers' such as DNA probes, microscopy does not allow for the distinction between what Schroeder and DeBoever referred to as '…morphologically different, but taxonomically unidentified' microorganisms (Schroeder & de Boever, 1970).

Recently, however, microscopy has experienced a resurgence, buoyed by the application of knowledge obtained from newer techniques and technologies. Combining the old with the new has recently enabled visualisation of the arrangement of members of oral communities in supragingival plaque (Mark Welch, Rossetti, Rieken, Dewhirst, & Borisy, 2016) as discussed later in this chapter.

2.2 BACTERIAL CULTURE

Traditional bacterial culture techniques involve the collection of bacterial samples from the oral cavity including saliva, dental plaque and swabs or scrapings from the soft tissues into an appropriate medium, transfer to the laboratory, suitable dilution and/or disruption and growth on a (semi)solid media that enables isolation of clonal colonies (Suchett-Kaye, Morrier, & Barsotti, 2001). There are myriad bacterial growth media and environmental conditions that can be used for the culture of bacteria and all have biases and limitations. Following growth of the resulting bacterial colonies, further tests enable the characterisation of species including identification of known species. Characterisation of a microorganism based upon phenotype obtained from culture such as colony morphology, cell-staining behaviour, metabolic activity, substrate utilisation and chemical characterisation of cell components makes traditional culture techniques pivotal to comprehensive taxonomy (Tindall, Rossello-Mora, Busse, Ludwig, & Kampfer, 2010).

2.2.1 Characterisation of the oral microbiota with bacterial culture

The characterisation of bacteria from the oral cavity has focused largely on the attempt to determine the causative bacteria of the diseases of the oral hard and soft tissues: dental caries and periodontal diseases. The sites in the oral cavity sampled for culture studies of oral bacteria reflect this, with much early investigation of oral bacteria focusing on those species found in the gingival crevice (in health) or periodontal pockets, resulting from tissue destruction during periodontal disease.

In 1890, without the use of strict anaerobic culture techniques, Miller claimed "…I have isolated over one hundred different kinds of bacteria from the juices and deposits in the mouth" (Miller, 1890). He hypothesised that the supreme difficulty he and others encountered in cultivating bacteria from the oral cavity was the result of their fastidious nutritional and environmental requirements, dismissing a theory circulating at the time that these organisms were strictly parasitic (Miller, 1890). It was evident that even with the use of anaerobic culture the number of cells enumerated following cultivation of subgingival dental plaque was much lower than the number seen microscopically (Socransky et al., 1963). The application of strict anaerobic techniques during sampling from the oral cavity, and the development of the anaerobic workstation paved the way for arguably the most comprehensive survey of oral bacteria using bacterial culture, carried out by W.E. Moore and L.V. Moore over the course of multiple studies (Moore et al., 1983; Moore, Holdeman, Smibert, Good, et al., 1982; Moore, Holdeman, Smibert, Hash, et al., 1982). Following a specialised sampling procedure in an attempt to maintain the viability of anaerobic species, clinical samples were transferred to a laboratory and inoculated onto plates and roll tubes, all under anaerobic conditions. Identification of isolates was carried out using a variety of tests, including subculture or selective media where necessary (Moore, Holdeman, Smibert, Good, et al., 1982). The expertise of this group of microbiologists is demonstrated by the report of only 3.5%–5.5% of the colonies selected for identification failing to grow in pure culture or surviving the identification process (Moore, Holdeman, Smibert, Good, et al., 1982; Moore, Holdeman, Smibert, Hash, et al., 1982). Approximately half of the taxa identified were unnamed species despite this comprehensive identification process (Moore et al., 1983; Moore, Holdeman, Smibert, Good, et al., 1982; Moore, Holdeman, Smibert, Hash, et al., 1982). Bacterial communities from subgingival biofilms in subjects with a healthy periodontium were found to be simple compared with those from gingivitis or moderate periodontitis, and were dominated by *Streptococcus*, *Actinomyces* and *Veillonella* species (Moore et al., 1983; Moore, Holdeman, Smibert, Good, et al., 1982). The development of the subgingival plaque microbiota over time was shown to occur via colonisation by additional species and their proliferation rather than replacement of existing species, resulting in a more diverse community of microorganisms as dental biofilms develop (Moore, Holdeman, Smibert, Good, et al., 1982). The cumulative data from this series of studies, however, indicated an overall complexity to the oral microbiota even when limiting the site sampled to the gingival sulcus or periodontal pocket.

Of 51,000 bacterial isolates from 300 subjects, 509 different taxa were detected (Moore & Moore, 1994). A figure impressively close to the 688 taxa currently in the HOMD (Chen et al., 2010).

For years, bacterial culture was considered the gold standard against which other techniques for microbiological identification and classification were compared; however, this has not been considered appropriate for some time (Loesche, 1992). The fastidious nature of many oral bacteria makes some difficult to isolate in pure culture, with others remaining uncultivable. For example, *T. forsythia* proved difficult to culture without the presence of other oral bacteria such as *Fusobacterium nucleatum* (Tanner, Listgarten, Ebersole, & Strezempko, 1986) until its obligate requirement for *N*-acetylmuramic acid was uncovered (Wyss, 1989). More recently, a TM7 phylotype isolated from the oral cavity demonstrated an obligate requirement for surface attachment to *Actinomyces odontolyticus* for successful laboratory growth (He et al., 2015), indicative of a parasitic lifestyle.

The recovery of bacterial species can be influenced by numerous factors including plaque sampling method (Renvert, Wikstrom, Helmersson, Dahlen, & Claffey, 1992; Wikstrom, Renvert, Dahlen, & Johnsson, 1991), sample transport and dispersion (Socransky, Haffajee, Smith, & Dzink, 1987) and media choice (Slots, 1986). While yet to be employed on oral samples, culturomics: the use of diverse culture conditions followed by molecular methods for identification (Lagier et al., 2015) has been employed on stool samples to identify almost 200 potentially new species thought to reside in the gut (Lagier et al., 2016). The requirement of a viable culture for description of a new species (Parker et al., 2015) may be partly responsible for this recent resurgence in microbial culture (Marx, 2017), although it is undoubtedly not possible to fully elucidate a microorganism's role in health or disease without it. Based on our emerging knowledge of the interdependencies of oral bacteria and their specific (micro)nutrient requirements, new techniques for culturing the 'unculturable' are being developed and applied to the study of the oral microbiome (Vartoukian, Palmer, & Wade, 2010).

2.3 GENOMIC METHODS

To overcome the limitations of cultivability of the many fastidious inhabitants of the human oral cavity, laboratory methods involving the detection and analysis of prokaryotic nucleic acid have overtaken bacterial culture as the most frequently employed means of surveying prokaryotic community membership. The use of DNA sequencing has allowed the detection, if not identification, of potentially hundreds of as-yet uncultivable species from the oral cavity in saliva (Lazarevic, Whiteson, Hernandez, Francois, & Schrenzel, 2010), from carious lesions (decayed tooth surfaces) (Simon-Soro et al., 2014) and subgingival plaque (Abusleme et al., 2013; Griffen et al., 2012; Hong et al., 2015). The ubiquitous 16S rRNA gene has been the target for the majority of molecular analyses to date (Nyvad, Crielaard, Mira, Takahashi, & Beighton, 2013).

2.3.1 The 16S rRNA gene

The 16S rRNA gene encodes the small subunit ribosomal RNA molecules of ribosomes, responsible for the essential process of converting genetic messages to functional cell components via the translation of mRNA to proteins. As such ribosomal RNA is a component of all self-replicating systems; it is readily isolated; and its sequence changes slowly with time, permitting the detection of relatedness among very distant species (Woese & Fox, 1977). Sequence analysis of the 16S rRNA gene and structural modelling of 16S rRNA revealed the sequence of the gene contains multiple conserved and variable regions. Evolutionarily conserved nucleotides appear towards the centre of the ribosome at functional sites, whereas variable nucleotides appear towards the surface of the ribosome where it is proposed nucleotide substitutions will not affect ribosome function (Wuyts, Van de Peer, & Wachter, 2001). The 16S rRNA gene serves as the molecular target for multiple laboratory methods for examining oral microbial community membership.

2.3.2 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) involves the separation of PCR products based on a difference in base pair sequence. Following amplification of DNA extracted from a microbial community sample with universal bacterial primers complementary to highly conserved regions of a gene, most often the 16S rRNA gene, the products from different taxa are separated by running them through a polyacrylamide gel containing an increasing gradient of DNA denaturants. Each different PCR product will move through the gel, stopping when it becomes denatured (Siqueira & Rocas, 2005). The point in the gel at which a DNA product will denature is dependent upon its nucleotide sequence and composition (Marsh, 1999). DNA bands on the gel can be compared to those of DNA bands from reference microorganisms run concurrently, and can be excised from the gel and their DNA sequence determined, allowing for the identification of known, and detection of unknown bacterial species in a sample. It is possible, however, for multiple species to be represented by a single band, and a single species to be represented by multiple bands (Fujimoto et al., 2003). Analysis of samples taken at different times can allow changes in microbial community structure associated with a move from health to disease to be visualised.

2.3.2.1 Characterisation of the oral microbiota with DGGE

A lower bacterial diversity in saliva as determined by DGGE has been associated with dental caries compared with health in young adults (Li, Ku, Xu, Saxena, & Caufield, 2005), and a decrease in diversity in supragingival plaque has been associated with a move from health to dental caries in young children (Tao, Zhou, Ouyang, & Lin, 2013). Diversity of subgingival microbial communities was not found to be associated with healthy patients as compared with patients with chronic periodontitis (Ledder et al., 2007); however, this may have been due to the choice of sites for sampling rather than the status of the patient. The influence of the local environment and disease status on microbial community membership can

be demonstrated by more complex microbial community profiles being found in deep compared to shallow periodontal pockets in subjects with periodontal diseases (Fujimoto et al., 2003). While the use of DGGE for the examination of oral microbial communities has now been superseded by other laboratory techniques, it has been employed more recently as a screening tool to assist in the development of growth media to sustain more diverse oral microbial communities in the laboratory that are more representative of the communities present in the oral samples (Tian et al., 2010).

2.3.3 16S rRNA gene sequencing

16S rRNA gene sequence comparison exploits the combination of highly variable and highly conserved segments of the 16S rRNA gene, enabling comprehensive examination of complex microbial communities such as those found in the oral cavity. The prokaryotic 16S rRNA gene contains nine hypervariable regions (V1–V9) found between highly conserved regions and is approximately 1.5 kbp in length. Universal PCR primers are designed to bind conserved regions and amplify across hypervariable regions. The resulting amplicons are then sequenced and either taxonomically identified by the best-matching sequence in a reference database, or the percentage sequence identity is used to cluster the bacterial sequences into operational taxonomic units (OTUs), using $\geq$97% sequence identity to approximate species level identification (Chen, Zhang, Cheng, Zhang, & Zhao, 2013; Stackebrandt & Goebel, 1994). Due to the high sequence conservation of the areas used for primer binding, no prior knowledge is required of the organisms present in the sample under examination, thus enabling the 'discovery' of the true breadth of bacteria present, regardless of whether they are cultivable or not. This is the great strength of 16S rRNA gene sequencing and why it is the preferred method for identifying the microbiota within a microbiome. Originally performed with Sanger sequencing which required the PCR product to be cloned with one clone sequenced at a time (de Lillo et al., 2006; Hutter et al., 2003), the advent of massively parallel sequencing methodologies has removed the requirement for cloning and has not only enabled the sequencing of a mixed DNA population simultaneously, but multiple mixed populations can be sequenced together thanks to barcode identification (Hamady, Walker, Harris, Gold, & Knight, 2008). This deep sequencing has required the development of software 'pipelines' such as MG-RAST (Meyer et al., 2008), mothur (Schloss et al., 2009), QIIME (Caporaso et al., 2010), MEGAN (Mitra, Stark, & Huson, 2011), VAMPs (Huse et al., 2014) and FROGS (Escudie et al., 2018) for deconvolution and analysis of the data using 16S rRNA gene sequence databases such as the RDP (Cole et al., 2009), SILVA (Pruesse et al., 2007), Greengenes (DeSantis et al., 2006), HOMD (Chen et al., 2010), HMP (Nelson et al., 2010) and CORE (Griffen et al., 2011). The increased sensitivity of this method has led to a rapid increase in identification of rare and/or uncultivated bacteria and a more complete picture of several microbiomes (Dewhirst et al., 2010; Huttenhower et al., 2012; Moitinho-Silva et al., 2017). However, there are several problems associated with the use of the 16S rRNA gene in general and when combined with next-generation sequencing (NGS) in particular.

The high complexity of microbiome samples predisposes multitemplate 16S rRNA gene PCR to artefacts and biases. These include the formation of chimeras, hybrid products between multiple parent sequences (Haas et al., 2011; Kalle, Kubista, & Rensing, 2014; Kanagawa, 2003) and heteroduplexes, heterologous sequences that anneal following denaturation (Kalle et al., 2014; Kanagawa, 2003). These can both be falsely interpreted as novel organisms, thus inflating apparent diversity. Templates with different starting concentrations will have an unequal probability of being amplified (Polz & Cavanaugh, 1998), and variations in the amplification efficiencies of each individual template occur due to slight differences in the DNA primary structure (Kalle et al., 2014). Bias can also result from misannealing of primers and misincorporation of bases by DNA polymerases (Kanagawa, 2003), differences in primer binding energy of degenerate primers (Polz & Cavanaugh, 1998) and the high G+C content of templates (Munson, Banerjee, Watson, & Wade, 2004). The multiple rounds of PCR amplification can exacerbate biases, thereby affecting the abundance of detected genera which can alter the apparent bacterial community structure (Bonnet, Suau, Dore, Gibson, & Collins, 2002). It is also important to use 16S rRNA gene databases that derived their reference sequences from genomic DNA and not PCR products.

2.3.3.1 Characterisation of the oral microbiota with 16S rRNA gene sequencing

Sanger sequencing of cloned PCR products is capable of providing full-length 16S rRNA gene sequences (Kumar, Brooker, Dowd, & Camerlengo, 2011), but the short read length from NGS means that instead of full-length sequences, particular variable regions must be targeted, such as V1&V2 (~350 bp), V3&V4 (440~470 bp), V5&V6 (~300 bp) and V7&V8&V9 (~420 bp) (Guo, Ju, Cai, & Zhang, 2013), as dictated by the read length of the NGS platform being used. This limits resolution since many bacterial taxa may share the same short amplified sequence (Fuks et al., 2018). Also, the various regions of the rRNA gene have different powers of taxonomic resolution as demonstrated by Kumar et al. (2011) upon examination of subgingival plaque. The genera *Prevotella*, *Fusobacterium*, *Streptococcus*, *Granulicatella*, *Bacteroides*, *Porphyromonas* and *Treponema* formed 65% of the community when the V1–V3 region was targeted, while *Streptococcus*, *Treponema*, *Prevotella*, *Eubacterium*, *Porphyromonas*, *Campylobacter* and *Enterococcus* accounted for the same abundance in the community generated by V4–V6 primers, and 65% of the V7–V9 community was formed by *Veillonella*, *Streptococcus*, *Eubacterium*, *Enterococcus*, *Treponema*, *Catonella* and *Selenomonas*. Among the predominant genera, *Fusobacterium* was not detected in any of the samples by the V7–V9 primers, while the V4–V6 primers did not detect the *Selenomonas*, *Mycoplasma* or TM7 *phylum* in any sample (Kumar et al., 2011). There is no hypervariable region of the 16S rRNA gene that is equally diagnostic for all genera (Table 2), hence using a combination of different regions is recommended (Griffen et al., 2012; Kumar et al., 2011; Szafranski et al., 2015). In light of this, and recognising the need for a simple and low-cost 16S rRNA gene-based profiling

Table 2 Primer Pairs Commonly Utilised for Next-Generation Sequencing of Oral Bacterial Communities

Target Region	Primer Pair Sequences	References	+ Indicates Possible Best Match − Indicates Region Possibly Not Suitable
V1–V2	(F) GTTTGATCMTGGCTCAG (R) CTGCTGCCTYCCGTA	Griffen et al. (2012)	+ Genus *Prevotella*
V1–V2	(F) AGAGTTTGATCMTGGCTCAG (R) CTGCCTCCCGTAGGAGT	Szafranski et al. (2015)	+ Class Deltaproteobacteria + Class Fusobacteria + Class Erysipelotrichia
V1–V3	(F) GTTTGATCCTGGCTCAG (R) GTATTACCGCGGCAGCTGGCAC	Kumar et al. (2011)	+ Genus *Prevotella* + Genus *Fusobacterium*
V4	(F) TNAYTGGGYRTAAAG (R) TACYVGGGTATCTAATCC	Griffen et al. (2012)	− Genus *Prevotella* + Genus *Acinetobacter*
V4–V6	(F) GTGCCAGCTGCCGCGGTAATAC (R) GGGTTGCGCTCGTTGC	Kumar et al. (2011)	− Genus *Fusobacterium* + Genus *Streptococcus* + Genus *Treponema* + Genus *Porphyromonas*
V5–V6	(F) GGATTAGATACCCBRGTAGTC (R) AGYTGDCGACRRCCRTGCA	Szafranski et al. (2015)	− Class Synergistetes + Class Clostridia
V7–V9	(F) GCAACGAGCGCAACCC (R) AAGGAGGTGATCCAGGC	Kumar et al. (2011)	− Genus *Selenomonas* − Genus *Fusobacterium* − Phylum TM7 − Genus *Mycoplasma* + Genus *Eubacterium* + Genus *Enterococcus* + Genus *Veillonella*

approach that harnesses short read lengths to provide a much larger coverage of the gene, Fuks et al. (2018) developed 'Short MUltiple Regions Framework' (SMURF), a method to combine sequencing results from different PCR-amplified regions to provide one coherent profiling. The method is based on independent PCR amplification and sequencing of several regions (actually any number of regions) along the 16S rRNA gene, which are computationally combined to provide a joint estimate of the microbial community composition.

The concept and definition of bacterial species has always been problematic and until recently a 97% identity across the 16S rRNA gene was considered to be adequate to separate distinct species. The 97% threshold to approximate species was reevaluated to 98.7% (Stackebrandt & Ebers, 2006); however, recent work by Edgar (2018) has indicated that it should be increased to ~99% for full-length sequences and ~100% for the V4 hypervariable region (Edgar, 2018). However, while 16S rRNA gene hypervariable region analysis is a powerful tool for bacterial taxonomic studies, it struggles to differentiate between closely related species. This is problematic as identification to the genus level does not necessarily distinguish between species associated with health or disease, an important consideration for the oral microbiota where several genera including *Streptococcus*, *Tannerella* and *Veillonella* have species associated with both health and disease. Small differences in the sequence of the 16S rRNA gene can be markers for substantial genomic and ecological variation between bacteria. Recently Eren et al. (2014) demonstrated that instead of clustering sequences into OTUs, nucleotide sites that show high variation (as determined using Shannon Entropy) can be concatenated and used to partition sequencing data into high-resolution groups while discarding the redundant information and noise in a process called oligotyping (Eren et al., 2014). Oligotyping allows the identification of taxa that differ by as little as a single nucleotide in the sequenced region. Applying oligotyping to the oral microbiome produced a more nuanced characterisation of the microbial communities inhabiting each oral site than had been obtained previously with genus- or even species-level analyses. Oligotyping demonstrated distinctive community compositions at different oral sites, showed that many genera and even some species-level taxa contain previously undetected site specialists, indicated the predominance of certain oligotypes within individuals, and provided correlations between sites within individual mouths (Eren et al., 2014).

In addition to high levels of sequence conservation among closely related species, the copy number of 16S rRNA genes may vary from 1 to 15 among eubacterial genomes. While it was generally believed that all the copies of ribosomal RNA in an organism are identical or nearly identical in nucleotide sequence (Liao, 2000), intragenomic heterogeneity is commonly detected (Clayton, Sutton, Hinkle, Bult, & Fields, 1995; Gürtler, Subrahmanyam, Shekar, Maiti, & Karunasagar, 2014). In fact, there is intragenomic heterogeneity found within some *Veillonella* species that is greater than interspecies variability (Marchandin et al., 2003; Michon et al., 2010). To overcome this problem, sequences of housekeeping genes of the genus *Veillonella*, such as *dnaK* (70 kDa heat-shock protein), *gltA* (citrate synthase), *gyrB* (DNA gyrase

subunit B) and *rpoB* (RNA polymerase subunit B), have been demonstrated to be more discriminatory between species than the 16S rRNA gene (Aujoulat, Bouvet, Jumas-Bilak, Jean-Pierre, & Marchandin, 2014; Mashima et al., 2018; Mashima, Theodorea, Thaweboon, Thaweboon, & Nakazawa, 2016). Such housekeeping genes are single copy and possess the same key attributes as 16S rRNA genes, namely, that they are common to all bacteria, that they have conserved as well as variable regions and that they function as an evolutionary clock (Dahllof, Baillie, & Kjelleberg, 2000).

Finally, the 16S rRNA gene sequence only provides information on microbial taxonomy; when coupled with well-designed prospective longitudinal studies this can provide convincing evidence of the bacterial species, genera and communities involved in the aetiology of chronic oral disease, and conversely those associated with health (Knight et al., 2018). However, the identification of these species does not identify the metabolic functions involved in the transition from health to disease, or the bacterial virulence factors involved. These have traditionally been identified in monoculture using a combination of biochemical analyses, genetic manipulation of the bacterium and animal models of disease. The application of metagenomics and metatranscriptomics is now addressing bacterial metabolism and virulence factors in the true in situ polymicrobial setting.

2.3.4 Metagenomics of the oral microbiome

The metagenome is the collection of genomes and genes from the members of a particular microbiota. This collection is obtained through shotgun sequencing of DNA extracted from a sample (metagenomics) followed by assembly or mapping to a reference database then followed by annotation (Marchesi & Ravel, 2015). 16S rRNA gene sequencing relies on the amplification and sequencing of taxonomic marker genes, and is not considered metagenomics; it has been suggested to call it metataxonomics (Marchesi & Ravel, 2015).

2.3.4.1 Characterisation of the oral microbiota with metagenomics

The metagenome of oral bacterial communities has been explored in terms of site-specific communities in health (Huttenhower et al., 2012; Xie et al., 2010), comparisons between health and disease in caries (Alcaraz et al., 2012; Belda-Ferre et al., 2012; Belstrom, Constancias, et al., 2017) and between health and periodontal diseases (Belstrom, Constancias, et al., 2017; Dabdoub, Ganesan, & Kumar, 2016; Duran-Pinedo & Frias-Lopez, 2015; Laksmana et al., 2012; Liu et al., 2012; Shi et al., 2015; Wang et al., 2013; Yost, Duran-Pinedo, Teles, Krishnan, & Frias-Lopez, 2015).

In their examination of healthy and carious sites using metagenomics, Belda-Ferre et al. (2012) determined that the *Veillonella* present in two healthy individuals were missing a 28-kbp genomic island that was present in individuals with caries, suggesting that genes found on this genomic island could be related to pathogenesis. Thus, metagenomics can detect differences between strains of the same species which would have passed unnoticed by 16S rRNA studies (Belda-Ferre et al., 2012).

Building on a 16S rRNA study where bacteria found in pooled subgingival plaque samples from individuals with chronic periodontitis were also found in their saliva (Belstrom, Sembler-Moller, et al., 2017), Belstrom, Constancias, et al. (2017) used salivary metagenomics to examine the bacterial composition of stimulated saliva from individuals with chronic periodontitis, caries or who were orally healthy. Results from this study reinforced the concept that pathogenic bacteria are shed from local periodontitis and caries lesions into saliva, which may increase salivary abundance of these bacteria in patients with periodontitis and dental caries (Belstrom, Constancias, et al., 2017). Low levels of *P. gingivalis* and *S. mutans* were also occasionally identified in saliva from orally healthy individuals, indicating that these bacteria are part of the resident oral microbiota (Belstrom, Constancias, et al., 2017).

Being a relatively new technique, there are fewer metagenomic studies on the oral microbiome than 16S rRNA gene analyses. In addition, there are also greatly increased costs associated with sequencing multiple genomes as well as the increased computational requirements to handle the huge amount of data generated. Metagenomic reads are more complex, owing to the nonclonal heterogeneous reads resulting from multiple strains differing only by partial regions or rearrangements, lower or uneven coverage across genomes, sharing of repetitive sequences among closely related species, and lateral gene transfer sharing similar entities between distantly related organisms (Kim et al., 2013). In addition, a high percentage of the genomic reads can come from human DNA contamination (Liu et al., 2012; Xu & Gunsolley, 2014). Due to the high cost, metagenomics projects are typically based on a small number of samples, which limits the statistical power of the studies and conclusions that can be drawn from them (Alcaraz et al., 2012; Belda-Ferre et al., 2012; Liu et al., 2012; Xie et al., 2010). To overcome this, Ai et al. (2017) combined the results of two previous studies resulting in an increased sample size which showed alpha diversity to be the single strongest predictor of a subject's periodontitis status, such that healthy subjects had the highest alpha diversity, while samples from stable sites in subjects with chronic periodontitis had the lowest (Ai et al., 2017). Marker species *L. gasseri*, *Campylobacter showae* and *Olsenella uli* were more abundant in progressing periodontitis samples, while the marker species *Gemella morbillorum*, *Veillonella parvula*, *Haemophilus parainfluenzae*, *Corynebacterium matruchotii*, *Neisseria flavescens*, *C. showae* and *S. sanguinis* were significantly more abundant in samples from healthy subjects (Ai et al., 2017). The phylogenetic profiles of these nine marker microbes could be applied to differentiate the states of periodontitis to an accuracy of 94.4% (Ai et al., 2017). Combining results from different studies, however, can make interpretation problematic if different criteria have been used to define disease, or if different sampling techniques or study methods have been utilised. This highlights the need for standardised protocols in order to combine data from different metagenomic studies.

2.3.5 Metatranscriptomics of the oral microbiome

Although metagenomics provides a partial glimpse into the functional profile of a microbial community through gene identification, the functional profile is better inferred using metatranscriptomics, which involves sequencing the complete

transcriptome of the microbial community (Aguiar-Pulido et al., 2016). 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 (McLean, 2014). 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 (McLean, 2014). A confounding parameter of metatranscriptomics though is the change in a given genome abundance between two samples that can skew differential gene expression when comparing sample sets such as between health and disease within in vivo collected samples. For example, if the abundance of a particular species increases, the number of mRNA copies will increase and thus appear to be differentially expressed genes between the sample sets (McLean, 2014). Both metagenomics and metatranscriptomics are also limited by the availability of complete reference genomes; the identification of a unique read to a given strain can only be accomplished when a reference genome of that strain is available (McLean, 2014).

Yost et al. (2015) identified changes to the abundance of transcripts encoding particular metabolic pathways in the microbial community associated with the initial stages of dysbiosis during chronic periodontitis progression (Yost et al., 2015). Regardless of the overall composition of the community, certain predicted metabolic signatures were consistent with disease progression, suggesting that the whole community, and not just a limited number of identified oral pathogens, is responsible for an increase in virulence that leads to disease progression (Yost et al., 2015).

Both metagenomic and metatranscriptomic analyses identified higher salivary abundance of specific oral bacterial species in chronic periodontitis and dental caries patients compared with healthy individuals (Belstrom, Constancias, et al., 2017). Expression of genes encoding specific metabolic pathways was significantly different in patients with chronic periodontitis and caries compared with healthy controls, suggesting that bacteria shed from local diseased lesions are still active when dispersed into saliva (Belstrom, Constancias, et al., 2017). The high activity (RNA/DNA ratio) of *F. alocis* and *Neisseria* sp. was associated with chronic periodontitis (Belstrom, Constancias, et al., 2017).

Unlike the 16S rRNA gene sequencing studies by Griffen et al. (2012) and Abusleme et al. (2013) which reported a greater alpha diversity within chronic periodontitis sites, Jorth et al. (2014) showed that bacterial alpha diversity in aggressive periodontitis-associated populations was significantly lower than health-associated populations, containing fewer overall species and lower species diversity (Jorth et al., 2014). Within each individual, disease-associated populations showed defined changes in expression of metabolic genes, suggesting that specific metabolic shifts were occurring in disease-associated communities. Specific pathways that showed enhanced gene expression in all diseased sites included lysine fermentation to butyrate, histidine catabolism, nucleotide biosynthesis and pyruvate fermentation. While population composition varied among plaque populations, enzyme expression was well conserved, suggesting that multiple organisms that varied among

populations were capable of filling conserved metabolic niches. While several oral microbes have the capacity to produce butyrate, the Gram-negative bacterium *F. nucleatum* was the sole bacterium responsible for community lysine degradation to butyrate in all patients. In contrast, several bacteria were responsible for enhanced expression of genes involved in histidine degradation and pyruvate fermentation during disease, and the bacteria differed between patients. The interchangeability of community members in each patient provides insight into why metagenomic analyses of the oral microbiome have displayed little conservation. The bacteria contributing to expression of known extracellular virulence factors varied between patients too, suggesting that in addition to metabolism, distinct microbes produced conserved virulence determinants in each individual (Jorth et al., 2014).

Nowicki et al. (2018) found significant shifts in the abundance of several genera occurred during transition from health to gingivitis, suggesting a dysbiosis due to development of gingivitis (Nowicki et al., 2018). Metatranscriptome sequencing analysis indicated that during the early stages of transition to gingivitis, a number of virulence-related transcripts were significantly differentially expressed in individual and across pooled patient samples. Upregulated genes included those involved in proteolytic and nucleolytic processes, while expression levels of those involved in surface structure assembly and other general virulence functions leading to colonisation or adaptation within the host were more dynamic (Nowicki et al., 2018).

Examination of supragingival dental plaque metatranscriptomes from caries-free and caries active twins enabled Peterson et al. (2014) to describe a metabolically active core dental plaque microbiota of 58 unique species from this cohort; however, there was no detected link between gene expression and disease status. They did, however, identify different networks made up of several genera/species displaying coordinated transcriptional activity. Interestingly, the supragingival dental biofilm microbiota devoted a significant amount of its transcriptional potential to stress response pathways that help deal with a low pH environment, such as the expression of proteins that substantially remediate superoxides and peroxides and H^+ produced by fermentative bacterial species (Peterson et al., 2014).

Thus, it appears that a common theme arising from metatranscriptomic analysis is the identification of different networks of bacteria that perform similar metabolic functions overall and could therefore represent a likely basis of the high interpersonal variation observed in oral microbiota.

2.3.6 The future of characterising the oral microbiome

Unprecedented visualisation of oral microbial biofilms has recently been achieved by Mark Welch and colleagues who elegantly employed metagenomic sequence analysis to identify key taxa for the selection and design of fluorophore-labelled oligonucleotide probes (Mark Welch et al., 2016). This enabled the overall structure of the complex but highly organised communities in supragingival plaque to be observed (Fig. 2). We echo the author's suggestion that the availability of metatranscriptomic analyses should not negate the importance of taxonomy. That the use of

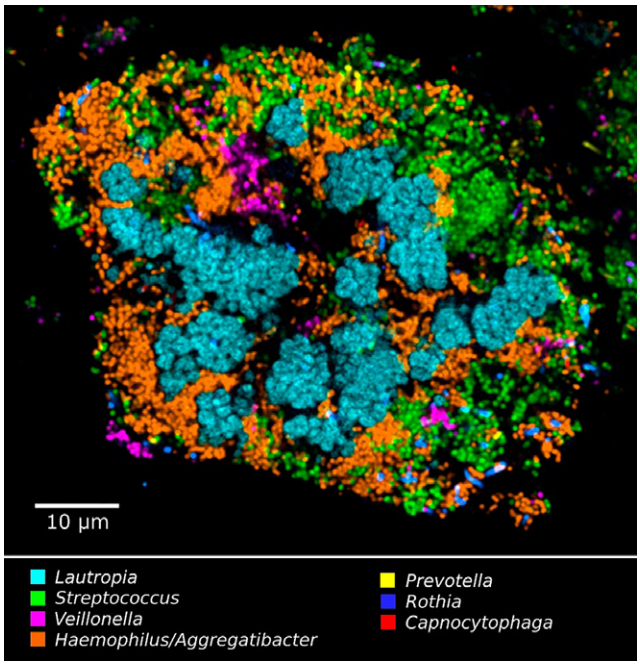

FIG. 2

A cauliflower structure in supragingival dental plaque composed of *Lautropia*, *Streptococcus*, *Haemophilus/Aggregatibacter* and *Veillonella*, with scattered cells of *Prevotella*, *Rothia* and *Capnocytophaga*.

Figure reproduced from Mark Welch, J. L., Rossetti, B. J., Rieken, C. W., Dewhirst, F. E., & Borisy, G. G. (2016). Biogeography of a human oral microbiome at the micron scale. Proceedings of the National Academy of Sciences of the United States of America, 113*(6), E791–E800.* https://doi.org/10.1073/pnas.1522149113.

such a combination of laboratory techniques to examine the oral biofilm following longitudinal studies of health and disease may shed further light on not only who is there, and what they are doing, but where they are which may impact on their ability to interact with one another and their host to maintain health, or inflict damage.

CONCLUSIONS

So where does this leave the characterisation of the oral microbiome? We have hope that the continued development and combination of novel laboratory methods coupled with comprehensive prospective longitudinal studies of oral health and diseases has the potential to expand our understanding of our microbial partners and how we might harness their power in an attempt to maintain oral health in a time of increased susceptibility to oral microbial biofilm-associated diseases.

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