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The salivary microbiome assessed by a high-throughput and culture-independent approach

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ABSTRACT

The lack of cultivation of a significant fraction of bacteria found in the oral cavity means that culture-independent approaches are needed for the study of the salivary bacterial community composition and diversity. Saliva is easily obtained and could provide an alternative to blood in diagnostics, forensics, epidemiology and population studies. Our goal in this review is to put together the findings from the handful of recent studies of human salivary bacteria derived from culture-independent high-throughput sequencing of the 16S rRNA gene and look for emerging trends in the resulting larger dataset. Differences in phyla and genera abundances between studies of the salivary microbiome may be due to individual (genetic and lifestyle) variations, geographic variations and biases introduced during the experimental steps. Nevertheless, seven major phyla found in all relevant studies may be arbitrarily assigned into three major categories according to their abundance: (i) Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were very abundant; (ii) TM7 and Fusobacteria were moderately abundant and (iii) Spirochaetes had the lowest abundance. The frequency of genera varied among the studies but many had a relatively consistent presence. Genera found in all of the four reported salivary microbiomes were generally present at a high frequency and contributed to 71-80% of all sequences in the corresponding datasets. Conversely, genera identified in only one available salivary microbiome generally showed a low abundance. Improvements in high-throughput sequencing technology will enable random metagenome fragment sequencing to become a powerful tool to study bacterial, archaeal, fungal, phage and human viral components of the salivary microbiome in parallel.

Keywords: Metagenomics; Microbiome; Microbiota; High-throughput Sequencing; Oral Bacteria; Saliva.

Human microbiota

The microorganisms harbored by the human body are organized in complex communities, called microbiota. They outnumber human cells by an order of magnitude [1] and their non-redundant gene set is on average more than 25 times larger than the host's gene complement [2]. Bacterial populations are not evenly distributed across the human body; they differ in density (Fig. 1), in taxa composition and abundance as well as in stability. It has been shown that microbiota variation between different habitats of the same subject is greater than interpersonal variation for the same habitat, whereas the smallest variation was observed within a given habitat over time [3].

The vast majority of bacteria colonizing the human body inhabit the distal part of the gastro-intestinal tract. Analyses of the intestinal microbiota revealed the existence of an individual core, representing the stable colonizers in a single subject, and also suggested that humans share some microbial species, the so-called universal core [4]. While the existence of a universal core was confirmed by analyzing a small number of individuals, recent studies of the intestinal microbiota including more than 100 subjects revealed that no single species-level 16S rDNA phylotype was found to be shared by all individuals [5, 6]. Therefore, it was suggested that the concept of a functional core microbiome defined at the level of shared genes, rather than shared species, would be more appropriate [6]. Yet, the issue remains a matter of the sequencing depth and definition. A deeper sample coverage and inclusion in the analysis of very rare phylotypes rather than only those occurring above a defined threshold is expected to increase the number of shared phylotypes. Indeed, deep sequencing of

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metagenomic libraries from human fecal samples revealed 18 species shared by all of the 124 investigated subjects [2].

Alteration of the human microbiotas is associated with different pathologies [2, 7-10], although formal evidence that microbiota changes are actually causing such health disorders is lacking. New tools for studying human microbiota are bringing us closer to this kind of evidence. For example, the presence of particular gut microbiota has been shown to prevent intestinal inflammatory disease [11, 12], while other specific gut microbiota appear to trigger multiple sclerosis [13]. Bioremediation of microbial imbalances arises as an attractive therapeutic approach [14] and in some cases is already happening. For example, a severe *Clostridium difficile* gut infection which did not respond to several anti-microbial approaches was resolved with a healthy faucal transplant from the patient's spouse [15].

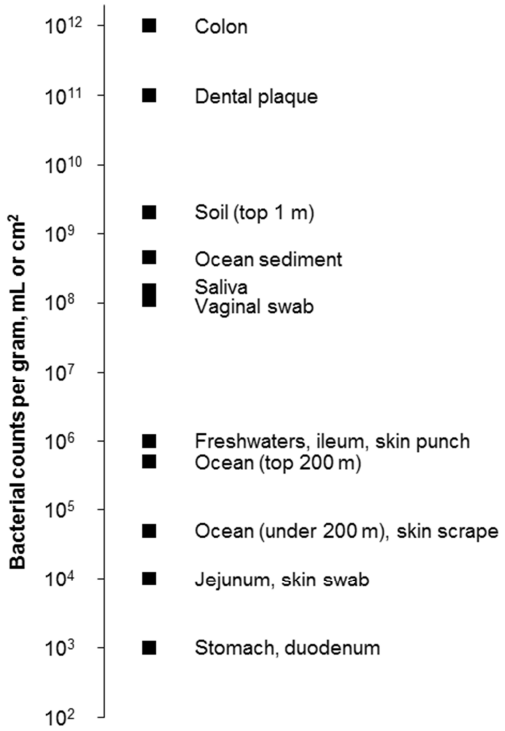

Figure 1. *Bacterial loads in different biotic and abiotic habitats.* Data were compiled from several sources in which different approaches, mainly culture-based, have been used to estimate bacterial counts [1, 16-20]. Bacterial counts on the y-axis are expressed per gram (soil, gastrointestinal and vaginal samples), per cm^3 (saliva, dental plaque, waters, ocean sediment samples) or per cm^2 (inner elbow skin samples).

Oral microbiota

Bacterial communities in the mouth have a significant impact on general health by either preventing or causing infections. Poor oral hygiene affects not only the health of the oral cavity but also the overall health of an individual by increasing risk of bacterial endocarditis and of respiratory infections [21-23]. Some oral pathologies may have a polymicrobial etiology and different types of infections appear to be associated with various mixed bacterial consortia [24]. A hypothetical causative relationship between oral microbiota profiles and oral diseases has been established [8]. Nine bacterial species were detected significantly less often in subgingival plaque samples from subjects with periodontitis compared to healthy control individuals [8]. Subjects with different forms of periodontitis can be distinguished by the frequency of 9 putative periodontal pathogenic and 15 additional species [8]. Dental caries in children are associated with a significant shift in the relative abundance of six genera in supragingival dental plaques [25].

The mouth is an entry site for passage of bacteria into the digestive tract, the respiratory tract, and the bloodstream. Since microorganisms in the mouth can translocate and colonize other parts of the body, the oral microbiota is important in the development of distant infections [26]. For example, *Streptococcus mitis* strain SF100 from the oral cavity is able to bind to human platelets with the help of phage-encoded proteins [27]. The results of a metagenomic survey of bacterial communities after the transplant of tongue bacteria to the skin suggested that different parts of the body vary in their susceptibility to colonization by oral bacteria [3].

Because of a high density and species richness, the oral cavity, including dental plaque and saliva, offers conditions that may favor genetic exchanges [28]. It has also been speculated that antibiotic-resistant oral bacteria could serve as a reservoir for the horizontal transfer of the resistance genes to other non-oral organisms that transit the oral cavity [28]. The naturally transformable bacterium *S. pneumoniae*, the major respiratory tract pathogen, may develop resistance to beta-lactam antibiotics through alterations of penicillin-binding proteins that result from genetic exchanges with commensal oral viridans streptococci [29].

More than 700 bacterial species have been identified in the human mouth and 35% of them are not yet cultivated [30]. The need to study complex oral microbiotas without culturing the bacteria prompted several research laboratories to use high-throughput sequencing of partial 16S rDNA genes amplified directly from oral bacterial communities. This approach currently provides the best compromise between sequence coverage, analytical speed and experimental costs.

Recent studies of oral microbiota using high-throughput sequencing of 16S rDNA amplicons estimate that the number of species-level phylotypes is between 540 and about 10,000 [31-33]. However, these figures were obtained using different sequencing coverage, sampling different anatomical sites and analyzing samples pooled from different numbers of individuals.

A study of the oral microbiota from three individuals, which included a mixture of samples from teeth, cheek, hard palate, tongue and saliva, supported the concept of a universal core [33]. Comparison of these oral microbiomes showed that 26.3% of distinct sequences (100%-ID phylotypes) and 47.3% of species level-phylotypes (97%-ID phylotypes) which were shared contributed to 66% and 93% of all sequence

reads of the pooled dataset, respectively. The phylotype overlap between these three oral microbiomes was significantly higher in comparison to the values reported for other body sites [5, 34], which was explained by relatively stable conditions in the human mouth [33]. In line with this observation, a survey of microbiota variation over space and time showed that the oral microbiota are less variable than those at other investigated body sites [3].

Co-occurrence analysis of bacterial taxa in data from ten individual subjects revealed genus pairs unlikely to appear together in the oral cavity [35]. By comparing the occurrence of phylotypes defined at 99% identity, evidence of possible competitive interactions between species (and/or strains) was found only within the phylum Firmicutes [35].

Salivary microbiota

Saliva contains between 10^7 to 10^9 bacteria per mL (Fig. 1), with an average value of 1.4×10^8 bacteria/mL and a higher abundance of anaerobes [18]. Since the average daily flow of saliva is between 1 and 1.5 L [36] around 10^{11} salivary bacteria may be swallowed daily. The number of bacteria attached to exfoliated oral mucosal epithelial cells was estimated to be about 2.6 times as many as those free in saliva [37]. In spite of high loads which approach those of the distal gut (Fig. 1), bacteria on the teeth and in the periodontal pockets make a small contribution to the salivary bacterial counts [38]. When 40 bacterial species were sampled on 8 oral soft tissue surfaces and studied using a DNA-DNA hybridization technique [39], salivary microbiota was found to be most similar, proportionately, to that of the dorsal and lateral surfaces of the tongue. This was confirmed by a pyrosequencing approach where the microbiota of saliva was more similar to mucosal than dental microbiotas [33].

Culture independent studies of the salivary microbiome

Several recent studies (Table 1) based on high-throughput sequencing of the preferred phylogenetic marker, the 16S rRNA gene, focused on the salivary component of the oral bacterial communities [31-33, 40, 41].

The existence of the universal core was specifically addressed in the study of the salivary microbiome from 5 individuals, each sampled at 3 time-points [41]. As expected, the size of the universal core was inversely correlated with the number of subjects sampled and the number of time points from the same individual (Fig. 2). When the three time-point samples of all individuals were taken into account, the universal core was represented by 0.3% of distinct 16S rDNA sequences and 1.9% of phylotypes defined at a 97%-ID cut-off, which corresponds to 23.3% and 37.6% of the full dataset, respectively [41]. These figures are below values reported in the study of three oral microbiomes at a single time-point [33], which had a higher sequence coverage but, besides saliva, included swabs from several oral surfaces.

The salivary microbial community appears to be relatively stable within individuals over time [41]. A large fraction of the salivary microbiome 16S sequences corresponds to the individual core. In the study which included five individuals, on average 89.0±6.1% and 77.9±7.5% of 16S rDNA sequences contributed to the individual core depending on whether the phylotypes were defined at 97 or 100% identity [41]. In the same study, time-point samples were grouped by subject using UniFrac [44]. The UniFrac metric clusters samples in terms of the phylogeny of their communities, where larger values are assigned to changes in more distant taxa. Within the same subject, samples taken at closer time intervals were not necessarily more similar than those from more distant time points, pointing to the fluctuation of some bacterial taxa between the time points. Additional analysis methods which do not give more weight to differences between more distant taxa may also be helpful in comparisons of communities, such as ANOSIM [45].

Salivary taxa abundances

We compared the abundance of phyla and genera in salivary microbiomes reported in different studies (Table 1). Comparisons were made using a pooled dataset for each study, because either samples from different individuals were not specifically labeled [31], or because the number of sequences from each individual was relatively low [40].

Differences in taxa abundance between studies may be due to individual (genetic and lifestyle) variations, geographic variations and biases introduced during the lysis procedure and PCR amplification. The 16S rRNA gene fragments were obtained by PCR amplification using primer pairs that differed between studies. Nevertheless, seven major phyla found in all studies may be arbitrarily assigned into three major categories according to their abundance (Table 1): (i) Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were very abundant (>6%); TM7 and Fusobacteria were moderately abundant (0.5-3.7%), and (iii) Spirochaetes had the lowest abundance (0.022-0.34%). In addition, very low abundance phyla were detected in some studies. For instance, many "cyanobacterial" sequences found at very low frequency may correspond to plant chloroplasts. They are likely transient colonizers linked to food intake or exposure to airborne pollen [3]. Members of 11 other very low-abundance phyla (<0.06%) were identified sporadically. The average abundances for the top 7 phyla of the four pooled salivary microbiomes [31, 33, 40, 41] were ranked in the same order as those found in a mixture of saliva and dental plaque specimens from 10 individuals [35]. Of all bacterial phyla previously found in the oral cavity [35, 46], only Chlamydiae and OD2 were not identified in the four salivary microbiomes investigated by high-throughput sequencing of the 16S rRNA gene. The possible reasons for this are low prevalence and/or low counts of members of these two phyla in saliva as well as biases introduced during PCR amplification.

Although the frequency of genera varied among the studies, many had a relatively consistent presence (Fig. 3A). Genera found in all of the four reported salivary microbiomes were generally present at a high frequency and contributed to 71-

Table 1. Comparison of phyla abundance in salivary microbiomes

Reference	[41]	[33]	[31]	[40]
Number of subjects	5	3	71	120
Remark	3 time-points each			10 locations
16S rDNA region amplified/sequenced	V1-3/V3	V5-6	V6	V4-5
Lysis procedure	Proteinase K/ Tween-20	0.1 mm zirconium beads/phenol	0.1 mm zirconium-slilica beads/phenol	Proteinase K/SDS
Sequences analyzed	31,169	18,182[1]	73,485	14,115
Sequencing platform	GS FLX	GS FLX	GS20	Sanger
Taxonomy analysis	RDP Classifier[2]	GAST/RDP	GAST/RDP	SeqMatch/RDP
Firmicutes	53.7	47.6	40.7	37.7
Proteobacteria	20.5	16.4	21	28.6
Actinobacteria	10.4	22.9	6.3	7.0
Bacteroidetes	9.7	10.4	27.2	20.4
TM7	3.4	1.4	1.9	0.51
Fusobacteria	1.69	1.1	2.9	3.7
Spirochaetes	0.28	0.022	0.2	0.34
Bacteria	0.109	0.1	0.2	0.4
Cyanobacteria	0.064	0.049	0.02	
SR1	0.045		0.014	
Tenericutes	0.016			0.06
Synergistes				0.04
Acidobacteria			0.049	
Planctomycetes			0.018	
Nitrospira			0.0039	
Chloroflexi			0.0039	
Deinococcus-Thermus			0.0023	
Thermotogae			0.0016	
OP11			0.0016	
OD1			0.0016	

80% of all sequences in the corresponding datasets. As expected, genera identified in only one salivary microbiome generally showed a low abundance. When the average and median frequency were calculated for all of the genera shared by the given number of microbiomes (1 to 4), a positive correlation was found (Fig. 3B).

Genus *Atopobium* showed the highest variation in abundance across the studies. While the fourth most abundant genus in one [41], its members were not identified in another study [31]. Inspection of the microbiomes of the five individuals in our previous study [41] revealed significant interpersonal variation: the average frequencies of *Atopobium* based on three time-points ranged from 0.23±0.21 to 14.2±3.6. Similarly, genera *Enterobacter* and *Serratia* showed a significant interindividual variation but also a geographic patterning [40]. Both genera had a relative abundance of >4% in the salivary microbiome study reported by Nasidze *et al.* [40]. However, in three other pooled microbiome datasets, they were either absent (*Serratia*) or found only once (*Enterobacter*) at more than a 100-fold lower frequency. The observed differences in *Serratia* and *Enterobacter* frequency across studies may be due to yet-unidentified cultural and/or environmental factors [40].

Studies of the salivary microbiome based on culture-

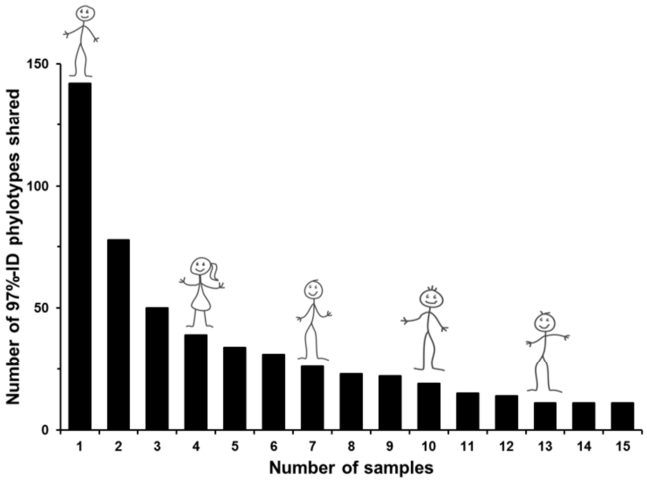

Figure 2. *Decrease in the number of species-level phylotypes shared as a function of the number of samples compared.* The bar with a stick figure above corresponds to the first time point of an individual; it is followed (to the right) by bars corresponding to the second and third time point of the same individual. The figure is based on data from our previous study [41].

independent high-throughput sequencing of partial 16S rDNA amplicons revealed genera and even higher-level taxa up to the phylum level that were not previously found in the human mouth and were not listed in the Human Oral Microbiome Database [46]. For instance, members of the class Sphingobacteria were found in saliva and/or on oral surfaces of some subjects [33, 41]. The 16S rDNA sequences corresponding to Sphingobacteria were found at a relatively low abundance (<0.11%), except in one saliva sample [41] where they represented 3.7% of all reads. Interestingly, in other saliva samples taken from the same subject at different time points within a one-month interval, Sphingobacteria were weakly present. This example provides evidence of fluctuations in oral bacterial taxa resulting in a very high relative abundance of otherwise apparently rare bacteria. Although the impact of very rare bacteria on the physiology of a bacterial community and their interaction with the host organism are not expected to be significant, these rare organisms may potentially become very abundant [41] and therefore should not be neglected a priori. Rare bacteria may also be useful as markers that are unique to individuals, as evidenced by improvements in separation of bacterial populations from individuals using a 100% sequence identity cut-off, as opposed to the standard 97% or lower cut-offs [41].

While the fraction of 16S rDNA sequences that could not be confidently placed at the phylum level is relatively small in available salivary microbiomes (Table 1), the sequences that could not be assigned at the genus level are significantly more abundant. They represent between 9.6% and 12.5% of the total number of sequences in relevant microbiome datasets [31, 33, 41]. The unassigned sequences may correspond to uncharacterized bacterial lineages confined or not to the oral niche.

Concluding remarks and outlook

After decades of culture-based investigation, culture-independent molecular techniques provide a new way to characterize salivary microbial communities. So far, these new approaches have been mainly focused on healthy adults. It will greatly benefit the field to conduct long-term surveys of a larger number of subjects in order to provide insight into the impact of different factors such as age, gender, smoking, dietary habits, oral hygiene, geographic location, use of antimicrobial compounds, immune status, underlying illness and human genetic composition on the salivary microbiota profile. Because of its relative stability, salivary microbiota may be potentially applied as an alternative or complementary approach in forensics for person identification, as recently proposed for skin bacterial communities [3].

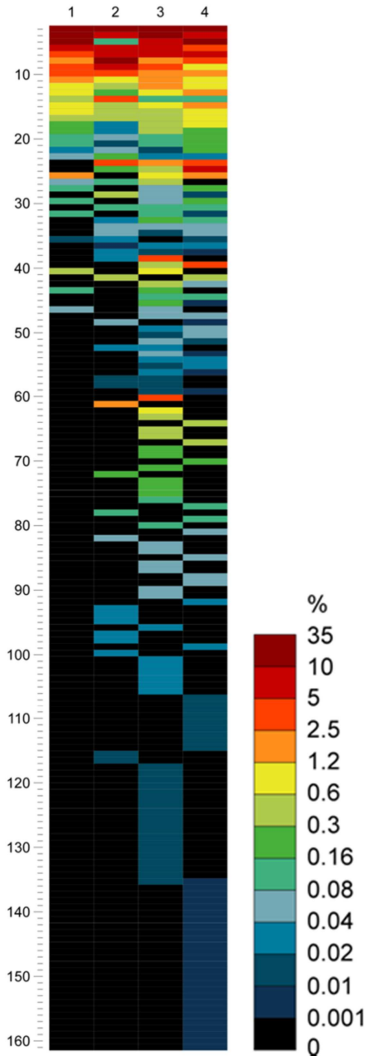

Figure 3A. *Relative abundance of bacterial genera across four (pooled) microbiomes.* Rows 1 to 161 correspond to genera listed in Supplementary Table 1 which also contains other relevant details. Genera are ranked first by the decreasing number of studies in which they were identified, then by the average frequency. The abundance is indicated according to the color scale at the right. Columns correspond to different studies as follows: 1, [31]; 2, [33]; 3, [40]; 4, [41].

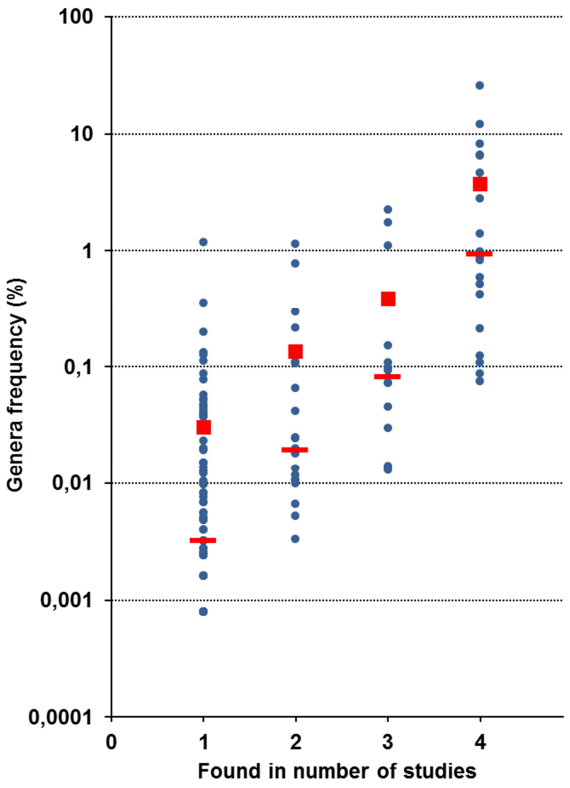

Figure 3B. *Relative abundance of bacterial genera across four (pooled) microbiomes.* Genera frequency as a function of number of studies in which they were detected. Blue circle, frequency of individual genera; red square, average genera frequency; red line, median genera frequency.

The salivary microbiome is a promising clinical diagnostic indicator of oral cancer, periodontitis [8-10] and possibly other diseases. The presence of specific pathogens and/or of a disturbed oral bacterial community might indicate the disease before symptoms are evident and may have clinical applications. Taxa which were proposed to be associated with periodontal disease before the use of high-throughput sequencing [8] are now found in healthy mouths of some individuals but often with lower abundance [35].

In addition, the human salivary microbiome may provide insights into human population structures and migrations [40], and studies with greater sequencing depth are an important background to establish what is healthy for different populations. Saliva is trivial to obtain, and can replace blood in some epidemiological studies and in diagnostics. A simple, scalable, non-invasive and cost-effective collection of saliva samples is expected to considerably increase the response rate in epidemiologic studies [47].

The fact that new taxa have been identified in each new study of the salivary microbiome and that the same genera have not been uniformly considered as universal core members across different studies, show that the our knowledge of the salivary microbiota is still developing. Therefore, larger-scale high-throughput approaches involving many time-points are required to better define the individual and universal core. Such studies, including relevant metadata records, may also allow us to understand if there are certain community structures that are more common than others and whether the oral microbial communities of healthy people can shift between alternate structures as shown for vaginal microbiota [48]. Analysis of 16S sequence fragments from oral samples taken from 300 individuals as part of the Human Microbiome Project by the National Institutes of Health, just becoming available online in 2010, may begin to establish these baselines [49].

Technological advancements in next-generation sequencing, including longer reads and paired-end strategy, may routinely provide full length 16S rDNA gene amplicon sequences in the near future, and therefore a better taxonomic resolution. This, combined with standardized DNA extraction procedures and PCR conditions, will enable more reliable comparisons of the results from different studies.

In order to minimize the impact of sequence errors, most bacterial community analyses rely on phylotypes defined at 97% identity cut-off which corresponds to the conservative estimate of bacterial species [50]. In view of the possibility that different individuals may be preferentially colonized by different strains of the same species, studies of the human microbiomes should also include analyses based on 100%-ID phylotypes [33, 41].

Ever-increasing sequencing throughput and computational power along with reference sequence databases will enable random metagenome fragment sequencing to ultimately become a standard approach for studies of oral bacterial (and other) communities. This approach has been validated and applied in the analysis of the human gut microbiome [2, 5, 51, 52]. Although a large fraction of DNA extracted from saliva corresponds to human DNA, the high sequence coverage of the whole metagenome using available HTS platforms allows us to obtain an appreciable number of bacterial and even bacteriophage sequences (unpublished). The taxonomic assignments of such identified bacterial sequences may be inferred from the BLASTN search [53] of the NCBI microbial database [54] which currently (as of December 2010) contains sequences of 1414 bacterial genomes, 879 of which are completed. Bacteriophage sequences will become increasingly identifiable as bioinformatic tools for identifying them improve; a new version of the RAST annotation engine focusing on phage annotation [55] will allow for rapid identification of known phage genes. In addition to providing a measurement of the relative abundance of bacterial taxa and the metabolic potential of the microbiota, the random sequencing of metagenomic fragments may help identify genes conferring resistance to antibiotics.

Here we have focused on bacterial communities in saliva, and we also look forward to obtaining more information about the archaeal, fungal and viral communities in the mouth, which undoubtedly play an important role in human health and will give us another angle to learn about the bacterial communities.

Archaea have not been identified in saliva so far, however, a

methanogenic archaeon *Methanobrevibacter oralis* was detected in dental plaques from a subset of patients with periodontitis. In addition, a direct correlation between the relative abundance of archaeal 16S rDNA and the severity of disease was observed [56]. The study of the archaeal component of the human oral microbiome based on the 16S rRNA sequencing requires PCR primers different from those used for bacterial identification [57].

Pyrosequencing of the internal transcribed spacer was used to characterize fungi present in the oral cavity of 20 healthy individuals [58]. Across all the samples studied, 74 culturable and 11 non-culturable fungal genera were identified, none of which was found in all sampled subjects.

By their ability to lyse bacteria, bacteriophages may play an important role in the dynamics of the oral microbiota. Bacteriophages infecting *Enterococcus faecalis* and *Proteus mirabilis* were isolated from saliva [59, 60]. Other phages, lytic for *Lactobacillus*, *Veillonella*, *Actinomyces*, *Actinobacillus*, *Streptococcus* and Proteus have been recovered from oral material including mouth wash (reviewed in [59]).

Saliva is documented to harbor several double-stranded DNA viruses (Herpes simplex virus 1, Epstein-Barr virus, cytomegalovirus and human herpesvirus 8) whose prevalence appears higher in HIV-seropositive patients [61]. Infection by viruses may have a profound effect on bacterial community composition, giving an invasive disease a chance to thrive. Viral sequences may be potentially identified in the whole salivary metagenome should the virus load be high enough and viral particles efficiently lysed.

Already the high-throughput sequencing which has been applied to the oral microbiome has revolutionized our understanding of the microbiota which reside there, giving us useful reference data for future studies of humans under diverse circumstances.

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