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Oral Microbiome: Metagenomics in Oral Health

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Abstract

The relationship between living organisms in our oral cavity and various diseases, such as dental caries or halitosis, has been well established for many years. In the past, traditional microbiology tools were only possible to deduce the relationships between certain specific bacteria and oral diseases. Today, the evolution of molecular biology, especially of DNA sequencing, and the progress of both bioinformatics and biostatistics allow the detection of uncultivable bacteria that might contribute to the diseases. The interaction among bacterial species can affect biofilm formation and behavior when it is confronted with adverse exogenous factors. Consequently, close investigation of oral microbiota using metagenomics is mandatory to fully understand how to prevent oral diseases related to dysbiosis. Some recent studies have also underlined the putative link between oral and systemic health, emphasizing the importance of understanding oral microbiota and its potential modulations. This review includes (1) the summary of various methodologies for assessing oral microbiota identification, characterization and development, (2) the relationship between oral microbiota and systemic health based on existing animal and human studies, and (3) the possible ways to modulate oral microbiota for positive effects on health.

Keywords: metagenomics, oral health, systemic diseases, polyols.

Introduction

It is now well known by the general population that our gastrointestinal tract is inhabited, that the human insides are colonized by billions of bacteria, and that we are not alone in our body. People are more and more aware of the presence of various microbiota in or on our body. The most well-known is the gut microbiota, but more recently oral, vaginal and skin microbiota have earned some attention. Some time ago, it was believed that the bacteria were deleterious, that they had to be removed from our lives. Soaps were very strong and bactericidal, toothpastes and mouth rinses aimed to remove 99.9% of oral bacteria. Nowadays, new care products have been developed to respect human skin, mucosa and their endogenous microflora. Some of them even try to influence these commensal ecosystems in a positive manner. This important change in the general population's way of thinking stems from a better understanding and knowledge of human microbiomes, thanks to high through-put technology such as metagenomics and sequencing. This review will focus on the oral microflora. Firstly, we will describe what metagenomics is in terms of evolution of technology, and then its use in the studies of the influence of living conditions. Secondly, the link between the oral microbiome and systemic health will be discussed. Finally, we will see how the oral microbiota can be modulated by health status or by external factors.

What do we Know about Human Oral Microbiota?

Metagenomics and Evolution of Technology

The term microbiome has been defined as the "ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body surface and have been all but ignored as determinant of health

and disease" [17]. The oral microbiome consists of several bacteria in small ecosystems such as the teeth, gums, tongue, lips and cheeks [17]. The human oral microbiome has been widely studied since it can be easily sampled from tooth or saliva [12], contrary to the gut microbiota. Bacterial survival is also better when collected from the oral cavity than from the colon, as living conditions are less harsh in the mouth. Close investigation into this complex biofilm was made possible with the development of molecular biology, and more precisely with the demonstration of high-throughput sequencing technologies [12]. Indeed, traditional bacterial culture studies were not sufficient to gain an accurate view of the biofilm since some species were not able to grow in the set culture conditions. These older technologies were used to isolate approximately 280 bacterial strains from the oral cavity when current molecular techniques can differentiate more than 600 species in the same sample [17]. A global research effort between several international teams led to the compilation of large databases such as The Human Oral Microbiome Database (HOMD) [12], by sharing their results on 16S ribosomal RNA gene sequence analysis. The aim of these large databases is to create a stable taxonomic structure to identify unknown oral taxa according to their 16S rRNA sequence. The larger the database, the more accurate the identification. Other projects aim to define oral microbiota as precisely as possible, such as the NIH Human Microbiome Project [2].

In order to sequence 16S rRNA from bacteria, high-throughput sequence analyzers were created in order to obtain faster and less costly sequencings than with the Sanger method. This technology is a combination of molecular material amplification and sequencing. This technology was made possible through improvement in the ability of bioinformatics software to analyze and assign each sequence for a sample. The strengths of these new techniques were compared with those of previous techniques such as microarray [2]. 16S rRNA is found only in prokaryotes and is considered to be the barcode which identifies specific bacteria without the culturable or non-culturable organism problem [2]. Microarray microbe identification is based on the design of specific probes for each bacterium, whereas the metagenomic approach is based on 16S rRNA gene sequence hybridization [2]. Consequently this type of analysis will only find what it is looking for. A study demonstrated that bacterial community profile analyses from 16S rRNA pyrosequencing or from microarray assay, produced similar results for the more common taxa at genus level. Nevertheless, pyrosequencing provided for a broader spectrum of taxa identification and greater detection sensitivity for minor species [2]. Firstly, pyrosequencing itself was improved with an increase in the length of rRNA sequences from 100 base pairs (bp) to more than 500 bp for long reads depending on sequencer manufacturer. This increase enabled clearer identification and quantification of common taxa [36] and broader ranging detection of minor taxa [15, 60]. With the improvement in sequencing techniques, bioinformatics tools had to be developed in parallel, such as biostatistical analyses. Various bioinformatic techniques were used in order to order and to gather RNA sequences from a biological sample such as OTU (Operational Taxon Unit) [86] or oligotyping [25, 26]. OTU's are poorly-defined clusters of sequences. Resolution is limited. It is better compared to genus, but leaves room for quite a few variations. Oligotypes are based on a mathematical procedure to discriminate between technical noise and biological (true) variation. This method provides for the best possible sequence data resolution [27]. These analyses produced equivalent results for samples that are significantly different, using standard biostatistical tools such as PLS-DA (Projection on Latent Squares – Discriminant Analyses). When there are minor differences between 2 biological samples or if we want to compare 2 time points of a unique ecosystem, biostatics has to be really precise and sensitive. Consequently some specific methods were developed in order to improve the statistical strengths of some analyses such as supervised machine-learning methods [59]. In addition to identification and quantification of taxa in biological samples, 16S rRNA sequencing can also be used to identify functional profiles for bacteria communities [45, 56, 67]. Indeed, PICRUSt technology can be used to demonstrate that phylogeny and function are sufficiently correlated to delivery functional prediction for a bacterial community from its 16S rRNA sequence [45]. Today, the resolution and the precision of taxa identification and bacteria quantification are further improved with total DNA sequencing [7]. These broad metagenomics studies also

improved the functional analyses and prediction of a bacterial community from its DNA sequence [46]. The joint analysis of data sequencing and pictures of biofilm structure will make it possible to understand biofilm community organization and function [54].

Influence of Age and Living Conditions on Oral Microbiome

These complex and useful tools were at first used to analyze and compare microbial communities in various populations. For instance, very isolated populations were studied in order to understand the development of their salivary microbiota [61]. An African hunter-gatherers tribe shares almost 2 thirds of its oral microorganisms with Western populations. In the same study, Nasidze*et al.* also compared populations sharing either geographical localization or way of life. They concluded that similar lifestyles and diet can lead to more similar oral microbiomes than habitat, although larger scale studies would be needed to further investigate these conclusions [61].

The influence of age on microbiota development was also an interesting outcome of metagenomics studies on oral bacteria, as was the influence of early nutrition and parents' oral hygiene [11, 14]. A first study demonstrated that the salivary microbiota in children from 3 to 18 years is still developing, and that longitudinal large scale studies would be preferred in order to identify early oral hygiene habits and nutritional behaviors that make for a healthy mouth [14]. The study of early oral microbiota showed that disease risk was not only related to presence of pathogenic bacteria: 10% of children with dental caries do not exhibit detectable levels of *S. mutans* for instance [1] and on the contrary, pathogenic bacteria were found in some healthy adults [14]. Delivery mode seems to have an influence as do living conditions (diet, siblings and even presence of pets) [11].

Major lifestyle changes can also significantly affect the oral microbiota. This has been seen among sailors during long sea voyages [99]. Voyages lasting more than 3 months with highly intense work, a different diet and circadian biorhythms in a humid and salty environment, induced a significant decrease in microbial diversity and in microbial metabolism [99]. This study shows that external factors can deeply impact our microbiomes. These results have to be taken into account, together with the concept of resilience. Indeed, it has also been largely demonstrated that if an ecosystem is in equilibrium state, the disturbance has to be highly significant to last a long time; otherwise the microbial system will tend to revert back to its equilibrium state. The influence of antibiotic treatments on oral microbiota has been widely investigated. The effects of various antibiotics on the ecology of both the gut and the oral microbiomes have been studied [98]. The salivary microbiome was found to be significantly more robust, whereas antibiotics negatively affected the fecal microbiome; in particular, health-associated butyrate-producing species became strongly underrepresented; understanding the mechanisms behind the resilience of the oral microbiome toward ecological collapse might have been proven useful in combating microbial dysbiosis elsewhere in the body [98]. Although large scale modifications in the oral microbiome are difficult to observe, there is great inter-individual variation in this ecosystem and even significant intra-day fluctuations, meaning that many precautions have to be taken for observational or interventional clinical trial sampling [73]. Sato *et al.* concluded that dental plaque sampling is better for preventing intra-day variations and that samples should be collected from the internal face of molars in order to ensure a sufficient quantity of DNA is collected. Nevertheless, due to oral microbiome resilience and inter-individual variations, if a study is investigating slight changes in oral microbiome following a minor disturbance, the time of collection needs to be highly specific, and control groups should be included in the study [73].

The key technical points for investigating oral microbiota development were described in this first section. Monitoring oral microbiota development is not only important in understanding the role of oral microbiota diversity in oral diseases, but it is also strongly linked to the potential influence and relationship that seems to arise between oral microbiota and systemic diseases.

Relationship between Oral Microbiota and Systemic Diseases

Plausible Mechanisms

Although the underlying mechanisms between the oral microbiome and systemic diseases have not been fully elucidated, several biologically plausible mechanisms have been put forward [32]. One of the accepted mechanisms is that oral microorganisms evade immune-mediated killing by subverting the host immune response in the oral cavity. They are then disseminated in the systemic circulation through the lesion in the oral cavity, causing bacteraemia and distant site infection [32]. Several oral microorganisms, such as *Streptococcus spp.*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans, and Prevotella intermedia*, have been identified in the blood [55], atheromatous plaque [35], and synovial fluid [55, 83], respectively. *P. gingivalis,* the keystone pathogen in periodontal diseases, is believed to be able to protect itself and other bacteria from immune-mediated killing by subverting the complement function, inactivating antimicrobial peptides, and suppressing microphage endocytosis and nitric oxide-dependent intracellular killing [32]. *F. nucleatum*, as a potential accessory pathogen, not only invades the host cell itself [33], but also could facilitate the invasion of other pathogens [34]. When these oral bacteria translocate to extra-oral sites, they are likely to stimulate the inflammatory response.

Systemic inflammation plays an important role in the pathogenesis of type 2 diabetes [21], and cardiovascular disease [50]. Triggering of systemic inflammation was put forward as an alternative mechanism for the role of oral bacterial infection in systemic disease development. Chronic exposure to the dysbiotic microbial community leads to a persistently inflammatory environment in the oral cavity. The locally-produced proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IL-6, are released into the circulation, leading to chronic inflammation, then they stimulate C-reactive protein (CRP) production by liver, further exacerbating systemic inflammation [32]. This hypothesis was supported by the observation of higher serum CRP levels in people with periodontitis compared with healthy controls, and the positive relationship between periodontal pathogens and CRP levels [63].

Another potential mechanism is changes in the composition of the gut microbial community, which has been associated with chronic diseases, such as obesity, metabolic syndrome, type 2 diabetes, and Inflammatory bowel disease (IBD) [28, 72, 75, 88]. The Human Microbiome Project (HMP) reported that the oral and gut microbial community could predict each other [18]. Using a high resolution method, Qin *et al.* compared the gut microbiome in liver cirrhosis and healthy controls [69]. Among enriched species in liver cirrhosis patients, 54% are species originating from the oral sphere and belonging to the *Streptococcus*, *Fusobacterium*, *Lactobacillus*, *Veillonella* and *Megasphaera* groups, suggesting that oral commensal flora invades the gut and becomes overgrown in patients with liver cirrhosis [69]. Animal studies have also shown that oral administration of *P. gingivalis* increases the portion of *Bacteroidetes* and decreases the portion of *Firmicutes* in the gut microbiota, and inhibits expression of epithelial tight junction protein in the small intestine [4, 57], resulting in increased gut permeability, which was associated with serum endotoxemia and systemic inflammation. *P. gingivalis* could only be detected in the jejunum and ileum 1 hour after oral administration, the quantity being decreased at 3 hours [4]. How *P. gingivalis* leads to changes in the gut microbiota needs to be identified.

Type2 Diabetes

It has been clearly demonstrated that there is a bi-directional relationship between periodontal disease and type 2 diabetes. On the one hand, the prevalence of periodontal disease is three to four-fold higher in diabetic patients than in healthy controls, which may be due to changes in the microbial community environment and host inflammatory response caused by hyperglycemia [64, 65]. On the other hand, baseline periodontal disease was positively associated with a higher risk of incident diabetes [16], and diabetic patients with periodontal disease had worse glycemic control than the diabetic patients without periodontal disease [64].

A few studies have been conducted to investigate the microbial composition of plaque in type2 diabetic patients compared with non-diabetic controls, but the results were not consistent [9, 10, 23, 37]. Using the checkerboard DNA-DNA hybridization method, Hintao*et al.* demonstrated higher *Treponemadenticola*, *Prevotellanigrescens*, *Streptococcus sanguinis*, *Streptococcus oralis* and *Streptococcus intermedius*levels in supragingival plaque samples, and more severe periodontitis, a higher plaque index and a higher prevalence and magnitude of root surface caries in type2 diabetic patients than non-diabetic controls, however, bacterial distribution in subgingival plaque was not significantly different between the two groups [37]. On the contrary, a higher prevalence of *P. gingivalis*, *A. actinomycetemcomitans*, and *Campylobacter spp.* in subgingival plaque samples was observed in Hispanic Americans with type2 diabetes [23]. Similarly, Campus *et al.* also reported a higher prevalence of *P. gingivalis* and *Tannerella forsythia* in type2 diabetics compared with non-diabetic controls using the polymerase chain reaction (PCR) [9]. With 16s RNA gene sequencing, a study in Brazil reported significant differences in subgingival microbiota when comparing type2 diabetic patients with non-diabetic controls, including higher percentages of *TM7*, *Aggregatibacter*, *Neisseria*, *Gemella*, *Eikenella*, *Selenomonas*, *Actinomyces*, *Capnocytophaga*, *Fusobacterium*, *Veillonella* and *Streptococcus* genera [10]. However, some well-established periodontal pathogens, such as *T. forsythia* and *P. gingivalis*, were more prevalent in non-diabetic controls than in type2 diabetic patients [10].

Although type 2 diabetes-related oral microbiota composition is still inconclusive, oral *P. gingivalis, F. nucleatum* and *P. intermedia* treatment-induced periodontitis led to periodontal microbiota dysbiosis, insulin resistance and glucose intolerance, which may be due to impaired adaptive immune response [6]. In addition, lipopolysaccharides from *P.gingivalis*could be a major molecular determinant responsible for *P. gingivalis*-aggravated high fat diet-induced insulin resistance [6]. Meanwhile, clinical trials also demonstrated an improvement in glycemic control in type2 diabetic patients with pre-existing periodontitis following non-surgical periodontal therapy [41, 64]. Combined non-surgical periodontal treatment and antibiotic treatments brought additional benefits [31, 64]. A meta-analysis of clinical trials reported a 0.66% decrease in HbA1c following non-surgical periodontal therapy alone and a 0.71% decrease with the addition of antimicrobials in type2 diabetic patients. However, this was notstatistically significant [41], which may be due to the small sample size.

Atherosclerosis

Atherosclerosis is a disease in which plaques build up inside the arteries, which leads to the development of cardiovascular diseases, for example myocardial infarction, stroke and coronary heart disease. The hypothesis as to the role of oral microbiota in the development of atherosclerosis is well accepted and supported by the identification of oral pathogens in the atheromatous plaques, including *A.actinomycetemcomitans, T. forsythia, P.gingivalis*, Non-c serotypes of *S.mutans*, *P. intermedia*, and *P. nigrescens [29, 35, 58]*. In addition, the abundance of *Fusobacterium*in the oral cavity was positively associated with levels of cholesterol and LDL-C levels, and *Neisseria* was negatively associated with HDL-C and ApoAI levels, while Streptococcus was positively associated with HDL-C and ApoAI levels [44].

Among these oral pathogens, *P. gingivalis* is one of the most prevalent pathogens, and some animal studies have explored the role of *P. gingivalis* in the pathogenic mechanisms of atherosclerotic diseases. Firstly, *P. gingivalis*has to avoid immune-mediated killing. *P. gingivalis*can avoid or antagonize TLR4-mediated bacterial activity in macrophages by enzymatically changing the lipid A moiety of its lipopolysaccharides [32, 78]. This also helps other TLR4-agonistic lipid A-expressing bacteria to avoid TLR4 activation [32, 78]. In the ApoE-/- mouse model, oral infection with the inert or antagonistic lipid A-expressing *P. gingivalis* could lead to vascular inflammation, macrophage infiltration and progression of atherosclerosis [78]. The oral bacteria then spread from the oral mucosa to aortic tissue. Three relocation strategies have been proposed [32]. In addition to a bacteraemic rout, *P. gingivalis* might invade recirculating macrophages and/or dendritic cells and then

direct these leukocytes to inflammatory aortic tissue, or it might bind to erythrocytes and then release into the circulation [32]. When *P. gingivalis* arrives in the aortic tissue, it invades the endothelial cells in a FimA fimbriae-dependent manner, where it increases expression of pro-inflammatory molecules [32, 81]. *P. gingivalis* lipopolysaccharides could stimulate the oxidized LDL-induced macrophage-derived foam cell formation [47, 49]. In addition, the cardiolipin-specific antibody, which could be induced by the epitopes present in *P. gingivalis* and *T.denticola* [13], is an autoantibody and cross-reacts with oxLDL [89], suggesting a molecular mimicry strategy is involved in the development of atherosclerosis. Similar molecular mimicry has also recently been reported for *A. actinomycetemcomitans* [93].

RA

RA is an autoimmune-mediated chronic inflammatory disease characterized by joint swelling, joint tenderness, destruction of synovial joints and the presence of autoantibodies such as the rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA), which is recognized as an early serological biomarker of RA, and its level is strongly associated with disease severity [3, 90]. Although extensive studies have been conducted to explore the relationship between periodontal disease and RA risk since the early 20th century, and a positive relationship has been observed, the underlying mechanisms are still unclear [74]. One hypothesis is that periodontal disease and RA may share some common risk factors, such as smoking, which is an established risk factor for both periodontal disease and RA [74]. Another is from recent research into the oral microbiota, especially the key pathogen in periodontitis, *P. gingivalis*, which provides further insight into the relationship between periodontal disease and RA. *P. gingivalis* hasaunique enzyme, Peptidyl Arginine Deiminase (PAD). The arginine gingipains, a proteinase and key virulence factor secreted by *P. gingivalis*, cleave the protein, such as bacterial protein and human fibrinogen and α-enolase, resulting in a peptide with carboxy-terminal arginine, which is then citrullinated by *P. gingivalis*-derived PAD, and subsequently generatesneoepitopes [95]. This potential mechanism could trigger autoantibody production (i.e. ACPAs), which could form immune complexes with the citrullinated host protein and exacerbate inflammation in RA [32]. Infection of mice with wild type *P. gingivalis* significantly increased levels of autoantibodies to collagen type II and citrullinated epitopes, and this effect depended on the expression of *P. gingivalis*-derived PAD [53].

In human studies, the DNA of periodontal bacteria, *P. intermedia*, *P. gingivalis*, *F. nucleatum* and *S. proteamaculans*, was detected in synovial fluid of patients with RA and periodontal disease [55, 83]. In addition, *Anaeroglobusgerminatus, Prevotella, Leptotrichia*and *Tannerella* species have been associated with either RA-related autoantibodies or early-onset RA [74]. Due to the limited sample size of previous studies, these findings need to be replicated in larger population studies, and the hypothesis that there is synergistic interaction among these bacterial species requires further exploration.

IBD

IBD groups a number of conditions leading to chronic inflammation in the gastrointestinal (GI) tract, mainly ulcerative colitis (UC) and Crohn's disease (CD). Extensive evidence suggests that dysbiotic gut microbiota is involved in the pathogenesis of IBD [28, 72]. As mentioned above, oral microbiota is believed to be able to change the composition of the gut microbiota. Therefore, it is not surprising to observe an association between the oral microbiota and IBD.

The specific oral *Campylobacter concisus* strain, enteric invasive *C. concisus* (EICC) strain, was detected in 50% IBD patients, but not in control patients, although this difference was not significant [40]. Lesser microbial diversity in tongue swab samples was observed in the CD patients, but not in UC patients. This was mainly attributed to the loss of *Fusobacteria* and *Firmicutes*, and to an increase in *Spirochaetes*, *Synergistetes* and *Bacteroidetes* [19]. Similarly, an increase in *Bacteroidetes* and decrease in *Proteobacteria* in the saliva samples of IBD patients was also reported in a recent study, and the results showed that *Streptococcus*, *Prevotella*, *Neisseria*, *Haemophilus*,

Veillonella, and *Gemella* contributed to the dysbiosis of salivary microbiota in IBD patients [71]. There are only few animal studies exploring the underlying mechanism. In a mouse model of dextran sodium sulfate-induced colitis, intravenous administration of the highly-virulent *S.mutans* strain isolated from UC patients, but not the standard *S. mutans* strain,was seen to aggravate colitis [43]. In keeping with this finding, *S. mutans* could also colonize in the hepatocytes of liversin the colitis mice, and increased expression of interferon-Ɣ in the liver was observed [74].

The relationship between oral microbiota and systemic health has been illustrated through various examples. An attempt will be made to understand how our oral microbiota could be influenced in the next part of this review.

How to Modulate Our Oral Microbiome?

Disease-Mediated Modulation

As previously described, the oral bacteria ecosystem is extremely strong and difficult to modulate whether positively or negatively. Chronic diseases are nevertheless able to do this. If we focus on the oral domain, it has been demonstrated that even a few volunteers were sufficient to discriminate Chinese adults with or without gingivitis [39]. A number of organisms were strongly associated with the gingivitis phenotype such as *Leptotrichia* and *Selomonas,* which could constitute good biomarkers for onset of gingivitis [39]. It is known that the bacterial biofilm is able to recover rapidly after professional prophylaxis [82], the order of bacteria succession regrowth being of importance for the "health status" of the future microbiome. The prevalence of species strongly correlated with periodontal pathogenesis was decreased during early subgingival recolonization. Indeed, recolonization order was the same in periodontal health and disease, suggesting that many factors have to be taken into account in order to orient the redeveloping biofilm towards "healthy" or "sick" status. Consequently, it seems possible to promote beneficial microbiome regrowth by clearly understanding the mechanisms that control the succession of bacterial recolonization [82]. Larger studies were performed to characterize the microbiota linked to periodontitis. The analyses in various countries identifiedthe same bacterial strains. *Prevotella*, *Porphyromonas*, and *Treponema* were mainly linked to this infectious state [87]. The technological improvements in pyrosequencing and the increasing power of biostatics made it possible to develop functional analyses for the bacteria identified. Further analysis of these bacteria linked to chronic periodontitis was useful in shedding light on functional genes and metabolic pathways that are particularly activated during acute disease phases [94]. These particular pathways were involved in bacterial chemotaxis, flagellar assembly and toxin biosynthesis.

One of the most frequently studied oral diseases remains dental caries. Conventional techniques were useful to identify and characterize some bacteria particularly linked to tooth decay such as *S. mutans* and *Lactobacillus* spp. [1]. Pyrosequencing enabled the identification and quantification of more strains present in healthy volunteers or in volunteers with active caries. Four different phyla were especially linked to dental caries such as *Bacteroidetes*, *Firmicutes*, *Fusobacteria* and *Proteobacteria* [97]. However, the non-systematic pathogenic nature of *S. mutans*was also revealed [14]. Disease is not linked to ONE bacterial presence but to multifactorial causes [92]. Consequently the microbial interaction between bacteria, and their way of interacting with external factors is mandatory to understand why they become pathogenic or why they remain harmless.

A harmless but socially deleterious disease has also been widely studied in order to be countered; indeed halitosis can be a genuinely embarrassing and chronic disease. Halitosis was linked to the presence of specific bacteria in saliva in Chinese children [70]. 16S rRNA gene pyrosequencing and metagenomic sequencing were used to examine oral microbial composition and its functional variations in children with halitosis. The tongue coating of subjects with halitosis was morebacteria-rich than that of healthy subjects. The relative abundance and prevalence of *Leptotrichiawadei* and *Peptostreptococcus stomatis* were higher in tongue coating samples

from children with halitosis as was *Prevotellashahii* in their saliva. It seems that tongue and saliva communities did not follow the same development in spite of their geographical proximity. Moreover, functional interpretation of 16S sequencing places the emphasis on the correlation between the presence of genes involved in polyketide metabolism and in hydrogen sulfide-related metabolic pathways with halitosis, suggesting that there was higher microbial production and less usage of H_2S in subjects with halitosis [70].

Modulation Induced by Exogenous Factors

If we consider the various external factors likely to have an effect on dental health, we have to consider cigarette smoking. It is already known that nicotine may have a strong impact on the growth of bacteria involved in tooth decay [38, 48]. In addition, an observational study looked at the oral microbiota of smokers among American adults [96]. Overall oral microbiome composition differed between former smokers and current non-smokers based on the relative abundance of some taxa and genera (*Proteobacteria*, *Capnocytophaga*, *Peptostreptococcus* and *Leptotrichia* were depleted, while *Atopobium* and *Streptococcus* were enriched during smoking) [96]. Functional analysis of the sequencing showed that bacterial genera depletion by smoking was related to carbohydrate and energy metabolism, and to xenobiotic metabolism [96].

We have demonstrated, using several examples, that oral microbiota could be negatively modulated by exogenous and endogenous factors. The challenge to be faced now is to positively modulate this microbiome in order to keep our mouth and our whole body in general healthy condition using nutritional vectors.

The effects of nutritional supplementation on oral microbiota have been widely studied, but mainly using traditional microbiology techniques. Many plant extracts were tested on bacterial strains associated with dental caries, mainly *S. mutans*. Where effects were observed, these extracts were considered to be bacteriostatic and anti-cariogenic. Various administration methods such as mouth rinses, candies and chewing-gums were used in interventional clinical trials or simple extracts in *in-vitro* studies [8, 66, 68].

Some preliminary studies were conducted in order to influence mouth microbiota using bacteria known to be probiotic. Indeed, dental diseases can be considered as microbial imbalances resulting from a shift from healthy oral microbiota to dysbiosis, and a shift towards communities which are dominated by acidogenic and acid-tolerant gram-positive bacteria in the case of tooth decay [5]. It has been believed that probiotic bacteria such as *Lactobacillus* or *Bifidobacterium* could be effective in restoring balance to the oral microbiota, thus preventing dental caries or inflammatory oral diseases such as gingivitis or periodontitis. The putative beneficial effects of probiotics on bad breath have also been evaluated, but further evidence is needed to fully explore the potential of probiotics for preventing bad breath [5]. Supplementation using *L. reuteri* was administered to healthy subjects for 12 weeks [77]. It was demonstrated that the supplementation was useful in implementing this probiotic in the saliva microbiota during the intervention, however *L. reuteri* was washed-out at the end of the study. Consequently, modulation of oral microbiota using probiotics was only temporary and did not last, showing once again the resilience of healthy oral microbiota [77]. Another attempt was made in a longitudinal analysis of oral and more particularly salivary microbiome, where volunteers received supplementation in the form ofa commercial probiotic product containing milk fermented with *S. thermophilus*, *L. bulgaricus* and *L. paracasei* [15]. A significant change in microbial community was observed mainly on *Streptococcus* and *Actinomyces* genera. The presence of the *Lactobacillus* strain administered was not detected, whichsupports the hypothesis that colonization is not a requisite for a modulation of the microbiota. A microbial ecosystem can be influenced without intrusion [15]. Nevertheless, this supplementation was acute and the long lasting effect on microbiota was not investigated. Consequently, we may doubt that this modulation is persistent. From these studies on probiotic supplementation, we learned that salivary microbiome modulations are not easy to observe and to characterize. Protocols need to be highly specific, as do sampling and analyses. It should also be noted that dental plaque microbiota is more sensitive to shifts than the salivary ecosystem.

Interactions between food products and microbiota have been widely studied, with a special focus on the gut microbiome and fiber interaction [76]. Concerning the oral flora, a close look was taken on the health effects of sugar-free confectionary components such as polyols. Indeed, sugar alcohols are known to have beneficial effects on oral health in that they are poorly or not metabolized by oral bacteria and that they enhance saliva [51, 52, 84, 85, 91]. They showed positive effects on remineralization [20, 22] and gingivitis [42]. Their inhibitory effects on oral bacteria were also tested *in-vivo* and *in-vitro* [24, 30, 80]. Xylitol is the most frequently studied polyol as much *in-vivo* as *in-vitro*, using various vectors and doses [62]. There are fewer publications on overall modulation of oral microbiotaby food products than on the gut microbiota. The scientific community has focused on the impact of diseases or of drugs on this specific ecosystem but not of food components. The only preliminary study for the time being was conducted by Soderling *et al.* [79] and did not conclude on any effect from xylitol gum supplementation on salivary microbiota in children. Nevertheless, the choice was made to study the salivary microbiome, whereas it is now known that microflora modulations are more difficult to observe in saliva during interventional studies than in dental plaque. For short-term exposure, only 5 weeks' consumption, the most sensitive ecosystem should have been chosen. Moreover, the control group included children chewing sorbitol gum which could also impact oral microbiota, as it is the same kind of molecule as xylitol. Consequently, a more inert placebo could have made it possible to see some changes in the microbiota, even for 5 weeks' exposure. Compared with other clinical interventions on gums, 6g of xylitol per day is a commonly used dosage that has already demonstrated significant effects, and compared with other metagenomics study in dental health, the statistical power should have been sufficient, n = 35 to 38 per group, for observing biologically-relevant modulation in the oral microbiota.

To study the impact of food products on the oral microbiome, as we expect slight modulations due to the resilience of this ecosystem, it would be preferable to work on large standardized populations with an accurate placebo control, modern high-throughput technology, and strong biostatistics tools with a specific focus on dental plaque microbiota. The sampling procedure and timing also need to be really appropriated.

Conclusion

It has been shown that investigating changes in the oral microbiota can actually be very difficult due to interindividual and even intraday variations. Consequently, clinical methodologies have to be firmly established and the latesthigh-throughput metagenomics, bioinformatics and statistics toolsshould be used. There is growing evidence to suggest a relationship between the oral microbiota especially periodontal disease bacteria, and systemic diseases. However, the sample size of most of these studies was too small to be able to come to a conclusion. More large longitudinal studies and clinical trials are needed. In addition, whether the dysbiosis of oral microbiota is a cause or a consequence of systemic diseases needs to be clarified. To explore etiopathogenesis, multiple "omics" methods, like metagenomics, metatranscriptomics, and metaproteomics, could be applied. If the causal relationship is confirmed, correcting the dysbiotic oral microbiota will not only be a means of preventing and controlling oral diseases, but will also provide additional benefitsin the treatment of systemic diseases.

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