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Review

The oralome and its dysbiosis: New insights into oral microbiome-host interactions

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ABSTRACT

The oralome is the summary of the dynamic interactions orchestrated between the ecological community of oral microorganisms (comprised of up to approximately 1000 species of bacteria, fungi, viruses, archaea and protozoa - the oral microbiome) that live in the oral cavity and the host. These microorganisms form a complex ecosystem that thrive in the dynamic oral environment in a symbiotic relationship with the human host. However, the microbial composition is significantly affected by interspecies and host-microbial interactions, which in turn, can impact the health and disease status of the host. In this review, we discuss the composition of the oralome and inter-species and host-microbial interactions that take place in the oral cavity and examine how these interactions change from healthy (eubiotic) to disease (dysbiotic) states. We further discuss the dysbiotic signatures associated with periodontitis and caries and their sequalae, (e.g., tooth/bone loss and pulpitis), and the systemic diseases associated with these oral diseases, such as infective endocarditis, atherosclerosis, diabetes, Alzheimer's disease and head and neck/oral cancer. We then discuss current computational techniques to assess dysbiotic oral microbiome changes. Lastly, we discuss current and novel techniques for modulation of the dysbiotic oral microbiome that may help in disease prevention and treatment, including standard hygiene methods, prebiotics, probiotics, use of nano-sized drug delivery systems (nano-DDS), extracellular polymeric matrix (EPM) disruption, and host response modulators.

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1. Introduction

More than three centuries ago, Antony van Leeuwenhoek was the first person to observe microbes, possibly bacteria, from his own dental plaque with the use a microscope that he constructed [1]; thereby laying down the foundations of microbiology. Since then, new discoveries have highlighted that these microbes interact with their human host. A recent estimate shows that 3.8×10^{13} microorganisms colonize the human body, accounting for about half of the total cells on a human body [2]. In aggregate, these organisms are known as the human microbiome [3,4].

2. Microbial habitats in the human body

The NIH Human Microbiome Project (HMP), which was launched in 2007, comprised 18 different studies to map and characterize the human microbiome and its role in human health and disease. It is well appreciated that the human microbiome is present in nearly every human body site [5], and the HMP has established, thus far, that there are 48 main microbial habitats in the human body [6]. According to the HMP [6], 34% of all primary microbial habitats were associated with human skin, 25% of all habitats were related to the gastrointestinal tract, and 20% of all habitats were associated with cavities of the head and neck region.

Among these main human habitats, the oral cavity is a challenging environment for microbial survival, since it undergoes high daily fluctuations in nutrient supply, temperature, pH, sheer and mechanical forces from mastication and hygiene practices, and chemical exposure from hygiene, pharmaceutical or toxic/smoking products [7]. Yet it retains a rich and complex ecosystem, harboring different micro-colonizers that thrive in this dynamic environment – the oral microbiome [8]

3. The oral microbiome and the oralome

The oral microbiome is defined as a community of microorganisms of up to 1000 total microbial species, comprising bacteria, fungi, viruses, archaea and protozoa that live in the oral cavity [1,9–12]. A bacterial predominance in the oral microbiome has hindered and limited the term "core microbiome" to that of a bacterial microbiome [9]. Thus, most reports usually focus only on the oral bacteria within the microbiome, while other oral biomes (i.e. fungi, archaea, protozoa and the viral biomes) receive much less attention. Although there is limited literature on these other biomes, they remain relevant [1,9,13,14]. Thus, we will discuss each of these biomes in the context of the oral cavity.

3.1. Oral bacterial microbiome – the bacteriome

Due to changes in shear forces, nutrient/energy supply, temperature, pH, and oxygen content in different environments, bacteria have evolved by leveraging specific survival strategies, including co-aggregation of different cells into communities embedded in extracellular matrices –biofilms [10]. Despite the existence of planktonic bacteria in the oral cavity, most of the oral microbiome exists in a biofilm state, known as the oral biofilm.

3.1.1. The oral bacterial biofilm

Biofilms have been defined as a structured community of aggregated bacterial cells (either from the same species or multi-species) embedded and enclosed in a self-produced extracellular polymeric matrix (EPM) and adherent to an inert or living surface [15-18]. Specifically, for oral bacterial biofilms (commonly referred to as "oral biofilms"), features of the oral cavity have shaped bacterial communities to adapt to high cell density, which, in turn create a micro-environment capable of modulating the pH, redox, and oxygen levels in its core [7,16,19].

Oral biofilms are formed by an initial adhesion of planktonic bacteria, known as "early colonizers" [20]. Typically, these early colonizers are saccharolytic aerobes and facultative anaerobes that primarily feed on oral glycoproteins and salivary mucins, with 80% of the early colonizers being represented by *Streptococcus* species [7,20,21]. Surface colonization occurs by bacterial attachment to an oral surface (e.g. dental surface) via specific surface adhesins [7]. After the initial colonization, the surface-attached bacteria change their metabolic and gene expression profiles to produce and secrete EPMs, which, for oral biofilms, is made up of polysaccharides, proteins, lipids and extracellular DNA (eDNA) [7,22-26]. Interestingly, different microorganisms have been shown to depend on eDNA to form biofilms in monocultures, which may indicate that deoxyribonucleases (DNases) can be used to disrupt and control biofilm growth [26-28]. Production and secretion of

EPMs provides several advantages to the oral microbiota, such as enhanced nutrient availability and protection from environmental stresses [29]. For instance, cells embedded in biofilm matrices are up to 1000-fold more tolerant to antibiotics compared to their planktonic counterparts, and therefore more difficult to disperse [30].

Overall, the oral bacterial biofilm experiences different levels of oxygenation within its own structure [31–34]. For instance, analyzing oxygenation levels of *ex vivo* supragingival plaque biofilms (~300 μm thickness), von Ohle *et al.* [34] found anaerobic conditions (<0.5% oxygen [35]) in layers deeper than 220 μm. In this context, *ex vivo* biofilms exhibit beneficial conditions for the growth of different microbes that thrive under different levels of oxygenation. Thus, within these biofilm structures, aero-tolerant taxa are present on the exterior, whereas the interior anaerobic compartments of the biofilm provide the environmental conditions for attachment and growth of proteolytic obligate anaerobes, including methanogens and sulfate-reducers, also known as "late colonizers" [7,20,21]. This oxygenation gradient also suggests the existence of potential nutrient gradients [33].

Using oligotyping analysis, Eren *et al.* [36] demonstrated that oral bacterial biofilm genera have different distribution/abundance relative to the three distinct habitat zones - dental plaque, tongue dorsum, and keratinized gingiva. This finding led to the site-specialist hypothesis for oral bacterial biofilms, which predicts that a particular strain will actively find its preferential growth site (e.g., dental plaque, tongue dorsum, and keratinized gingiva) and thereby thrive [33]. Further discussing this hypothesis, Mark Welch *et al.* [33] point out that some genera can be either generalists, such as the *Veillonella* genus (e.g. *Veillonella atypica* seems to be a tongue dorsum specialist, *Veillonella parvula/dispar 2* seems to be a dental plaque specialist, and *Veillonella rogosae* seems to be a keratinized gingiva specialist) or specialists that strongly specialize to one site, such as *Capnocytophaga* and Corynebacterium genera that are specialized to dental plaque sites.

In general, bacterial biofilms display a mushroom-shaped, whereas under turbulent flow, biofilms display a more elongated structure that is capable of rapid oscillations [15,37,38]. However, recent studies on dental plaque and togue dorsum biofilms demonstrated different shapes.

For supragingival biofilms, the "corncob" structure - long filamentous structures formed by *Corynebacterium* and/or actinomyces coated with cocci at the edges - has been reported for over 40 years [33,39]. Recently, Mark Welch *et al.* [32] demonstrated the "hedgehog" structure, in which, filaments of *Corynebacterium* spp. radiate outward from the dental surface, forming a structured habitat inhabited by other taxa at specific positions. For example, *Streptococcus* spp. occupy an outer shell forming the "corncob" structure and they share the habitat with *Porphyromonas* spp. and *Haemophilus* spp. or *Aggregatibacter* spp. Microaerophilic taxa, such as *Fusobacterium* spp. and *Leptotrichia* spp., on the other hand, possibly occupy the anaerobic layers close to the base of the structure.

For subgingival biofilms, four different layers were found by Zijnge *et al.* [40]. The basal layer (close to the dental surface) was formed by *Actinomyces* spp. and other unidentified species (due to low fluorescence outcome), followed by a second layer comprised of spindle-like bacteria, such as *F. nucleatum* and *Tannarella spp*. The third layer was formed by filamentous, rod-shaped and coccoid-shaped cells belonging to the *Cytophaga-Flavobacterium-B acteroides* cluster. Finally, on the top of the biofilm, the authors found a 'palisade'-like lining layer, which was in close contact with eukaryotic cells. Unfortunately, subgingival biofilms are not easily analyzed without the loss of structural integrity [40]. Thus, more studies using novel strategies are still necessary to better understand the structure of subgingival plaque biofilms and to specifically unveil how shear forces and different flow parameters in the mouth shape both the supragingival and subgingival dental plaque bacterial biofilm structure.

For the tongue dorsum, Mark Welch *et al.* [33] found a third different structure, but with similar degree of organization as the other two structures. The human epithelial tissue occupied the central core of the biofilm followed by a layer of *Actinomyces* spp.. *Streptococcus spp.* were located in the exterior layer and in stripes in the interior of the biofilm. Other taxa, such as *Rothia spp*., *Neisseria spp*., *Veillonella spp*. were present in clusters and stripes in the interior of the biofilm as well, suggesting that the biofilm grew outwardly from the central core.

Unfortunately, we were unable to find any articles describing biofilm structures on keratinized gingiva; thus, more studies are required in the field. For more details on the biogeography of oral bacterial biofilms and the site-specialist hypothesis, please refer to Mark Welch *et al.* [32,33].

Approximately 700 bacterial species have been identified in the oral cavity [7,41,42], making the oral cavity the second largest bacterial community in the human body, after the gut [1]. Aas *et al.* [41] detected 141 bacterial species in the oral cavity; among them, the most common species belonged to the *Gemella, Granulicatella, Streptococcus, and Veillonella* genera.

3.2. Oral viral biome – the virobiome/virome

Human oral viral biome, also known as the "oral virome" or "oral virobiome", is a highly conserved [43] and highly personalized community; to such a degree that its composition can vary depending on the host's sex [44]. The vast majority of viruses in the oral cavity are bacteriophages [44–46], which exhibit a very stable lytic/lysogenic cycle. This lytic process has the potential to exterminate bacterial species in the community or impart new functions on the oral bacteria, and thereby completely change these human bacterial communities [44]. Some of these bacteriophages are associated with *Veillonella spp*. and *Streptococcus* spp., two of the main commensal bacterial genera in the oral cavity [44].

In healthy patients, the main bacteriophage families found in the oral cavity were *Siphoviridae, Myoviridae,* and *Podoviridae*; all belonging to the *Caudovirus* order [45,46]. *Myoviridae* and *Podoviridae* are predominantly lytic viruses that rapidly eliminate their bacterial hosts, while *Siphoviridae* are largely lysogenic viruses, establishing a dynamic equilibrium with their associated host species [47]. Interestingly, this suggests that the oral virome may play a big role in controlling the bacterial population in the oral cavity, since this bacteriophage dominance in the virome seems to be correlated with the bacterial dominance of the oral microbiome, and phages are thought to account for 20–80% of total bacterial death; thus, representing a profound bacterial growth limiting factor. Also, in 2018, de la Cruz Peña [48] found eight new viruses that are naturally abundant in saliva of healthy patients, such as the unculturable virus 92-C13, which seems to belong to the *Caudovirales* order as well.

So far, eukaryotic viruses, such as *Herpesviridae*, *Papillomaviridae*, and *Anelloviridae* are among the most common eukaryotic virus families present in healthy patients. Among them, *Human Papillomavirus* (*HPV*), Human cytomegalovirus (CMV), Herpes simplex virus type-1 (HSV-1), and e *Epstein-Barr Virus* (EBV), have been found in asymptomatic healthy individuals [47].

However, only few studies have evaluated the oral viral community [43–45,48,49] and, thus, more studies are still necessary to evaluate the role of the virome in the oral microbiome.

3.3. Oral fungal microbiome - the mycobiome

The human oral cavity is also colonized by fungi/yeast species [50]. Ghannoum *el al.* [13], in 2010, first described the "basal oral

mycobiome". The authors found a total of 101 oral fungal species, among 74 culturable- and 11 nonculturable-genera. Among those identified, three (i.e. *Aspergillus*, *Fusarium* and *Cryptococcus*) are known to be pathogenic in humans and are not known to be oral colonizers. The authors proposed that the pathogenicity of these fungal species might be controlled by other commensal oral fungi.

Following this study, Dupuy *et al* [14], in 2014, identified 5 additional fungal genera in the oral mycobiome. Among those, the *Malassezia* genera has been described as a skin commensal and an opportunistic pathogen associated with scalp disorders [51] and, now, is being considered a predominant member of the commensal "basal oral mycobiome" [52,53]. Recent research by Peters *et al* [54], in 2017, increased the total number of commensal fungal species to 154.

The most common commensal fungi and members of the basal oral mycobiome are the *Candida* genera [55], which are found in 70% of healthy patients [13] as confirmed by Ghannoum *el al.* [13] and Peters *et al* [54]. The most abundant species in this genera is *C. albicans*, which is found in 40–80% of healthy individuals, followed by *C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. stellatoidea, C. kefyr, C. khmerensis* and *C. metapsilosis* [55,56].

C. albicans and C. glabrata are also important pathogens under certain conditions. The difference between commensalism and pathogenicity for *C. albicans* seems to be the result of a fine balance between fungal virulence and host defense mechanisms, and *C. albicans* is one of the most prevalent pathogens in mucosal and systemic fungal infections [57,58]. In contrast, *C. glabrata*, which has been historically considered as a commensal saprophyte, has been reported as an opportunistic oral pathogen. Specifically, it has been identified as a co-infecting agent along with *C. albicans* in oral candidiasis in immunocompromised populations, and these co-infections tend to be more severe and more difficult to treat than single infections of *C. albicans* [58–60].

However, little is still known about fungal colonization succession and fungal biogeography in the oral cavity [50,53] and their role in host health and disease. Also, further evaluation is still required to determine whether the species found so far are indeed functional residents or mere transient species [53]. Diaz *et al.* [53] recommend large longitudinal studies that include seasonal changes to help separate transient environmental fungi from permanent residents. For more details on the current and future perspectives on the oral mycobiome, please refer to Diaz *et al.* [53].

3.4. Oral protozoa biome – the protozoome

Historically, protozoa have been considered parasitic and assumed to have detrimental effects on the host [61]. However, a recent analysis revealed the presence of more than 15 known commensal protozoa genera in the human intestinal tract [62]. In the oral cavity, however, their roles are still unclear. Kofoid [63], in 1928, reported *Trichomonas tenax* and *Entamoeba gingivalis* as mouth parasites. However, in 1956, preliminary studies found these oral species frequently rated as "clean and healthy". *T. tenax* and *E. gingivalis* have been found in increased numbers in subjects with poor oral hygiene [21,64] and in patients with gingivitis and periodontal disease [65–68]. Recently, *T. tenax* has also been associated with *P. gingivalis*, *T. denticola and Eubacterium nodatum* [68]. However, these results have recently been explained as related to protozoal nutrient factors, and with no impact on the host's health. Specifically, it's thought that poor oral hygiene and periodontitis increase protozoal nutrient sources - the amount of bacteria and food debris in the mouth [21,69], and, thus, it is reasonable to find them associated with proteolytic bacteria. Currently, these species are regarded as harmless saprophytes and part of the oral microbiome [21,65,69].

3.5. Oral archaea biome – the archaeome

Archaea are organisms that are morphologically similar to bacteria but they share more molecular features with eukaryotes and they comprise the third domain of life [70,71]. Some archaea species, classified as methanogenic, produce methane from carbon dioxide as an energy source for growth [72]. Five different methanogenic archaea genera have been identified in the oral cavity of healthy patients - *Methanobrevibacter, Methanosphaera, Methanosarcina, Thermoplasmata* and *Methanobacterium* [73]. Among these, *Methanobrevibacter oralis, Methanobacterium congelense/curvum* and *Methanosarcina mazeii* are the main archaea species found in healthy patients [73–77], with *M. oralis* dominating over other species at a prevalence of 40% [72,76,78]. Archaea are members of the oral microbiome, however, they are considered less abundant and diverse than oral bacteria [79]. More studies are needed to evaluate archaea's role in the oral microbiome of healthy patients.

Archaea species have also been implicated in oral diseases, as they are known to form biofilms, and can interact with the human immune system [80]. Several studies identified increased numbers of *M. oralis* in periodontitis, *peri*-implantitis and root canal necrosis cases [73,75,76,78,81], possibly implicating the species in those diseases. Further, studies have demonstrated that archaea coexist with periodontal pathogens, such as *Treponema denticola*, *Tannarella forsythia* and *Porphyromonas gingivalis* [72,75,78]. These findings suggest a possible role for archaea as terminal degraders of host components, favoring the continuation of the catabolic cascade [72,79]. However, their specific role in disease pathogenesis remains unclear and more studies are necessary to evaluate archaea's pathogenic potential. For more details on the oral archaeome, please refer to Belmok *et al.* [79]

3.6. Interspecies interactions

All of these micro-organisms live in close proximity, forming a complex relationship among them. These relationships establish a unique microbiome, known as the oral microbiome [82]. This close-proximity results in a wide range of interspecies interactions, which can be categorized into synergistic, signaling, or antagonistic interactions [19,82,83], as shown in Fig. 1.

Oral microorganisms have a natural tendency to adhere to other microbes, facilitating the formation of multi-species biofilms [82,83]. This process, called coadhesion, significantly changes the gene expression of both cells involved, usually enhancing biofilm formation [82,84]. For instance, yeasts, such as *C. albicans*, can also coaggregate with oral streptococci, forming a synergistic partnership in which the bacteria enhance *C. albicans's* invasive properties, while the yeast enhances streptococci biofilm formation [85,86].

These coaggregated cells and the EPM around them obstruct the free movement of molecules, slowing down the diffusion of nutritional factors and oxygen, resulting in nutritional gradients throughout the biofilm [83]. Interestingly, these nutritional gradients also promote nutritional interactions when the metabolic byproducts of one organism become the food source for another organism. These interactions can even develop whole food webs in the biofilm, resulting in efficient and complete catabolism of complex molecules, such as glycoproteins, to their simplest metabolic end-products, such as CO_2, CH_4 and H_2S [82]. Another functional consequence of the coaggregation is the protection of facultative and oxygen-tolerant anaerobes in anoxic pockets inside of the biofilm that result from neighboring oxygen-consuming species [31,82].

Horizontal gene transfer (HGT) is a process that many bacteria use to gain access to novel genes, enabling them to acquire new traits, such as virulence factors and antibiotic resistance [87]. There are three canonical mechanisms that bacteria use to transfer genes

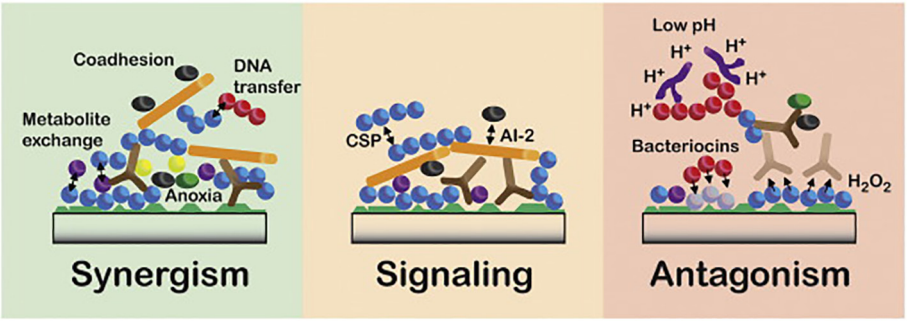

Fig. 1. Interactions between microorganisms that may drive the community assembly of oral biofilms. Different interactions are designated as having a primary function in synergy, signaling or antagonism. However, in many cases, components may have multiple roles with both positive and negative impacts depending on the situation. Reprinted from Jakubovics [83].

to other individuals – conjugation, transformation, and transduction [88–90]. Briefly, conjugation is the direct transfer of DNA through cell surface pili or adhesins between two cells, in a process requiring cell-to-cell contact; transformation is the uptake and incorporation of free extracellular fragments of genetic material; and transduction is the gene transfer via bacteriophages [89,90]. HGT is a common occurrence in the oral microbiome [90–92]. For instance, it has been revealed that *S. mutans* cells transform 10- to 600-fold more in biofilms than in their planktonic state [87]. Also, Tribble *et al.* [93] revealed the natural competence for transformation by several *Porphyromonas gingivalis* strains, which plays a major role in the overall survival and persistence of the species in the oral cavity, with conjugation playing a minor role. Besides, Chi *et al.* [94] demonstrated that regions of the pbp2x gene are shared between *Streptococcus pneumoniae* and other oral streptococci, such as *Streptococcus oralis* and *Streptococcus mitis*, thereby increasing penicillin resistance of these species. This finding indicates that there is both intra-species and interspecies HGT in the oral cavity.

Regarding signaling interactions, there are two major *quorum sensing* signaling systems in the oral microbiome – Autoinducer-2 (AI-2) and the competence-stimulating peptide (CSP).

Autoinducers are a group of molecules capable of eliciting phenotypic changes in bacteria. These molecules are produced by many bacterial species and they accumulate in the extracellular environment until a critical concentration threshold for detection is reached, after which downstream signaling and effector responses develop [95]. These molecules can induce bacterial group behaviors, known as bacterial *quorum sensing* [95]. Among these molecules, AI-2, a molecule derived from (S)-4,5-dihydroxypen tane-2,3-dione and synthesized by the enzyme LuxS, appears widespread among prokaryotes. It is produced by over 50% of all sequenced bacterial species, including both Gram-positive and Gram-negative, so it has been described as the "common-language" among bacterial species [82,95,96]. In the oral cavity, AI-2 seems to regulate several phenotypes, including virulence and biofilm formation [96,97]. Intriguingly, this molecule seems to induce biofilm formation in some species, whereas, in others, it inhibits formation. For instance, knocking out the AI-2 gene from *S. oralis* prevents *Actinomyces naeslundii* biofilm formation, whereas purified AI-2 from *Veillonella tobetsuensis* prevents *Streptococcus gordonii* biofilm formation [98,99]. Further, Jang *et al.* [100] reported that AI-2 produced by a strain of *Fusobacterium nucleatum* had very different outcomes in two different species of the same genera – it enhanced biofilm formation in *S. gordonii* but inhibited biofilm formation in *S. oralis*. These data indicate that there are differences not only in the species that release AI-2 molecules, but also in how the molecules are perceived by each species. Remarkably, AI-2 may even participate in inter-kingdom communication, since the molecule is able to regulate fungal morphogenesis, germination, apoptosis, biofilm development, and pathogenicity [96]. For instance, Bachtiar *et al.* [101] demonstrated that AI-2 produced by *Aggregatibacter actinomycetemcomitans* inhibits both hyphae and biofilm formation of *C. Albicans.* Despite many studies on AI-2, it is still poorly understood and thus further studies are needed to better elucidated its biology [83,96].

Another *quorum sensing* system is based on the competence-stimulating peptide (CSP) molecule. CSP is a peptide encoded by the comC gene. CSP is expressed then cleaved and exported out of the bacterial cell by the ABC transporter ComAB. Once CSP concentration reaches a certain threshold, the molecule binds to and activates the transmembrane histidine kinase receptor ComD. The activated ComD receptor can alter several bacterial transcription factors and protein synthesis related to biofilm formation, cell competence, bacteriocin synthesis, stress resistance, and autolysis [82,102]. In the oral cavity, some streptococci can inhibit *S. mutans* biofilm formation by inactivating *S. mutans* CSP [103,104]. Interestingly, CSP was found to be ubiquitous in streptococci, and important for controlling the acquisition of genetic material from the environment, biofilm formation, bacteriocin production, and, in pathogenic streptococci, virulence factor production [102]. Recently, Nagasawa *et al.* [105] demonstrated that CSP may be involved into releasing eDNA in *S. mutans* biofilms, contributing to firmer and more stable *S. mutans* biofilms. Counterintuitively, *S. gordonii* mutants, lacking the comC gene, produced more eDNA and more stable biofilms in the presence of *C. albicans* than the isogenic wild-type counterpart [106]. Thus, the regulatory pathways underpinning these interactions are far from clear [83],

In terms of antagonistic interactions, microbes use bacteriocins or hydrogen peroxide (H_2O_2) or they decrease a niche's pH to gain a competitive advantage or even create a selective pressure on other species [82,83].

Bacteriocins are part of a larger group of molecules named antimicrobial peptides (AMP). AMPs are peptides consisting of 12–100 amino acids that are synthesized by almost every form of life on earth (from bacteria to amphibians and humans) and they are released extracellularly to inhibit the growth of microbes [107]. Bacteriocins are AMPs produced by both gram-positive and gram-negative bacteria to kill or inhibit the growth of other prokaryotes, especially different bacterial strains in the same environment, thereby conferring to the bacteriocin-producing bacteria a competitive survival advantage over other strains in the same niche [82,107].

Similarly, hydrogen peroxide (H_2O_2) can be produced to kill or inhibit the growth of other species. The deleterious effects of H_2O_2 on bacterial cells arises from the generation of hydroxyl radicals in the presence of Fe(II). This leads to a reaction of the hydroxyl radicals with other molecules, resulting in cellular damage by degradation of iron-sulfur clusters on enzymes, leading to their inactivation, or by oxidation of macromolecules, including

DNA and proteins [108]. Certain oral Streptococci species can produce H_2O_2 at concentrations that inhibit the growth of a range of gram-positive bacteria *in vitro* and under aerobic conditions [82,83,108,109]. Despite hydrogen peroxide being one of the most well studied agents in dental biofilms, its impact on the oral microbiota is complex and difficult to predict [82].

Sugar-fermenting species can create an antagonistic environment for other strains by decreasing the local pH, thus selecting strains that are either tolerant to low pH (aciduric) or even acid-producing (acidogenic) bacteria [83,110]. Nevertheless, several commensal species of the oral cavity are well adapted to live in low pH microenvironments [83].

For more details on oral microbiome interspecies interactions, please refer to Bowen *et al.* [19], Jakubovics [83], and Marsh & Zaura [82]. The oral microbiome also forms a close symbiotic relationship with human host cells in the oral cavity and this will be discussed in the next section.

3.7. Oral host-microbial interactions – the oralome

The oral microbiome with all its interspecies interactions forms a complex ecosystem that thrives in a very dynamic environment that is the oral cavity. Thus, the oral microbiome not only mediates microbial interspecies interactions, but also interactions with the oral cavity and, thus, it creates a symbiotic relationship with the human host [4,8] - the microbial composition is significantly affected by interspecies and host-microbial interactions, which in turn, impact the health and disease status of the host [7]. We propose the term **Oralome** to describe all the interactions that take place between the oral microbiome and the host. For instance, it is well stablished that the oral microbiota are important for the maturation and development of an appropriate oral immune response, as the host immune system has to defend the host against pathogenic microbes, but also harmonize and protect commensal oral microbes [111–113]. For example, EBV stimulates a strong host immune response by eliciting the production of antibodies, such as immunoglobulin (Ig) G, IgM and IgA. These immunoglobulins recognize some EBV targets, such as EBV glycoproteins, as well as lytic and latent antigens. However, both CD4 + helper and CD8 + cytotoxic T cells mediate the robust response against EBV [114]. At the same time, the immune system has to avoid producing antibodies against oral commensal microbes.

Nevertheless, the host immune response must balance between inflammation for pathogen eradication and prevention of an unwanted immune response against the host's own tissue and commensal organisms, as mounting an aggressive immune response against microbes that pose no threat would be unnecessary, metabolically wasteful, and potentially damaging to host tissues [115]. In exchange, some oral commensals can act as a pathogen 'Sensor', 'Mediator' and 'Killer' and have coordinated roles in initiating the antagonistic action against a pathogen to prevent the colonization and integration of pathogens, a phenomenon referred to as colonization resistance [116]. For instance, *Streptococcus salivarius* antagonizes the main etiological agent of pharyngitis, *Streptococcus pyogenes,* preventing its colonization and growth in the pharyngeal mucosa, thus preventing pharyngitis [117–119]. This mutual protection is one indication that the host immune system evolved to tolerate and maintain some beneficial bacteria [120]. Also, some mucosally-adherent species can protect the host from carcinogenic metabolites [121,122].

In addition to benefitting the oral cavity, the oral microbiome also exerts an important role on the systemic health of the host [123]. For instance, some oral bacteria also participate in an entero-salivary nitrate-nitrite-nitric oxide cycle in which dietary nitrate in the saliva is reduced to nitrite and nitric oxide [124], which may assist in cytoprotection, vasodilation, antithrombotic effects, immune modulatory effects, blood pressure modulation, and may improve outcomes of myocardial infarction, heart failure, pulmonary hypertension, and vascular hypertrophy in terms of infarct size, cardiac function and hypertrophy [125,126]. Interestingly, high abundances of *Rothia* and *Neisseria* genera were shown to be beneficial for the maintenance of nitric oxide host homeostasis and cardiovascular health, while high abundances of *Prevotella* and *Veillonella* genera were detrimental to homeostasis.

Moreover, 16S rDNA studies have shed light on the existence of specific microbial patterns that are considered "healthy oral microbiota" [127,128]. In this sense, a healthy oralome (i.e. symbiotic host-microbiome interactions between humans and these microorganisms) would be an an example of *eubiosis* [4,8].

3.7.1. Eubiosis

Although the classification of ecological relationships (commensalism, parasitism and mutualism) is well defined, the boundaries between each category are fluid [129]. In this context, the oral microbiome, in a healthy host, under normal conditions, maintains balanced symbiotic/commensal relationships, which have been defined as a "microbial homeostasis" or "eubiosis" [4,8,130,131]. This balance promotes beneficial mutualism and/or commensalism without causing harm to either the microorganisms or the host. Despite this homeostasis, this does not mean homogeneity of the oralome in all individuals, as recent studies report that up to five different clusters can be found in the salivary oralome of different healthy individuals [132–134]. However, the largest populational-based study to establish the salivary microbiome (with more than 2,300 individuals) reported only two salivary community types, one associated with oral health and the other associated to oral diseases [135]. These results suggest that the difference between health (eubiosis) and disease (dysbiosis) may be complex and more than just natural heterogeneity in healthy individuals.

3.7.1.1. Dysbiosis. Although the oral microbiome has resilience (i.e. capacity of an ecosystem to deal with perturbations without shifting its state of symbiosis [136]), insults or changes, such as tobacco use, can shift the eubiotic balance from mutualism/commensalism to a unbalanced parasitic/pathogenic state, thus, promoting disease in the host [129,137]. This specific parasitic/pathogenic state wherein microbials promote disease in the host is known as "dysbiosis" [111]. This is also known as an "unbalanced microbiome" [138,139]. According to Peterson *et al* [111], dysbiosis can be characterized by three different scenarios that are not mutually exclusive and may occur simultaneously. i) the overall loss of microbial diversity; ii) losing the beneficial microbes; and iii) expansion of the pathogenic microbes.

3.7.1.2. Loss of microbial diversity. The general ecological concept of loss of biodiversity within a community indicates a decline in the number, genetic variability, and/or variety of species of a biological community at a determined location [111]. This loss of biodiversity can lead to the breakdown of that ecosystem. In the context of caries, several reports indicate a loss of diversity as the severity of the disease increased, suggesting that increased acidification of the oral micro-environment is accompanied by loss of diversity and a reduction in the levels and metabolic activity of beneficial bacteria, leading to the rise of cariogenic bacteria [140–145].

In terms of periodontal disease, changes in microbial diversity remain controversial, with some reports indicating loss of microbial diversity with periodontitis [146–149], and others indicating the opposite (i.e., that periodontitis is associated with increased levels of microbial diversity compared to healthy control levels; as a consequence of the increased amount of nutrients derived from host's tissue degradation [135,139,140,149–151]; and even

others reporting no significant difference between the groups (i.e., periodontitis vs. healthy patients) [152,153]. For instance, Almeida *et al.* [148] reported significantly lower taxonomic diversity in subgingival microbial samples from both gingivitis and chronic periodontitis patients compared to healthy individuals (total of 110 participants). However, Griffen *et al* [150] reported a significantly higher taxonomic diversity for chronic periodontitis individuals compared to healthy controls (58 participants in total). Interestingly, this discrepancy may be due to sampling from different probing depths, as Ge *et al.*[154] indicates that deeper pockets (>5 mm) contain significantly higher richness and diversity levels compared to shallower ($\leq$3 mm) pockets in patients with chronic periodontitis. Thus, further studies are needed to clarify these contradictory findings.

Interestingly, a meta-transcriptomic study of mature oral microbiomes demonstrated an over-expression of genes related to natural genetic transformation, indicating high functional redundancy in the oral microbiome [155]. However, under dysbiosis, this redundancy may be lost due to loss of beneficial microbes or growth of pathogenic bacteria, suggesting a general frailty in the oral microbiome related to the diversity and composition of its microbiota [156].

3.7.1.3. Loss of beneficial microbes. One of the main characteristics of dysbiosis is the loss of some of the benefits acquired from an established healthy oralome. As discussed before, the oral microbiota are important for the maturation and development of an appropriate oral immune response [111,112]; protecting the host against oral pathogens [117] and from carcinogenic metabolites [121,122]; and are part of the nitrate-nitrite-nitric oxide pathway [126], thereby offering several benefits to the host. Thus, losing those beneficial microbes may lower the host's ability to fight off pathogenic bacteria and respond to an excessive immune response against the host's own tissue, exposing the host to carcinogenic metabolites and detrimental vascular changes [111,112]. This loss is particularly important in the context of periodontal disease, wherein excessive chronic inflammation leads to loss of supporting tissues around teeth, including alveolar bone loss, which leads to tooth loss in the severe forms of the disease [157].

3.7.1.4. Expansion of pathogenic microbes. In a eubiotic environment, the oral microbiome contains opportunistic pathogens at such low levels that they do not cause any issues to an immune-competent host [111,122]. However, an outgrowth of these pathogens represents a risk for the host. Particularly, this may increase the risk for dental caries, periodontal disease and may even be correlated to several systemic diseases, such as atherosclerosis, Alzheimer's disease, and cancer. Specifically, *P. gingivalis* and/or *F. nucleatum* have been associated with periodontal disease [56,130,139], head and neck cancer [130,158], pancreatic cancer [159], colorectal cancer [160-163], Alzheimer's disease [164-167], atherosclerosis [168] and pre-term births [169,170].

In the next section, we will discuss the oral dysbiosis signatures of some these diseases.

4. Oral biofilm dysbiosis signatures of common diseases

4.1. Dental caries and periodontal disease

The search for the cause of tooth decay dates back to 5000 BCE, when Sumerian texts described a "tooth worm" as the causative agent of dental caries. In the late 1600s, Anthony van Leeuwenhoek was the first person to report microorganisms living in his own dental plaque; laying down the foundation for microbiology. In 1890, W. D. Miller proposed the "chemoparasitic" theory for the origin of caries, which explained that "in susceptible hosts who frequently consumed fermentable carbohydrates, oral microorganisms would convert these carbohydrates into acid, which would result in the demineralization of teeth"; thereby laying down the foundation for modern dental research [171,172]. However, due to limitations in culturing bacteria in the 19th century, Miller was unable to identify any specific causative agents of caries. Based on the work of Miller and G. V. Black, it was believed, at the time, that the quantity and not any specific pathogens were responsible for periodontitis. In this sense, the disease would only develop if the bacteria were able to surpass the threshold capacity of the body to detoxify bacterial products. Thus, idea has been known as the "non-specific plaque hypothesis" [173].

Interestingly, the turn of the century brought new techniques to isolate and identify bacteria. In 1924, J. K. Clarke identified a caries-causative agent – *Streptococcus mutans*. Unfortunately, Clarke was unable to directly demonstrate that *S. mutans* caused caries; this was later demonstrated by R. J. Fitzgerald and P. H. Keyes in the 1960′s.

In the 1970′s, W. J. Loesche and colleagues reported that the antibiotic kanamycin was particularly effective against cariogenic bacteria [173,174]. In 1976, the "Specific Plaque Hypothesis", was postulated, which holds that dental caries is an infection of specific bacteria present in dental plaque, namely "mutans streptococci" (including *S. mutans* and *Streptococcus sobrinus*) and lactobacilli. At the same time, several studies started comparing health- and disease-associated plaque [173,174]. Among those, M. A. Listgaten, in 1976, for instance, observed distinct qualitative differences between them using electron microscopy [175]. These studies identified several different species related to periodontal disease, leading some researchers to the conclusion that specific bacteria (i.e., periopathogens) could initiate and drive the disease. Thus, extending the "Specific Plaque Hypothesis" to periodontitis [173,174,176,177].

In 1978, the term "biofilm" gained importance after J. W. Costerton's publication described how bacteria stick [178], referring to the matrix-enclosed bacterial communities that are key to understanding how bacteria interact with the environment [179]. After this, other studies have shown that bacteria within biofilms usually display different phenotypes compared to their planktonic counterparts [30,179,180]. Within biofilms, there are extensive metabolites exchanged, signal trafficking, and different levels of interactions among different species [179]. The introduction of biofilm theory into oral microbiology provided an impetus for researchers to take a closer look at dental plaque [172,179].

Recent studies have also shed light on possible interdomain interactions between bacterial and fungal species that can drive periodontitis. For instance, *C. albicans* can interact with and adhere to *P. gingivalis;* thereby potentially increasing *P. gingivalis's* virulence [181]. Furthermore, the fungus can enhance *P. gingivalis* invasion of epithelial and gingival fibroblast cells via an unknown mannoprotein-*b*-glucan complex-dependent mechanism [182]. Another example is the interaction between bacterial and viral species. Several studies have reported a higher *P. gingivalis* abundance in EBV-positive patients [183–187]. In addition, *P. gingivalis* [187] and *Porphyromonas endodontalis* [188] can reactivate latent EBV and induce its lytic cycle via histone epigenetic modifications [114].

Recently, our group introduced a new polymicrobial mouse model of periodontal disease in a common mouse strain (BALB/cBy), which revealed a widening of the periodontal ligament space, alveolar bone loss, and an increased host immune response after a polymicrobial infection [49]. This model may be useful for assaying microbial biofilm interactions *in vivo*. Interestingly, using murine models, Payne *et al.* [189] demonstrated the natural transmission of the dysbiotic oral microbiome from a periodontally-

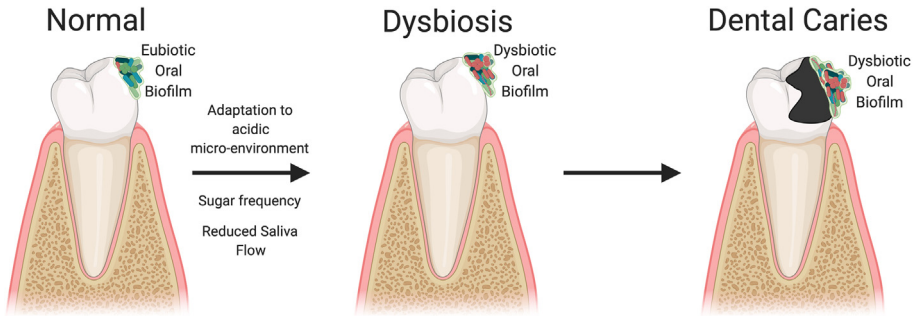

Fig. 2. Oral biofilm dysbiotic signature leading to dental caries.

diseased animal into a healthy one, leading to establishment of dysbiosis in the recipient, and suggesting that a dysbiosis may act as a conventional transmissible infectious disease agent by transmitting and stablishing dysbiosis in healthy individuals, with concomitant effects on its pathology.

With regards to dental caries, one of the most accepted theories about its etiology is known as the "ecological plaque hypothesis" [190]. This hypothesis states that frequent sugar ingestion drives an adaptation of the commensal bacteria to a more acidic micro-environment, favoring the succession of aciduric bacterial species (especially streptococci and lactobacilli), while inhibiting beneficial organisms that preferentially grow at neutral pH [141,191,192]. This adaptation to a dysbiotic microbiome is enabled by an enrichment with certain species of *Streptococcus, Lactobacillus*, and *Actinomycetes* genera, which are believed to be responsible for the formation of dental caries in adults (Fig. 2). For instance, compared to caries-free children, children with severe early childhood caries exhibit enhanced levels of *Streptococcus (*specially *S. mutans), Leptotrichia, Bifidobacterium, Porphyromonas, Leptotrichia, Stomatobaculum, Prevotella* and *Selenomas* genera. Specifically, *S. mutans* has been heavily studied for its cariogenic properties and is, today, regarded as one of the main pathogens associated with caries [56,130]. Yet, it should be noted that other species may also drive the disease, as *S. mutans* can persist in the oral cavity without evidence of detectable demineralization and caries, and caries can occur in the apparent absence of the species [190].

Recently, Hong *et al.* [52] found a high association between a Candida mycotype and aciduric bacterial species, whereas genera correlated with periodontitis, such *Fusobacterium*, *Porphyromonas* and *Treponema*, were highly associated to the Malassezia mycotype. Interestingly, the authors also found that the Candida mycotype was positively correlated with both caries and plaque, which suggests that both mycotypes may play a role in the link between periodontitis and caries. Thus, more studies are needed to better understand the role of both mycotypes in both diseases. Interestingly, these species have also been associated with caries sequelae, such as irreversible pulpitis. Recently, Zargar *et al.* [193] found 18 species in 41 root canals from patients with irreversible pulpitis. Among them, *Dialister invisus*, *P. gingivalis*, *S. salivarius*, *T. denticola*, *C. albicans* and HSV-1 were the ones with highest prevalence; *Lysinibacillus fusiformis* was detected for the first time in the root canals. This suggests a potential ecological succession of these species following caries progression.

The dysbiotic microbiome, led by *S. mutans,* would lead to the production of a wide range acids (predominantly lactic acid [194]) that partially demineralize the surface of the tooth. This mineral loss increases enamel porosity, widening the spaces between the enamel crystals and softening the surface, which allows the acids to diffuse even deeper into the tooth. This results in further demineralization of the layers below the surface, producing tooth decay. Once enamel is compromised, the pathogenic bacteria can eventually invade tooth dentin and pulp, causing pulpitis [195], as *S. mutans* has been frequently reported to be isolated in inflamed pulp associated with severe dental caries [196,197].

Once inside of the pulp, the dysbiotic bacteria have access to the circulatory system, which could lead to a transient bacteremia. Interestingly, oral streptococci, especially *S. mutans*, *Streptococcus sanguinis* and *Streptococcus mitis*, are thought to be important causative agents in infective endocarditis [196,197]. For instance, Nomura *et al.* [197] recently found significantly higher rates of *S. mutans* in the heart tissue of rats with $\geq$5 M containing dental caries extending to the pulp compared to rats with $\leq$4 M with similar dental caries. However, the authors reported that there is no direct evidence supporting a causative relationship between *S. mutans* from dental caries and the onset of infective endocarditis, as it remains unknown whether *S. mutans* can reach heart tissues through the bloodstream from advanced dental caries lesions [197].

Unlike caries, there is not a single hypothesis for the etiology of periodontitis [173] and this may explain why the initiation of periodontal disease and the microbes that drive periodontitis are still not completely established, despite being examined for decades [114,173].

To date, there have been five main hypotheses proposed for the initiation and pathogenesis of periodontitis (Fig. 3). We briefly discussed the first two previously above (i.e. the non-specific and the specific plaque hypotheses). The third is the "ecological plaque hypothesis" developed by Marsh in 1994 [190,198]. Marsh combined key concepts from the Specific and the Non-specific Plaque Hypothesis and concluded that the disease is the result of an imbalance in the microflora promoted by ecological stress, resulting in an enrichment of some periopathogens. Briefly, the hypothesis holds that the eubiotic microbiome can only harbor extremally low levels of periodontal pathogens, as the pathogens are unable to outcompete the predominant eubiotic saccharolytic bacteria. Due to external factors, the bacterial load in the subgingival microbiome can become incompatible with health and resulting in the activation of the host inflammatory response (gingivitis). This inflammatory response causes an increased flow of gingival crevicular fluid (a serum-like exudate), altering the subgingival nutrient status. This alteration drives an enrichment of proteolytic Gram-negative bacteria, such as *Prevotella intermedia*, *F. nucleatum*, *P. gingivalis*, *T. forsythia*, and *T. denticola* [56,130,139]. This enrichment drives host tissue degradation, promoting an even greater host inflammatory response and the cycle of destruction continues with the deregulation of the host inflammatory response, leading to periodontitis [139,190,198]. However, this hypothesis does not address the role of host genetic factors that significantly contribute to the composition of dental plaque and to susceptibility to disease [173].

The fourth hypothesis is the "keystone pathogen hypothesis" proposed by Hajishengallis and colleagues in 2012 [199].

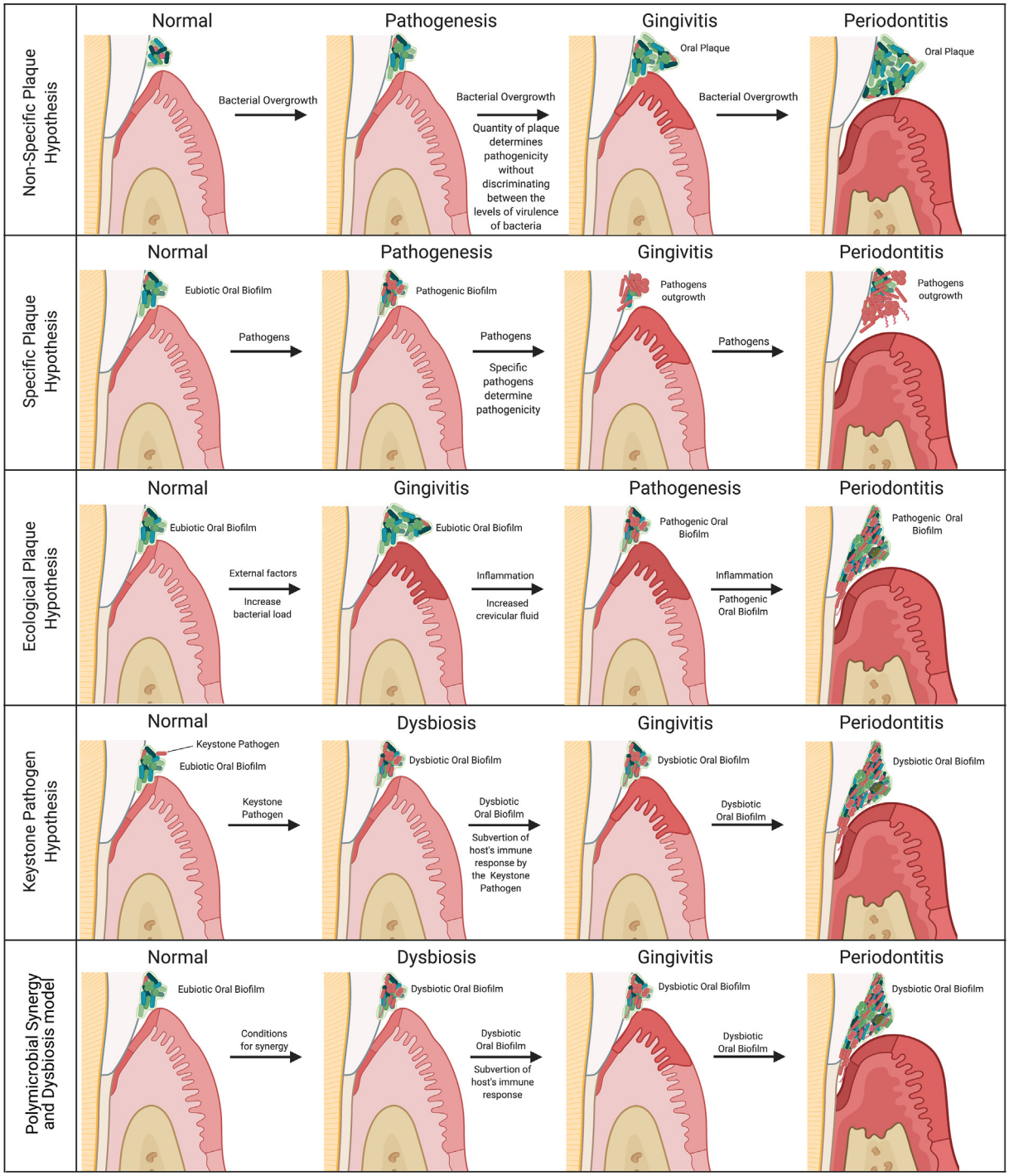

Fig. 3. Main hypotheses proposed so far for the initiation of periodontitis.

This hypothesis is based upon the ecological concept of "keystone species" coined by Robert Paine in his seminal work in the 1960's [200,201], which propose that some species are capable of holding their community together, despite their lower abundance and higher hierarchy in the food web; thus, having a disproportionately large effect relative to their abundance in their communities. Briefly, the "keystone pathogen hypothesis" holds that a certain low-abundance microbial pathogen can orchestrate a complete oralome remodulation into a dysbiotic state and attack the hosts defenses, including epithelial cells (as a nutritional source).

An important factor in this hypothesis is that the keystone pathogen be able to subvert the role of the immune system, as a wall of neutrophils stand between the plaque and the epithelial cell surface. In this context, Hajishengallis and colleagues [199,202] argue that the "keystone pathogen" for periodontitis is *P. gingivalis* since the bacterium has developed sophisticated strategies to subvert and impair the host immune response via several mechanisms, including Toll-like receptor response manipulation and interleukin 8 subversion.

Others have argued that the keystone pathogen is, in fact, a viral infection that disturbs the oralome into a dysbiotic state [203].

For instance, it has been demonstrated that CMV and EBV can inhibit macrophages by significantly downregulating their TNF-α production and TLR-9 expression, and inhibiting macrophage phagocytotic activity [204]; indicating that these viruses may inhibit the initial macrophage response, allowing the pathogenic bacteria enough time and space to initiate host aggregation and, thus formation of periodontitis. Interestingly, Slots [205] found CMV, EBV and HSV-1 in 3%, 7% and 12% of healthy periodontal sites, whereas their prevalence increased to 40%, 32% and 45% in subgingival chronic periodontitis sites and 49%, 45% and 63% in subgingival aggressive periodontitis sites. However, the viral keystone pathogen concept remains contested in the literature as several studies failed to find an association between these viruses and severe periodontal diseases [46,47,206].

Yet, no specific pathogen has been significantly associated with gingivitis, but the amount of plaque present and its bacterial load has been correlated with disease severity [21,177]; pointing out that the trigger may be more related to the abundance of organisms and their ecological succession rather than a specific pathogen driving the whole process from the beginning. In addition, certain periopathogens, such as *P. gingivalis*, can also be detected in periodontally healthy individuals, though less frequently, but without driving the disease [207–209]. Thus, Hajishengallis and Lamont updated the "Keystone Pathogen Hypothesis" to the "Polymicrobial Synergy and Dysbiosis" hypothesis in 2012 [140,202,210]. The concept of polymicrobial synergy in periodontitis has been established by animal models, which consistently demonstrated that significantly higher pathogenicity was found when several periopathogens were combined compared to the monospecies infection [140]. Interestingly, several important pathogenic functions require the expression of specific molecules, such as the appropriate adhesins, receptors, proteolytic enzymes and proinflammatory surface ligands, which cannot be found in one specific keystone pathogen, rather, the combination of these molecules act as community virulence factors to sustain a heterotypic, proinflammatory and dysbiotic microbial community that elicits a non-resolving and tissue-destructive host response [210,211]. Consequently, it has become apparent that pathogenic periodontal microbes only exert their fully pathogenicity when conditions favor synergism [210,211].

This model is consistent with the participation of both gram-negative and gram-positive bacteria in the pathogenesis of the disease, as long as they can provoke and/or tolerate inflammation [140,202,210]. Also, mixed microbial communities may provide opportunities for competitive and cooperative interspecies interactions, and such interactions can shape the nature and function of the entire microbiome [140,202,210]. For example, Passariello *et al.* [212] found a positive association between CMV, EBV and HSV-1 and several periodontal pathogens, including *P. gingivalis*, *T. forsythia*, *Fusobacterium periodonticum* and *Staphylococcus aureus*. Recently, our group, demonstrated an association between increased viral diversity and periodontal disease. Indeed, our data demonstrated that several viruses, such as *Gammaretrovirus* and *Porcine type-C oncovirus*, were also associated with alveolar bone loss and widening of the periodontal ligament *in vivo*, thereby implicating them in periodontitis [49]. Interestingly, Zhao *et al.* [213] demonstrated that hepatitis B virus (HBV) may also contribute to oral dysbiosis, as they found that a higher *Neisseriaceae* family abundance was positively associated with higher HBV titers on the tongue dorsum, whereas the virus was not present in healthy patients. Taken together, these data indicate a positive role for the virome in periodontal disease, but further studies are needed to determine all the critical triggers for periodontitis.

Currently, the subgingival dysbiotic microbiome signature most frequently identified includes a higher abundance of the red complex triad (*Treponema denticola*, *Tannarella Forsythia*, *and Porphyromonas gingivalis)* [140], as well as, the orange complex triad *(Fusobacterium nucleatum, Prevotella intermedia*, and *Parvimonas micra*), *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*, *Eikenella corrodens*, *Bacteroides forsythus* [114], *Filifactor alocis, Peptoanaerobacter* stomatis, *Firmicutes phylum* [140], *Methanobrevibacter oralis* [214], the archeon phylotype *Thermoplasmata* [81], *C. albicans* [54,215], CMV and EBV [205]. Thus far, no protozoan has been associated with the periodontitis signature.

The dysbiotic bacteria associated with periodontitis have access to the bloodstream, thereby creating the possibility for bacteremias. Bacteremia is defined as a transient or continuous presence of bacteria in the bloodstream [216]. In a double-blind, placebo-controlled study with 290 subjects, Lockhart *et al.* [217] found a positive bacteremia in 56% of patients after tooth extraction and 32% after toothbrushing. A similar rate of bacteremias was found in a more recent systematic review that included 9 observational studies, which showed that the most frequently identified bacteria in bacteremia were *Streptococcus viridans*, *A. actinomycetemcomitans*, *P. gingivalis*, *Micromonas micros*, and *Actinomyces* species [216]. These bacteria in the circulatory system could lead to the colonization of other hosts tissues and, thus, the association of the oral microbiome with systemic diseases.

4.2. Atherosclerosis

The link between dental disease and cardiovascular diseases was first established in 1993, when De Stefano *et al* [218] reported an increased risk (25% higher) of atherosclerotic plaque formation in patients with periodontitis. A recent study [168] demonstrated the presence of 23 oral pathogenic bacteria within atherosclerotic plaques in patients undergoing carotid endarterectomy, catheter-based atherectomy, or similar procedures. Of these 23 bacteria, 5 (*Campylobacter rectus, P. gingivalis, Porphyromonas endodontalis, P. intermedia, Prevotella nigrescens*) were unique to coronary plaques, while the other 18 were additionally present in non-cardiac organs and associated with over 30 non-cardiac disorders.

At least five mechanisms have been proposed for the contribution of oral dysbiosis to atherosclerotic plaque formation - i. bacteremia from the pathogenic oral microbiome invades the arterial wall and promotes plaque formation. For example, it has been demonstrated that oral bacteria can invade endothelial cells and phagocytic cells in the atheroma, leading to pathogenic changes and progression of the atheroma lesion [219]; ii. oral sites with inflammation release inflammatory mediators into the blood stream, which promote plaque formation; iii. autoimmunity to host proteins caused by the host immune response to specific components of oral pathogens promote plaque formation; and iv. oral pathogenic bacteria release specific bacterial toxins with pro-atherogenic effects [220]; v. via dyslipidemia, since patients with chronic or aggressive periodontitis have elevated serum levels of low-density lipoprotein and triglycerides, and decreased levels of high-density lipoprotein [219]. For a detailed review on this subject, please refer to Aarabi *et al* [220] and Schenkein *et al.* [219].

4.3. Alzheimer's disease

The first hint of a possible link between oral pathogens and Alzheimer's disease was shown in 1993, when Miklossy reported the presence of spirochetes in the blood, cerebrospinal fluid, and brain of 14 histopathologically confirmed Alzheimer's disease cases, but not in controls [164]. In 2002, Riviere *et al* confirmed this and reported the presence of 6 out of 7 *Treponema* species in the brain of Alzheimer's disease *postmortem* tissues. Interestingly, not only was the prevalence of *Treponema* DNA in brain cortex much higher among Alzheimer's disease samples than in controls, but Alzheimer's disease subjects also had more *Treponema* species

present. In 2012, a study that examined a longitudinal cohort found that poor oral hygiene was strongly linked to the development of dementia [221].

Recently, *Borrelia* genera and *T. denticola, P. gingivalis* and *Escherichia coli* [164–166] bacterial species and *Fusarium, Alternaria, Botrytis, Candida,* and *Malassezia* fungi genera [222] have been implicated in the etiology of Alzheimer's disease.

In 2019, *P. gingivalis* DNA was found in 7 out of 10 patients diagnosed with Alzheimer's disease, and a *P. gingivalis* gingipain protease was co-localized with Tau proteins in these patients' tissues. The authors further showed that mice orally infected with *P. gingivalis* also demonstrated brain infection and induction of the stereotypical Alzheimer's disease marker, amyloid beta 1–42 oligomers [167], suggesting that a bacteremia might have driven these pathogens to the brain. However, the role of these pathogens in this neurodegenerative disease remains unclear [223].

4.4. Diabetes

Type 1 and type 2 diabetes affects the periodontium of both children and adults, with an increase in periodontal inflammation and enhanced periodontal bone loss. The mechanism of action includes alterations in osteoclasts and osteoblasts of the periodontium by increasing the expression of inflammatory mediators, such as tumor necrosis factor (TNF), by increasing the receptor activator of nuclear factor kappa-B ligand (RANKL)/osteoprotegerin (OPG) ratios, and by enhancing advanced glycation end product (AGE) and oxygen reactive species (ROS) levels [224,225].

In 2001, a "two-way" relationship between diabetes and periodontal disease was proposed. This hypothesis was supported by investigations of individuals in the Gila River Indian community, where severe periodontitis at baseline was associated with an increased risk of poor glycemic control (HbA1c > 9.0%) at follow-up (minimum 2 years), suggesting that severe periodontitis was a risk factor for compromised diabetes management [226].

In 2007, Hintao *et al* [227] found an increased frequency of *T. denticola, Streptococcus sanguinis, Prevotella nigrescens, Staphylococcus intermedius,* and *S. oralis* in the supragingival plaque of individuals with type 2 diabetes.

Furthermore, several studies in 2011 showed that the treatment of periodontal disease influenced glycemic control in individuals with both type 1 and type 2 diabetes [228]. In addition, shifts in the oralome of patients with diabetes compared to healthy controls have also been reported [229]. Oral microbial dysbiosis is highly associated with the development of periodontal disease, which can induce higher levels of inflammation locally and systemically, and in turn, aggravate hyperglycemia [229].

In 2013, a consensus report from the European Federation of Periodontology and the American Academy of Periodontology found significant evidence demonstrating that mechanical periodontal therapy was associated with a 0.4% reduction in HbA1C levels after 3 months. The authors argued that this reduction was clinically equivalent to adding a second drug to the pharmacologic regime of diabetes patients [230]. Since the report found inconsistent evidence that diabetes type 2 significantly impacts the oral microbiota [225], this diminished the concept of the "two-way" relationship between diabetes and periodontal disease.

A 2019 study [229] demonstrated that *Leptotrichia, Staphylococcus, Catonella*, and *Bulleidia* genera were significantly enriched in patients with diabetes with very high glucose levels, suggesting that dysbiosis of the oral microbiota may be a typical feature of hyperglycemia and a potential contributor to progression of hyperglycemia. However, the oral microbial signatures that mediate the progression of hyperglycemia are not clear. Adding complexity to the issue, growing evidence suggests that gut microbiome dysbiosis may also play a role in diabetes [231,232]. For instance, recent metagenomic studies demonstrated a lower abundance of butyrate-producing microbes and an enrichment of *Bacteroides caccae*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium symbiosum*, *Eggerthella lenta* and *Escherichia coli*; the latter are known gut pathogens that cause intra-abdominal infections and bacteremias in type 2 diabetes patients. Thus, butyrate-producing bacteria may play a potential protective role.

Interestingly, Xiao *et al.* [233] demonstrated that diabetes can increase oral microbiome pathogenicity through IL-17 *in vivo*, bringing back the possibility of the "two-way" relationship between diabetes and periodontal disease. The pathogenic oral microbiome from diabetic mice was able to significantly increase periodontal inflammation and bone loss when transferred to germ-free mice, compared to an oral microbiome from healthy mice. Taken together, these data even suggest a possible "three-way" relationship where the oral microbiome, the gut microbiome dysbiosis, and diabetes exacerbate each other.

However, more studies are needed to identify the specific oral microbes that contribute to diabetes pathogenesis and to determine the underlying mechanisms by which both an oral and gastrointestinal microbial dysbiosis relate to diabetes. For more details on how diabetes can affect the oral microbiome, please refer to Graves *et al.* [225]

4.5. Pregnancy complications

The placenta is usually considered to be sterile, however, recent studies have shed light on a possible placental microbiome [169,234], and this microbiome might be involved in pregnancy complications. For instance, preterm birth rates were significantly higher when bacterial invasion and an IL-6 immune response was present [169]. However, no association has been found between preterm birth and a "healthy" placental microbiome (i.e. without histologic evidence of infection) [169]. Combs *et al* [169] investigated the microbes found in preterm births and found that oral microbes, such as *F. nucleatum, Bergeyella* sp., *Clostridium* sp., *Actinomyces* sp., *Peptostreptococcus* spp., and *Candida albicans* were present in half of the woman diagnosed with preterm labor and infection. Aagaard *et al* [234] compared the placental microbiome to the oral, skin, nasal, vaginal, and gastrointestinal microbiome of nonpregnant women and reported that the bacterial taxonomic profile of the placental microbiome was more similar to the oral microbiome, suggesting a possible bacteremia from the oral cavity to the uterus. However, comparing the microbiome of pregnant subjects to that of nonpregnant subjects may not be an ideal comparison, since a previous study from the same group found a significant difference in the vaginal microbiome between pregnant and nonpregnant subjects [235].

A significant association has also been established between microbes in the amniotic fluid and previous pregnancy complications, such as miscarriage, intrauterine death, neonatal death, preterm delivery and premature rupture of membranes. In all of these cases where *F. nucleatum* was detected, previous pregnancies resulted in one or more miscarriages [170].

FadA adhesin is a small (111 amino acids) helical peptide from *F. nucleatum* that has been proposed as a virulence factor involved in *F. nucleatum*-mediated cell attachment and invasion of host cells [236,237]. Ikegami *et al.* demonstrated that the deletion of the fadA gene resulted in >1000-fold lower bacterial titer in mice placenta compared to the control after 24 h of infection *in vivo*. Interestingly, restoration of the deleted gene on the strain also restored *F. nucleatum's* ability to invade and colonize placental tissue, indicating that the peptide is highly involved *F. nucleatum's* pathogenicity. However, fadA deletion did not completely eliminate placental invasion, thus suggesting the involvement of other proteins in the process.

The existence of a placental microbiome, however, has been recently scrutinized. Some studies demonstrated that the bacteria found in the uterus may have been due to contamination of laboratory reagents with bacterial DNA and lack of appropriate controls [238,239] and, thus, the concept of a uterine/placental microbiome has become controversial.

4.6. Head and neck cancer

Head and neck cancer (HNC) has a complex etiology. Risk factors for HNC include alcohol and tobacco use and betel quid chewing with or without tobacco [240,241]. Furthermore, alcohol consumption and smoking have a synergetic effect and these increase HNC risk, especially for oral and pharyngeal cancer [242]. In addition, alcohol [243] and tobacco [244] use negatively influence oral microbiome composition. For instance, Kumar *et al* [244] observed higher persistent rates of oral pathogens from *Fusobacterium, Cardiobacterium, Synergistes,* and *Selenomonas* genera in oral biofilms of smokers compared to non-smokers.

Molecular studies and meta-analysis have also pointed out that some viruses, such as *Human Papillomavirus* and *Epstein-Barr virus*, are associated with HNC progression [242]. Several molecular studies have found associations between *HPV* and oropharyngeal cancer (OPC) [245,246]. Thus, *HPV* was recognized in 2005 as a risk factor for OPC by the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) [246]. It has been estimated that HPV accounts for 30–60% of OPC and 12% of pharyngeal cancer [245,247]. Mechanistically, in HPV-induced OPC, p53 is present in both upstream and downstream pathways, but at low levels (10% or less) [245,248]. This is mainly due to p53 degradation by the HPV E6 enzyme [248–250].

Epstein-Barr Virus (*EBV*), a member of the herpesvirus family, was the first virus to be directly associated with carcinogenesis. It is transmitted through saliva and replicates in the epithelial cells of the oropharynx [251]. A recent meta-analysis demonstrated a significant association between *EBV* and HNC [252,253], although there has been no evidence of a direct role for *EBV* in HNC progression [253,254]. A higher frequency of oral sex and a greater number of sex partners are thought to be the reason for this increased risk for viral-related HNC, especially related to the oropharynx [245]. Mechanistically, EBV upregulates programmed cell death protein 1 ligand (PD-L1) via CTAR family proteins; decreasing p53 stability and increasing secretion of matrix metalloproteases (MMPs), thus upregulating the expression of cancer stem cell markers and facilitating cancer cell invasion and tumorigenesis. However, little is known about the potential carcinogenic role of *EBV* in HNC [253].

The specific link between viral infections and HNC led to a paradigm shift in understanding HNC risk, especially when *HPV* infection was previously associated with alterations of the oral microbiome in both infants [255] and adults [256]. This is particularly noteworthy, as recent cohort studies demonstrated that poor oral health affects the survival of patients with HNC [257] and good oral hygiene, such as annual dental visits and daily tooth brushing, may reduce the risk of HNC [258].

Culture-based studies revealed an increase in salivary bacterial counts in oral cancer patients, such *Capnocytophaga gingivalis, Prevotella melaninogenica and S. mitis*. In addition, DNA sequencing studies have demonstrated that different bacterial species colonize oral tumors compared to healthy sites, and high fusobacterial and low streptococcal levels may be a potential signature for the transition from health to HNC [259–263]. These findings suggest that unique microbial signatures may be potential diagnostic indicators for HNC, although they do not directly address the relationship of the oralome to the development of HNC.

In 2016, Guerrero-Preston *et al* [260] demonstrated that increases in Lactobacillus and loss of *Haemophilus, Neisseria, Gemellaceae* and *Aggregatibacter* in saliva may be biomarkers for HNC compared to healthy controls. Also, the authors demonstrated a shift in the microbial community of HPV-positive HNC tumors, with an enrichment in certain *Lactobacillus* and *Weeksellaceae* strains, and an abundance of *Eikenella, Neisseria,* and *Leptotrichia* in the HPV-negative tumors.

In 2018, a nested case-control study with 129 HNC patients [264] demonstrated that commensal bacteria, *Corynebacterium* and *Kingella,* were associated with a reduced risk for HNC, and data trends tended to be stronger in cases with a history of tobacco use. The authors also reported that oral *Corynebacterium* and *Kingella* genera were potentially mediating xenobiotic biodegradation, including metabolism of toxicants found in cigarette smoke. This finding suggests that a potential HNC prevention strategy may include promotion of these bacterial strains, especially for those with a history of tobacco use.

P. gingivalis has been associated with a variety of cancers, such as esophageal squamous cell carcinoma, colorectal, pancreatic and oral cancer [130]. For oral cancer, *P. gingivalis* infection was positively associated with late TNM stages, poor differentiation and lymph node metastasis [265]. One study showed that *P. gingivalis* elevated the level of Cyclin A, diminished the level and activity of p53 and activated the PI3K pathway to promote the proliferation of gingival epithelial cells [266]. Another study demonstrated that incubation with *P. gingivalis* for 72 h led to a dramatic increase in α-defensin, thus boosting oral squamous cell carcinoma (OSCC) cell proliferation by 125% [158]. Furthermore, *P. gingivalis* can induce activation of β-catenin and disassociation of the β-catenin destruction complex by gingipain-dependent proteolytic processing, thereby contributing to cancer pathogenesis [267].

A study by Kamarajan *et al* [268] demonstrated that multiple periodontal pathogens (*P. gingivalis, T. denticola, and F. nucleatum)* promote oral cancer aggressivity *in vivo*. The mechanisms involved included TLR/MyD88-Integrin/FAK crosstalk, which promoted cancer cell migration and invasion. Furthermore, this periodontal pathogen-mediated carcinogenesis was therapeutically responsive to treatment with a probiotic bacteriocin. Therefore, this study established a potential paradigm shift in the treatment of cancers by focusing on antimicrobial-based therapeutics for cancer.

Recently, Hong *et al.* [52] showed a significant increase in the *Candida* mycotype with a significant reduction of the Malassezia mycotype in patients with cancer (most frequently OSCC) compared to control patients. Although the patients age and recent history of chemotherapy did not correlate with any mycotypes, the *Candida* mycotype did emerge as a potential player in OSCC tumorigenesis and even chemotherapy resistance and recurrence. However, the authors also showed that receiving steroid premedication (administered to subjects prior to cancer sampling), and the subsequent higher neutrophil counts were both also correlated to higher *Candida* counts. This could indicate that higher Candida counts were present before the steroid administration or that the steroid administration and the higher neutrophil counts could be driving the higher *Candida* counts. Interestingly, when the authors normalized the data for steroid administration, the difference in *Candida* counts became non-significant, indicating that the later hypothesis (steroid administration drives the higher Candida count) may be the correct explanation of the results. Thus, further studies are needed to test this possibility.

Several mechanisms have been proposed to explain how carcinogenesis might be mediated by oral microbial dysbiosis [121] (Fig. 4). i. pathogenic bacteria, such as *Porphyromonas* and *Fusobacterium* species can upregulate cytokines and inflammatory factors (e.g. IL-6, matrix-metalloproteinases and TNF-α), leading to chronic inflammation and alterations of different molecular

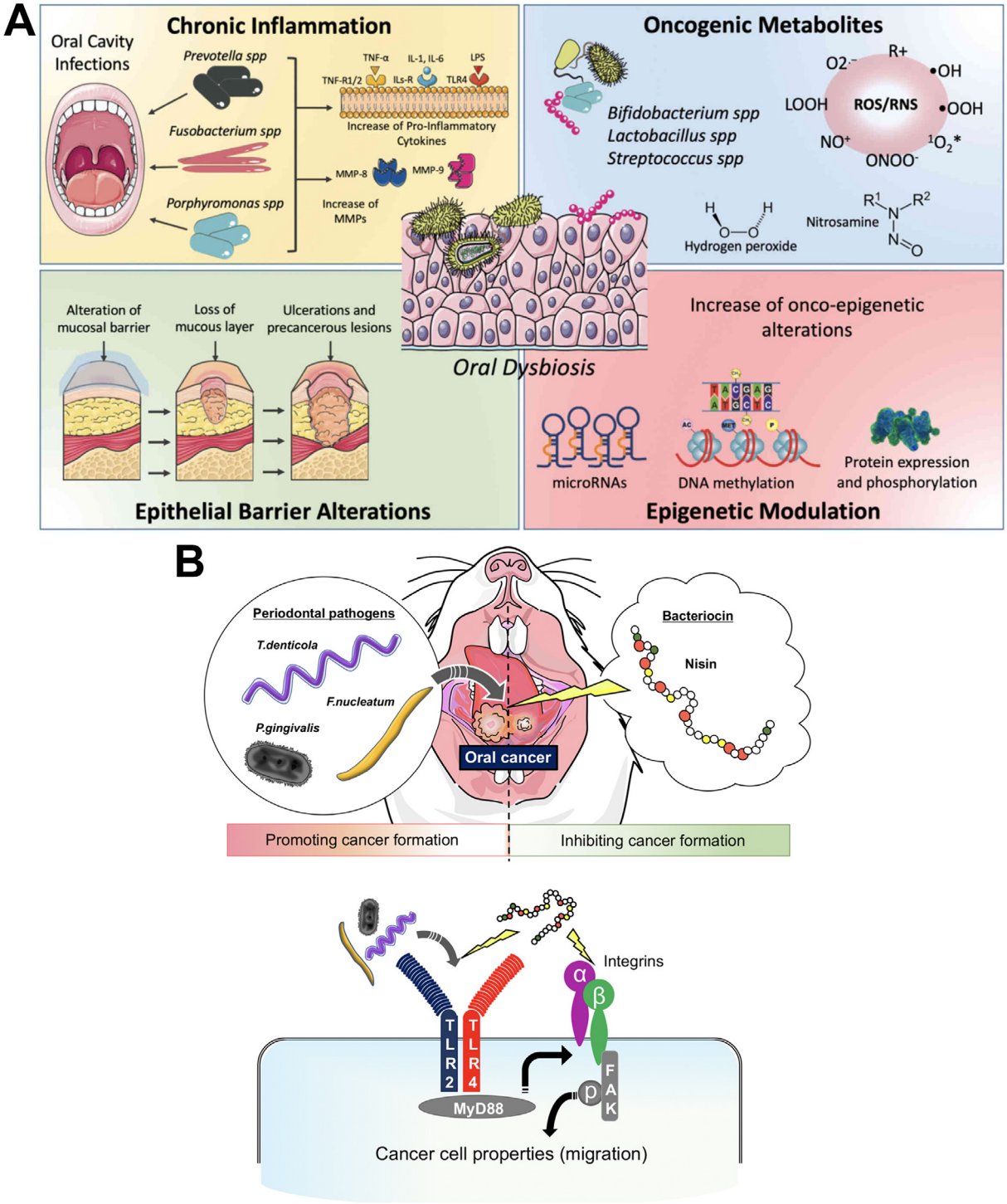

Fig. 4. Oral microbial dysbiosis is associated with oral cancer development through different mechanisms. A - Oral infections and dysbiosis are responsible for promoting a pro-inflammatory microenvironment, wherein inflammatory cytokines and matrix metalloproteinases favor the development and progression of tumors. Furthermore, the bacteria in the oral cavity produce oxygen and nitrogen reactive species, as well as oncogenic metabolites (e.g., nitrosamines) to induce genetic damage to cells within the oral mucosa. Another mechanism by which neoplastic transformation is mediated by oral dysbiosis is via the alteration of epithelial barriers, which predispose the oral mucosa to the development of chronic pre-cancerous lesions. Oral dysbiosis is responsible for several epigenetic alterations, which promote the development of tumors (e.g., alteration of onco-miR or DNA methylation phenomena). Figure reprinted from "Association of oral dysbiosis with oral cancer development" by La Rosa *et al* [121] licensed under CC BY 4.0; no changes were made to the figure. B - A recent report by Kamarajan *et al* [268] documents an *in vivo* model in which periodontal pathogens, i.e. *Treponema denticola* (ATCC 35405), *Porphyromonas gingivalis* (ATCC 33277) and *Fusobacterium nucleatum* (ATCC 25586) and, their lipopolysaccharides activate TLR/MyD88-Integrin/FAK cross talk to promote cancer cell migration and invasion. Interestingly, nisin Z (Handary, Belgium), a bacteriocin produced by *Lactococcus lactis* inhibits the cancer formation. Image courtesy of Drs. Pachiyappan Kamarajan and Ryutaro Kuraji.

pathways responsible for cell metabolism and proliferation [269–272]; ii. Several substances produced by pathogenic oral bacteria, such as ROS, sulfides and nitrosamines induce pro-tumoral genetic damage [273–278]; iii. oral biofilm dysbiosis can alter the homeostasis of epithelial barriers, leading to barrier dysfunction [279–281]; iv. recent studies have demonstrated the ability

of the microbiota to modulate hosts gene expression via miRNAs; these epigenetic changes were associated with the development and progression of tumors [282–284]. For a more detailed review of these mechanisms, please refer to La Rosa *et al* [121] and Radaic *et al*. [12]. Although there is evidence that different oral pathogenic species promote carcinogenesis, direct evidence of the mechanisms involved is still emerging [127,268].

4.7. Other cancers

Periodontal pathogens have also been associated with pancreatic and colorectal cancer. Next, we will briefly discuss these associations. For a more detailed review of the current epidemiological and microbiological evidence linking a dysbiotic oral microbiome to these and other types of cancer, please refer to Radaic *et al.* [12].

4.7.1. Pancreatic cancer

Fan *et al*. [285] reported that the presence of *P. gingivalis* and *A. actinomycetemcomitans* in the oral cavity was associated with a 60% increased risk of pancreatic cancer (hazard ratio = 1.60–95% confidence interval 1.15 to 2.22) in a nested case-control study with 732 participants. Interestingly, the authors reported that patients positive for *P. gingivalis* in the oral cavity have a 59% greater risk of developing pancreatic cancer than those who were negative for the pathogen.

Remarkably, Pushalkar *et al*. [286] demonstrated that pancreatic tumors harbor a specific microbiome, distinct from a normal pancreatic microbiome. Specifically, there is an increase in the genus *Brevibacterium* and order *Chlamydiales* in pancreatic cancers compared to normal controls. Despite a lack of direct evidence of oral microbial pathogens in pancreatic cancer, *P. gingivalis* is able to invade and survive inside of pancreatic cancer cells, enhance pancreatic tumor growth *in vivo* and cause higher rates of mutation in the tumor suppressor protein p53 and Kirsten rat sarcoma viral oncogene homolog (KRAS) gene in pancreatic cancer *in vitro* [159,287–290]. Thus, more studies are needed to evaluate whether *P. gingivalis* can directly colonize pancreatic tissue *in vivo*.

4.7.2. Colorectal cancer

Among all species in the gut microbiome found in the large intestine, *F. nucleatum* seems to be increasingly reported in gut infections, such as intestinal abscesses and acute appendicitis [291], with a high prevalence *F. nucleatum* infections in colorectal cancer [292]. In addition, *F. nucleatum* was found to enhance colorectal cancer growth and progression *in vitro* and *in vivo* via Toll like receptor 4 (TLR4) and myeloid differentiation primary response 88 (MyD88) protein activation and upregulation of microRNA 18a and microRNA 4802 [160,161,291] and is able to inhibit immune natural killer cells [293]. All these findings support that *F. nucleatum* may also play a major role in colorectal tumor growth and progression.

5. Current computational techniques to evaluate oral biofilm dysbiosis

Computational techniques, such as *in silico* models, can be used to understand biological systems as well as to select, complement, and inspire laboratory experiments [294–296]. For example, certain computational approaches can be used to estimate the immunogenic response of an antibody to a particular virulence factor [297] or evaluate the competition between two different bacterial species [298].

Dos Santos-Lima *et al* [297] used an *in silico* approach to analyze peptide epitopes from *P. gingivalis* virulence proteins for their potential immunogenic/IgG-mediated host response with relevance to periodontitis. Through this analysis, the authors were able to predict and select immunoreactive peptides from the pathogenic enzymes – lysine gingipain (KgP) and neuraminidase (also known as sialidase) before synthesizing them. Although, the authors confirmed the IgG immunoreactivity of the synthetized proteins, both peptides elicited very different immunoreactivity levels; KgP showed a high immunoreactivity, whereas neuraminidase showed very low immunoreactivity.

In another study, Valdebenito *et al* [298] also used an *in silico* approach to analyze the effects of competition between *Streptococcus sanguinis* and *S. mutans* in dental biofilms. The study focused on characterizing whole-genome differences between the species to explore potential metabolic advantages in a direct competition. The authors found that *S. sanguinis* had competitive advantages over *S. mutans*, primarily due to glutathione peroxidase activity and the capacity to undergo gluconeogenesis via genes present in the former but not in the later species.

Head *et al* [141] used *in silico* modelling to investigate how the frequency of and total sugar intake can promote the growth of more cariogenic species within oral biofilms. The authors

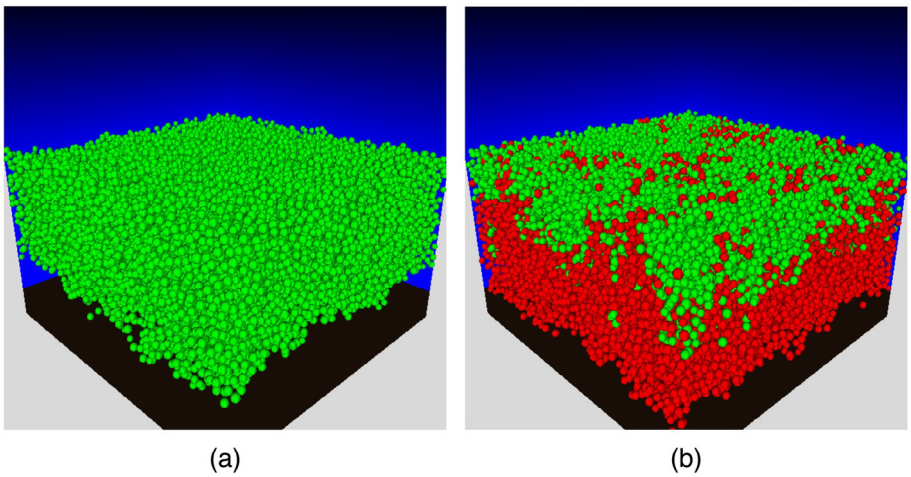

Fig. 5. Snapshots of biofilm composition at t = 60d receiving 6 glucose pulses per day, and a total daily amount of (a) 10 g/L/d and (b) 20 g/L/d. (a) Green spheres represent particles of a non-aciduric cell type (NA), and (b) red spheres represent those of an aciduric type (A). The computer simulations were initiated with 5% particles of type A and the remaining of type NA. Reproduced from Head *et al* [141]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

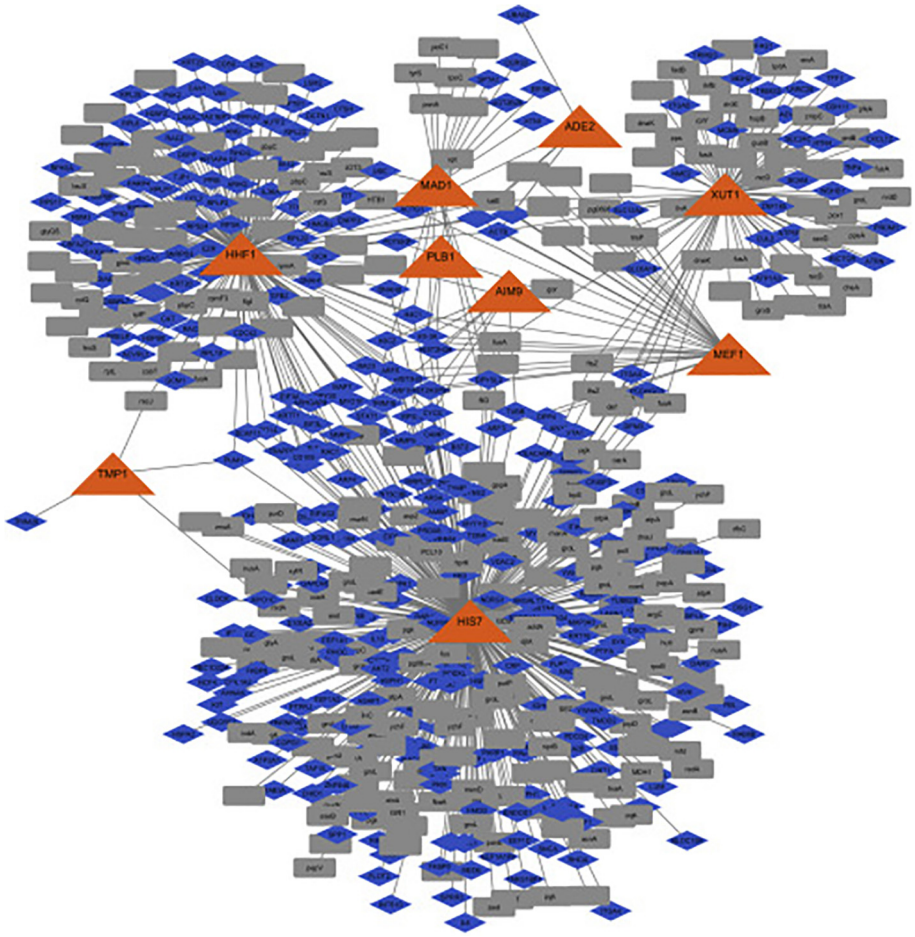

Fig. 6. Networks of protein–protein interactions (PPIs) between *Candida albicans* (orange triangles), human oral proteins (blue diamonds), and proteins from other oral microorganisms (gray rectangles) are identified by gene name. Reprinted from Rosa *et al.* [302]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concluded that total sugar intake was a determining factor in the promotion of cariogenic bacteria; with a low sugar intake, plaque was never cariogenic, whereas with a high sugar intake, oral plaque always promoted cariogenic bacteria, independent of frequency (Fig. 5). Furthermore, Marsh *et al* [299] also used *in silico* modelling to investigate how small effects, such as fluctuations in pH after sugar ingestion, can affect oral bacterial competitiveness. Their results demonstrate that variations in the buffering capacity of the plaque fluid modulate biofilm composition from a "healthy" state (i.e. low fraction of aciduric bacteria) to a dysbiotic state (i.e. dominated by acidogenic bacteria). This change resulted in a lower pH in the plaque fluid and an increased risk for enamel demineralization.

Computational techniques are not limited to *in sillico* models. Meta-analyses have revealed that metagenomics, metabolic networks, and interactomes have also been used to study the oralome.

Miossec *et al.* [300] evaluated 8 popular taxonomic profiling pipelines (i.e. MetaPhlAn2, metaMix, PathoScope 2.0, Sigma, Kraken, ConStrains, Centrifuge and Taxator-tk) that use different strategies for taxonomic profiling (e.g. marker-based, k-mer search, and read reassignment) to determine which factors or combinations affect time and robustness for perfect base calling. The different taxonomic profiling strategies were tested against 426 complete genomes stored in the Human Oral Microbiome Database to simulate various experimental conditions, including read length (75–1000 bp reads), sequence depth (100 K–10 M), metagenomic composition, number of species present (10, 100, 426), and species distribution. The authors found that taxonomic profilers, such as PathoScope 2.0 and Metamix, provided the most sensitive profiles but these took more computational time to execute the analysis. Other tools, such as Kraken and Centrifuge, required significantly less computational time, but found a lower microbial abundance. Thus, the more time-intensive pipelines may be the most beneficial tools.

Bernstein *et al.* [301] used a percolation theory to metabolic network analysis to estimate which metabolites, such as biomass components, are synthesized by the oral microbiome to better understand the metabolic interactions within the oral microbiome. To do so, the authors reconstructed the metabolic networks from the genome of 456 strains (371 different species) from the oral microbiome and generated an atlas of 88 different essential biomass metabolites from these organisms. This analysis can be further used in the field to estimate inter-microbial metabolic distances and correlate them with microbial co-occurrences.

Recently, Rosa *et al.* [302] developed a computational pipeline to specifically evaluate the oralome interactome – a specific network of protein–protein interactions (PPI) within a cell or even an organism. The authors reported that most PPI studies focus on the interactome of a particular organism, however, this analysis can be used to evaluate inter-organism interactions, such as PPI related to dysbiosis and symbiosis. Using the proposed pipeline and the SalivaTecDB, a database dedicated to annotated proteins, miRNAs and microorganisms present in the oral cavity and associated with oral or systemic mechanisms, the authors found

697 human-*C. albicans* protein interactions predicted with high confidence (score >0.9) (Fig. 6). The protein interactions found were primarily involved with cellular processes, such as metabolism, regulation of gene expression and cell cycle, and with pathogenesis, such as the phospholipase B enzyme, which is known for its role in virulence in mouse models of systemic infection [303]. Upon further analysis of the 317 human proteins that interact with *C. albicans* proteins, the authors also found an enrichment in leukocyte and neutrophil activation, inflammatory response, cytokine production and signaling, immune cell vesicle-mediated transport, secretion and exocytosis, as well as cell migration, which highlights a potential functional role for immune system interaction networks. Interestingly, this analysis suggests that both the yeast and host are aware of each other and that the host immune system is prepared to protect itself in case of a yeast infection.

6. Current and novel techniques for modulating oral biofilm dysbiosis

Despite the harmful effects that can be mediated by a dysbiotic oralome within the host, a complete elimination of the oralome is not the answer, since a eubiotic oralome provides health benefits to the host. Rather, techniques to modulate the dysbiotic oralome to re-establish a eubiotic state would be preferred. Among the current and novel techniques being studied, we discuss the use of traditional oral hygiene techniques, antimicrobial peptides (AMPs), nano-sized drug delivery systems, prebiotics, probiotics, EPM disruption techniques, and modifiers of the inflammatory response.

6.1. Oral hygiene

Although there are no specific pathogens associated with the early stages periodontal inflammation (i.e. gingivitis), the bacterial load and the amount of plaque present and its maturity have been correlated with disease severity [21,177]. Thus, frequent oral hygiene is an important technique for controlling the microbial load on the dentition and oral cavity and to prevent periodontitis and bacteremias [304,305].

A variety of oral hygiene measures have been advocated for removing dental plaque, however, brushing with toothpaste is the most highly recommended and primary method for oral hygiene in industrialized countries [306]. Substantial data supports that individual plaque control techniques, such as conventional tooth brushing with a fluoridated dentifrice and the use of chemical anti-plaque mouthrinses, significantly improves gingival inflammation and lowers plaque scores, provided that cleaning is sufficiently thorough and performed at appropriate time intervals [306,307]. When practiced regularly, oral hygiene keeps dental plaque in an immature state and in relatively small amounts that significantly decrease the chances of an bacteremia event compared to conditions where dental plaque is not effectively removed, as in patients with periodontal disease [21]; thereby, potentially avoiding the additional risks for systemic diseases associated with periodontal disease.

However, the frequency and duration of tooth brushing that is required to eliminate plaque and prevent periodontal disease is still not entirely clear, since longitudinal randomized controlled clinical trials are needed to fully evaluate this, yet these are challenging to carry out. Thus, we have to rely on information gained from either observational studies or from short-term studies conducted on gingivitis patients [306]. In general, the recommendation is to brush twice daily with a fluoride-containing toothpaste for 2 min, and the literature seems to endorse this [306]. For instance, a recently published 11-year-long prospective study with more than 1000 adults demonstrates a dose–response relationship between toothbrushing frequency and reduction in number of teeth with periodontal pocket depths (PPD) $\geq$ 4 mm - participants who reported brushing twice or more a day developed fewer teeth with PPD $\geq$ 4 mm than those who reported brushing less [308]. However, Chapple *et al.* [307] pointed out that expert opinion indicates that brushing for 2 min is likely insufficient for periodontitis patients, especially when considering the need for additional use of interdental cleaning devices.

Interestingly, a meta-analysis of 56 trials found in the Cochrane Central Registry of Controlled Trials and MEDLINE databases demonstrated a significant reduction in plaque index scores in both short-term (11% in 1-3 months) and long-term studies (21% >3 months) when using mechanical toothbrushes compared to manual toothbrushes [309], suggesting that mechanical toothbrushing may be helpful in reducing plaque-associated sequelae. However, the authors point out that there is a possible risk of bias, due to a great heterogeneity among the studies regarding brushing duration and frequency. Also, the effects of mechanical toothbrushing on periodontal disease progression remains unclear [306].

In terms of caries, a recent meta-analysis of three randomized trials, including more than 700 early teens (age 10–13), concluded that the oral hygiene technique by itself (without fluoride) may not prevent or reduce carious lesions [310]. Although the conclusion may be not be directly extrapolated to adults, since adults may have a different dental restorative status, gingival recession, and differences in saliva flow or systemic diseases [311], this analysis indicates that oral hygiene techniques may have a lesser role in preventing caries compared to fluoride. It is widely known that fluoride favorably shifts the balance towards enamel remineralization [312], whereas oral hygiene may only temporarily disrupt the acidogenic biofilm; and cariogenic dysbiosis is majorly driven by lifestyle factors, such as frequent sugar ingestion [195,311].

6.2. Antimicrobial peptides (AMPs)

Oral biofilm dysbiosis can be modulated through the use of antibiotics, however, antibiotics eliminate both pathogenic and commensal bacteria [313]. Also, due to their incomplete absorption by humans and animals, a large amount of ingested antibiotics are excreted into the environment via the feces or urine, contributing to environmental and multi-drug resistance concerns [314]. Therefore, new antimicrobial molecules are needed to effectively modulate microbial dysbiosis to address these concerns. In this regard, antimicrobial peptides (AMP) have shown promise in modulating oral biofilm dysbiosis. Due to their broad-spectrum antimicrobial activity, some AMP have the potential of becoming the next generation of antibiotics, and they may be useful in dealing with the crisis of multi antibiotic-resistant bacteria [107].

Among AMPs produced by bacteria, bacteriocins, like nisin, which is produced by *Lactococcus lactis*, are active against both gram-positive and gram-negative bacteria, including *S. aureus,* and *Listeria monocytogenes*. Due to its broad anti-microbial potential, nisin was approved as a food additive by the U.S. Food and Drug Administration (FDA) in 1988. To date, nisin is the most utilized bacteriocin and is the only bacteriocin used in food preservation in more than 50 countries. Our group has demonstrated that nisin disrupts oral biofilms without exhibiting cytotoxicity to oral cells [315]. Specifically, we demonstrated that nisin (from 1 to 50 μg/mL) inhibits the planktonic growth of oral bacteria, retards the development of multi-species biofilms, and disrupts biofilm biomass and thickness in a dose-dependent manner. These same concentrations did not affect primary periodontal ligament cells, gingival fibroblasts, primary oral keratinocytes, and osteoblast-

like cells; showing no signs of apoptotic changes up to 200 μg/ml for 24 h.

Among AMPs found in amphibians, K4-S4 (1–15) showed bactericidal effects against *S. mutans*, *Aggregatibacter actinomycetemcomitans,* and *F. nucleatum* (the latter two related to periodontal disease), both in their planktonic and biofilm forms [316].

Among human AMPs, LL-37 is expressed by several immune and epithelial cells and is directly involved in the host cellular response to microbial attacks. LL-37 has anti-fungal, antimicrobial and anti-biofilm properties, and can act as a chemoattractant for human peripheral blood neutrophils, monocytes, and T-cells, and is even capable of inhibiting Kaposi's sarcoma-associated-herpesvirus. Recent research demonstrates that LL-37 opsonizes *A. actinomycetemcomitans* and inhibits its formation into biofilms [317], plus inhibits the growth of *F. nucleatum* [316].

However, AMPs face some challenges. For example AMP resistance mechanisms develop within bacteria and these mechanisms have been associated with the virulence potential of pathogens [318]. These mechanisms include proteolytic degradation or sequestration by secreted proteins, exopolymer and biofilm matrix molecule inactivation, alterations of the cell wall and/or membrane composition via cell envelope regulatory networks (e.g. VanRS and LiaR), and AMP removal by efflux pumps [107,318]. In addition, AMPs can act as host sensitizers/allergens, which may lead to immunogenicity, especially after repeated exposure [107]. In this context, one alternative is to combine AMPs with nanoscale drug delivery systems (nano-DDS) [107,318–321].

6.3. Use of nanosized-drug delivery systems

Nanoparticles are colloidal particles made out of a variety of materials, such as lipids, metals, and polymers, that have sizes between 1 and 1000 nm [321,322]. At this size range, these nanoparticles possess unique features compared with their bulkier counterparts – they can offer a high surface area, increased reactivity, reduction in thermal resistivity, intracellular delivery of therapeutics depending on the particle shape, size, surface area and charge, and release of high levels of ions at low incorporated amounts, distinguishing them from their bulkier size and micro-sized materials [16,321,322]. Indeed, an inverse relationship has been demonstrated between the size of the metallic nanoparticles and their antimicrobial activity; particles in the size range of 1–10 nm have the greatest biocidal activity against bacteria [323,324], with silver nanoparticles, ranging from 5 − 40 nm being able to inactivate most microorganisms, including HIV-1 [325]. Thus, due to their antimicrobial ability, nanoparticles made of silver, copper, titanium oxide, and zinc oxide have been incorporated into polymer matrices as filler particles to control biofilm growth [16].

These unique features have also been used to improve therapeutic agents, which led to the development of nanomedicine [107,322]. Nanomedicine seeks to improve the use of proteins, genetic materials, and low-weight molecular agents for diagnostics and treatment of diseases through the use of nano-sized drug delivery systems (Nano-DDS) [322]. Specifically, this improvement is related to more specific targeting, controlled release of the compound, lower toxicity and better bioavailability [321]. For instance, Horev *et al.* [326] developed a polymeric nano-DDS capable of releasing farnesol in a pH-dependent manner, releasing the drug only at acidic pHs (4.5–5.5). This is very relevant to caries biology, as this mechanism may specifically disrupt only the aciduric dysbiotic biofilm. The nano-DDS formulation did disrupt *S. mutans* biofilms 4-fold more effectively than the free drug *in vitro,* and it significantly reduced both the number and severity of carious lesions *in vivo*, compared to free farnesol, which presented no *in vivo* effects.

With regards to AMPs, Nano-DDS can potentially protect AMPs from degradative enzymes and improve AMP immunogenicity by helping to evade the host immune response when the AMP is incorporated inside of the nano-DDS matrix, thus making AMP more efficient. For instance, Bernegossi *et al.* [327] encapsulated the synthetic AMP KSL-W in a lipid liquid crystalline nano-DDS made of oleic acid, polyoxypropylene-polyoxyethylene-cetyl alcohol and poloxamer 407 and they found higher growth inhibition of human saliva-derived oral biofilms using the KSL-W-loaded nano-DDS compared to the free peptide and the nano-DDS alone; reaching up to 100% growth inhibition after 7 days. Thus, the use nano-DDS show promise, since they can overcome the main drawbacks of AMP, and thereby making them more relevant for clinical practice [107,321]. However, very little has been tested in the area of oralome dysbiosis, as most of the studies focused on testing AMP-loaded nanoparticles against food-spoilage bacteria. For instance, of all the studies in the latest 4 reviews evaluating AMP-loaded nano-DDS [107,319–321], only one study, Bernegossi *et al.*[327] tested the efficacy of AMP-loaded nano-DDS in a saliva-derived oral biofilm model *in vitro* and none used *in vivo* models.

However, there are still some challenges and limitations that need to be addressed. For instance, some nanoparticle synthesis methods can expose AMPs to procedures that could modify and/or damage their structure and, thus, reduce their potential activity [107]. Also, nanoparticles are inheritably variable and extremely dynamic and, thus, are difficult to synthesize uniformly, creating batch differences [107,328,329]. Despite these challenges, more studies are needed to test the efficacy and safety of AMP-loaded nano-DDS, namely, studies demonstrating the *in vivo* use of nano-DDS to deliver AMP, especially studies demonstrating that nano-DDS can overcome AMP pharmacokinetics, pharmacodynamics and immunogenicity issues [107].

For a more details on the recent advances on nano-DDS for AMPs delivery, please refer to Radaic *et al.* [107], Martin-Serrano *et al.* [319], Makowski *et al.* [321] and Teixeira *et al.* [320]. For more details on the recent use of nanoparticles in dentistry, please refer to Jiao *et al.* [16] and Allaker &Yuan [325].

6.4. Prebiotics

Prebiotics are natural or synthetic food ingredients or supplements that modulate the microbiome to benefit the host [120]. This concept has been applied to the gut microbiome over the last decade, and, only recently, has it been applied to the oral microbiome. In this context, arginine has been shown to prevent dental caries by buffering the acids produced by a dysbiotic oralome [330,331]. The amino acid is metabolized by commensal arginolytic species, producing ATP and ammonia. ATP gives the arginolytic commensals a bioenergetic advantage over the acidogenic/aciduric bacteria [19,331], and ammonia raises the pH of the biofilm, preventing acidic damage to enamel [120,331]. Interestingly, Agnello *et al.* demonstrated by 16S sequencing that arginine pretreatment 20 h before sucrose addition resulted in a shift in the oral microbiome towards "health", with increased *Prevotella* genus and *Veillonella dispar* and *Streptococcus anginosus* species, and an increased biofilm diversity compared to the non-pretreated biofilms.

Nitrate is an ecological factor that can induce rapid changes in the structure and function of polymicrobial communities, thus it may be a potential prebiotic. Rosier *et al.* [332] demonstrated that supplementation with nitrate can be useful against both caries and periodontitis. Despite not affecting oral biofilm growth, nitrate supplementation (6.5 mM) was able to significantly increase ammonium production and alter the pH of the oral biofilm, as well as significantly reduce periodontitis-associated species.

Recently, Slomka *et al*. screened for 742 nutritional compounds and identified β-methyl-D-galactoside and N-acetyl-D-mannosamine as promising oral prebiotics, as they selectively triggered the growth of commensal oral bacteria and shifted biofilm communities towards a eubiosis *in vitro* [333]. Next, the group compared N-acetyl-D-mannosamine with two other promising prebiotics (succinic acid and Met-Pro) and found that N-acetyl-D-mannosamine was the most promising prebiotic among them, by showing a clear dose-dependent effect on the oral biofilms, such that treatment with1.0 and 1.5 M resulted in biofilms with 97% beneficial species [334].

Despite these advances, prebiotics for the oralome are still very limited, with limited evidence showing prebiotic effects against oralome dysbiosis. Thus, additional studies are needed to clarify the potential benefits of prebiotics [335].

6.5. Probiotics

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit for the host. In the case of the oralome, oral administration of probiotics may also benefit oral health by preventing the growth of harmful microbiota and/or by modulating mucosal immunity in the oral cavity [336,337].

In vitro studies have demonstrated that Lactobacilli and Streptococci strains isolated from healthy oral cavities have antibacterial activity against *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans* and *F. nucleatum* [338–341], which could lead to potential treatments for periodontal disease. However, Lactobacilli and Streptococci seem to use different antibacterial mechanisms. Streptococci may inhibit anaerobic species through the production of lactic acid and other organic acids [342], whereas Lactobacilli generate hydrogen peroxide via several different enzymes, including pyruvate oxidase [343], lactate oxidase, NADH oxidase [344] and NADH flavin-dependent reductase [345]. Interestingly, some studies have shown that different lactobacilli strains can inhibit the ability of multiple microbial species. For example, *L. fermentum* is active against microaerophiles, and *L. gaseri* is active against anaerobes [340].

Recently, our group demonstrated that a nisin-producing probiotic, *Lactococcus lactis*, prevents and disrupts oral biofilm formation *in vitro*. We also demonstrated that the probiotic modulated oral biofilm composition, by reducing the levels of *Heamophillus influenzae, F. nucleatum, T. forsythia* and *Klebsiella pneumoniae* (bacteria related to periodontal disease), while enriching for *Neisseria flava* (an oral commensal) within dysbiotic *in vitro* human saliva-derived oral biofilms; driving the composition of these biofilms back to control levels [31].

Pre-clinical and clinical studies have shown that probiotics are able to reduce gingivitis, plaque, alveolar bone loss, and modulate pro-inflammatory effects [346]. Specifically, chewing gum containing *Lactobacillus reuteri* led to reductions in gingivitis and plaque in patients with moderate to severe gingivitis [347], whereas *Bacillus subtilis* (CH201) reduced bone loss and attachment loss in rats with ligature-induced periodontitis [348]. Interestingly, Chatterjee *et al*. [349] suggested that the mechanisms of probiotic action are due to a combination of both local and systemic effects, including modulation of the local and systemic host immune response, antibacterial effects via different mechanisms, and stimulation of osteoblastic function.

Although probiotics have many health benefits, these benefits can only be realized if a high number of probiotic cells reach the intended target site, the periodontal pocket or caries surface. However, many probiotic cells die in food products after exposure to low pH and oxygen during refrigeration, distribution and storage, and the viability/survival rate of probiotic bacteria is strain-specific [350–353]. Thus, encapsulation of probiotics in micro- and nano-sized drug delivery systems (nano-DDS) may be an alternative that has been shown to protect cells from environmental factors [107,350]. For a more detailed review about nanoparticles and nano-DDS for targeting microbial biofilms, please refer to Koo *et al.* [354].

Also, one important consideration regarding probiotics is that changing the biofilm composition might alter the normal oral flora that is beneficial and/or critical for oral health [346]. However, studies detailing how probiotics interact with other microbes and how they interact with food in the oral cavity are still lacking [350]. Thus, further studies on long-term microbial changes in the oral cavity and probiotic interactions with food are still required. For a more detailed review of the current findings of probiotics as relates to periodontal health, please refer to Nguyen *et al.* [337,346].

6.6. Extracellular polymeric matrix (EPM) disruption

As previously discussed, EPM is the matrix produced by the oral bacteriome that forms the oral biofilm. This matrix is made up of polysaccharides, proteins, lipids and eDNA [7,22–26], and it protects the microbes from environmental stresses[29], such as from antibiotics. Nonetheless, controlling biofilm formation by disrupting the EPM can prevent the formation of dysbiotic biofilms and/or assist other therapies in biofilm disruption. Currently, there are two main strategies to disrupt EPM - disrupting EPS synthesis and secretion and targeting its composition and structure.

Disrupting EPS synthesis and secretion can be achieved by the use of cyclic-di-GMP or cyclic -di-AMP, as there is evidence that these molecules are responsible for governing the transition from planktonic state to a biofilm state [355–357]. For instance, Karaolis *et al*. showed that these compounds inhibited *S. aureus* cell-to-cell adhesion and reduced more than 50% of the species ability to form a biofilm. Similarly, Yan *et al*. [357] found that exogenous cyclic-di-GMP inhibit *S. mutans* adhesion and biofilm formation. Interestingly, deleting the gene that produces the molecule exacerbates matrix production. Taken together, these reports indicate the bacteria sense cyclic dinucleotide levels in the environment and use these as signaling molecules to decide whether to remain in a planktonic state or adhere to each other and promote biofilm formation [358]. Another approach is through the inhibition of glucosyltransferases, the enzymes responsible for initiation and elongation of matrix glycan chains [359]. From a virtual screening of about 150,000 molecules, Ren *et al*. [360] found that a particular quinoxaline derivative was a potential glucosyltransferase inhibitor. The authors then demonstrated that this compound could selectively antagonize glucosyltransferase in *S. mutans* by inhibiting its biofilm formation. Moreover, the authors evaluated the compound in an *in vivo* caries model and found that the molecule significantly reduced the incidence and severity of caries.

Disrupting EPS composition/structure can be achieved by the use of exopolysaccharide-degrading enzymes [361–363]. Some examples include glucanohydrolases, such as dextranase, mutanase, and dispersin B. Glucanohydrolases have been used successfully to degrade mixed-species *S. aureus* and *Pseudomonas aeruginosa* biofilms both *in vitro* and *in vivo* in models of chronic wounds by significantly dissoluting the biofilm matrix and reducing its biomass, thereby increasing the efficacy of subsequent antibiotic treatment [362]. Similar results has also been reported for dispersin B [361].

Despite the progress in the field, there is still limited knowledge about these techniques, with very few reports focused on the oralome. Thus, more studies are still needed [364]. For a more detailed review EPM disruption in microbial biofilms, please refer to Koo

et al. [354]. For more details on cyclic di-nucletides in the oral microbiome, please refer to Gürsoy *et al.* [364]

6.7. Host response modulators (HRM)

Since the host response mediates periodontal destruction through inflammation, use of host response modulators (HRM) may be an important technique against the disease. Currently, there is one commercially available product, called Periostat®. The main active ingredient in Periostat® is doxycycline, which downregulates the activity of matrix metalloproteinases (MMPs). MMPs are key destructive enzymes in periodontal disease, thus Periostat works by inhibiting collagenase activity. This is the first FDA approved systemic drug for host response modulation and it is currently indicated as an adjunct treatment to scaling and root planning in periodontitis [365]. In the same category of HRM, another heavily studied compound is the chemically modified tetracycline-3. So far, tetracycline-3 has been shown to reduce the collagenolytic activity induced by *P. gingivalis in vitro* [366] and it inhibits alveolar bone loss by inhibiting MMPs *in vivo* [367].

Another category of HRM is anti-inflammatory drugs, such as steroidal (SAID) or nonsteroidal anti-inflammatory drugs (NSAID). While, NSAID have been extensively studied in the field, evidence of the use of SAID in periodontitis are still scarce [368]. In 2016, Cavagni *et al.* [368] reviewed most of the current evidence for both anti-inflammatory drugs, including *in vitro* and *in vivo* assays and human clinical trials. For NSAID, the authors concluded that, despite the drugs ability to improve inflammatory parameters for both gingivitis and periodontitis, evidence showing that these drugs can indeed treat or prevent periodontal diseases is still lacking. For SAID, the authors concluded that most reports found no significant difference between SAID and controls, and effects on the periodontium remained controversial, thus further evidence is needed.

As previously discussed, the oral microbiome has an immunomodulatory effect on the host, such as priming immune responses to ensure rapid and efficient defenses against pathogens [118]. In this context, the oral microbiome produces metabolites that can immunomodulate the host response and contribute to the balance of pro- and anti-inflammatory responses, such as short chain fatty-acids (SCFA), proteins and even nucleic acids [118]. For instance, Santos Rocha *et al.* [369] demonstrated that a *Lactobacillus delbrueckii* surface protein/peptide has an anti-inflammatory effect. The authors found that the peptide was able to do this by either preventing IκB phosphorylation or promoting IκB dephosphorylation and, thus, reducing NF-κB activation. Recently, Corrêa *et al.* [370] demonstrated that SCFA can significantly reduce the expression of TNF-α, Cxcl2, and IL-10 *in vitro* and *in vivo* in *A. actinomycetemcomitans*-stimulated neutrophils.

Despite their potential in modulating the inflammatory host response and suppressing tissue destruction [371], HRM are limited in that they do not directly affect the oral dysbiosis. Thus, recurrence of periodontitis is always possible. Also, these molecules may be detrimental to the host, if not used properly, as they inhibit neutrophil effector functions, thus inhibiting inflammation [370]. Consequently, it is important to apply these locally. Finally, only limited studies have investigated possible side effects of HRM agents in humans, thus further studies are needed [372]. For more information on HRM therapy in periodontal disease, please refer to Preshaw [371] and Bartold & Van Dyke [373].

7. Clinical trials

Although the oral microbiome has been implicated in all these diseases, there are few clinical trials on this topic. Searching the clinical trials database ClinicalTrials.gov for the term "dysbiosis" in the field "condition or disease", we found 109 clinical trials in the database. Upon closer inspection, we found that the majority of these clinical trials were focused on the gut microbiome. When limiting the search to "oral dysbiosis", we found only 4 clinical trials that are actively evaluating the dysbiotic oral microbiome - two observational studies and two interventional studies. One observational study (NCT03225950), resulted in the publication by Damgaard *et al.* [374], which demonstrated that *P. gingivalis* was significantly associated with aggressive and chronic periodontitis. Although the relative abundance of the pathogen was different between the conditions, this difference was not sufficient to discriminate both diseases. A second observational study (NCT04251650) was trying to define the oral microbiome profiles of periodontitis patients then correlate this with the host inflammatory profile using crevicular gingival fluid. However, no results have been reported. One interventional study (NCT03863249) is trying to develop a microelectronic biosensor system (xCELLigence) to monitor and select effective antibiotic treatment for severe/chronic periodontitis patients. The other interventional study (NCT04027179) is looking to evaluate the effects of mouthwashes on the arterial blood pressure of periodontitis patients by measuring nitrite levels in saliva, bacterial levels on the tongue, arterial blood pressure over 6 months. No results have been reported for either trial.

Nguyen et al. [337], recently reviewed clinical trials using probiotics as both monotherapy and adjunctive treatment for periodontitis and gingivitis. The authors reported that the majority of clinical trials tested probiotics as monotherapy for treatment and prevention of periodontitis and gingivitis, whereas the majority of adjunctive therapy studies focused on using probiotics with scaling and root planning in the treatment of chronic periodontitis.

For probiotics as monotherapy, the authors reported mixed results from the clinical trials. Some studies demonstrated that probiotics improve several periodontal parameters, such as bleeding on probing, plaque and gingival indices, and gingival crevicular fluid volume, whereas others found no significant improvement on these periodontal clinical parameters. Thus, further clinical trials are needed to address these different findings.

In terms of adjunctive use of probiotics for periodontitis, most studies found significant clinical improvements in periodontal pocket depths, especially in the deeper periodontal pocket depths, clinical attachment levels, bleeding on probing, sulcus bleeding index, gingival and plaque indices, and/or percentage of sites with plaque. Also, several studies demonstrated that adjunctive probiotic treatment lowered the levels of matrix metalloproteinase-8, tissue inhibitor of metalloproteinase-1, and pro-inflammatory cytokines, including tumor necrosis factor-alpha, interleukin-1beta, and interleukin-17. However, the authors advised caution in extrapolating or generalizing these results, as some of these clinical trials had several limitations, such as small sample size, lack of appropriate randomization, blinding, appropriate controls, and significant heterogeneity in terms of probiotic strains, doses, duration of use, mode of delivery, and patient population studied [337].

Greber and Dawgul [375], Costa et al. [376] and Koo & Seo [377] have recently reviewed clinical trials using AMPs. The studies found a plethora of clinical trials using AMP from spermicide to infections in skin, ear canal and eyes. Among them, only one clinical trial has evaluated AMPs for any of the diseases discussed in this work - The AMP C16G2 has been investigated for dental caries by C3 Jian Corporation in phase I and II clinical trials. C16G2 is a Specifically Targeted AMP (STAMP), synthesized specifically to kill *S. mutans*, by selectively disrupting the bacterial membrane, without having an effect on other streptococci [377,378]. According to Baker et al. [379], C16G2 was first tested in a randomized, double-blind, placebo-controlled phase I clinical trial that focused

on evaluating safety and pharmacokinetics. The study enrolled a total of 127 subjects and tested several formulations, including a mouth rinse, an oral gel, a toothpaste, a varnish, and a tooth strip (NCT02509845, NCT03004365, and NCT03052842). The study reported no severe adverse events and that a single varnish application outperformed the other applications, followed by the toothpaste. The varnish formulation is a product candidate for in-office treatment, similar to fluoride varnishes commonly applied by dentists or hygienists for the treatment of high-risk, caries-prone populations. The phase II trial (NCT02594254) enrolled 135 patients and focused on the effectiveness of the C16G2 varnish. It showed a significant reduction in *S. mutans* abundance and *S. mutans* remained low even after the cessation of the treatment. Also, no adverse effects were reported, thereby validating the previous results. An additional phase II clinical trial of C16G2 is ongoing and it's goal is to determine the long-term effectiveness of the treatment.

Costa et al. [376] reported on a phase I/II clinical study evaluating whether hLF1-11, a synthetic antimicrobial peptide derived the human lactoferrin [380], can be used as a treatment for *Staphylococcus epidermidis* bacteremia from the oral cavity. However, the trial was closed prior to enrolment.

8. Summary and future outlook

Since the first observation of microbes in dental plaque by Antony van Leeuwenhoek in the early 1670's, the role of oral microbes has evolved from being considered simply "passengers" on the human host to one of benefiting and promoting diseases in their human hosts. The balance between health and disease has been explained by the eubiosis-dysbiosis hypothesis, which suggests that the oralome can shift from a healthy to diseased state with destructive host-pathogen interactions. Although the reasons for this shift are still unclear, *in silico* studies have shed light on this, indicating that small changes, such as pH alterations in specific oral sites, might drive this imbalance.

Oral dysbiosis has been linked to several host diseases, such as diabetes, atherosclerosis, Alzheimer's disease, and Head and Neck Cancer. These reports demonstrate the presence of oral microbes in several parts of the human body, linking these systemic diseases to oral dysbiosis and, ultimately, leading to new insights into those diseases.

Finally, the development and discovery of novel antimicrobial peptides and probiotics may help modulate the oralome from a diseased state towards a healthy state, and thereby, potentially improve the host's health or prevent diseases, such as head and neck cancer or Alzheimer's disease.

CRediT authorship contribution statement

Allan Radaic: Conceptualization, Investigation, Writing - original draft. **Yvonne L. Kapila:** Conceptualization, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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