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REVIEW ARTICLE

# Periodontal microbiology and microbial etiology of periodontal diseases: Historical concepts and contemporary perspectives

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## 1 | INTRODUCTION

The etiology of a disease refers to the causative trigger(s), whereas pathogenesis refers to the mechanism(s) by which the disease progresses. Over the past century, we have appreciated that periodontitis is of a microbial etiology and an inflammatory pathogenesis, albeit the coordination of the contributing factors for the initiation and progression of the disease may vary from an epidemiological perspective.[1] In other words, while the microbial biofilm developing on the tooth surface constitutes a necessary etiological factor, its mere presence is insufficient for the initiation of the disease. Further risk factors, such as host genetics, lifestyle, stress, and systemic conditions, that dictate the immunopathogenesis are crucial for the transition from a healthy to a diseased state. Such factors will be addressed in other papers within this special issue.

Whether it is one form of disease manifesting with different degrees of progression and severity, or different forms of disease exhibiting similar clinical manifestations, has long been a topic of public curiosity and scientific endeavor for mankind. The historical and contemporary knowledge established by pioneering researchers around the globe has led to paradigm shifts in our understanding of the etiology of the disease. This article discusses the continuum of seminal discoveries in the field, while highlighting the European contribution and its universal impact.

## 2 | ETIOLOGICAL HYPOTHESES AND MODELS FOR PERIODONTAL DISEASES

At the cradle of European civilization, ancient Greeks had already been able to identify the signs of periodontal disease and used their sense of smell as a diagnostic aid. Hippocrates refers to his scripts that the "evil malodor" is as result of "pitius" and even proposed oral rinsing with a solution of natural herbs as a treatment method.[2] Centuries later, the Romans observed "wobbly" teeth to be a diagnostic sign of the disease, which was attributed to the hard "calculus" deposits on the tooth surface, a dogma that dominated until the 18th century. Then, French pathologist Pierre Fauchard concluded that periodontal pathology is "a distinct type of scurvy" of local rather than systemic causes, whereas later that century, Scottish physiologist and surgeon John Hunter supported that gingival inflammation is the cause of alveolar bone dissolution, while introducing for the first time the term "periodontosis".[3] In late 19th century American dentist John Riggs historically named the disease "pyorrhea alveolaris" (also known as "Riggs' disease"), describing it as a suppurative inflammation of the gingiva and the alveolar process, while strongly advocating for hard calculus as the single local causative factor.[4] This theory coincided with the time of an unparalleled evolution of the scientific field of microbiology, leading to the contemporary notion that bacteria residing within the dental plaque deposits are

indeed the causative factor of "pyorrhea alveolaris". At that time, Willoughby D. Miller, an American dentist, studied in greater detail oral microorganisms at the lab of Robert Koch in Berlin. Based on his observations, he introduced the "chemo-parasitic" theory for the endogenous causation of oral diseases, according to which dental and gingival tissues are susceptible to being challenged by the bacteria inhabiting the mouth.[5]

The role of microbial dental plaque in the primary etiology of periodontal disease was revised and modernized after the second half of the 20th century. Danish researchers led by Harald Löe showed in a human volunteer "experimental gingivitis" cohort that abstinence of oral hygiene leads to dental plaque accumulation and development of gingival inflammation, which is diagnosed clinically as gingivitis. Subsequent reinforcement of oral hygiene and removal of dental plaque causes inflammation to subside and subsequently restore gingival health.[6] Characteristic microbiological changes accompanied these clinical observations, primarily as a switch from a sparse plaque consisting of Gram-positive cocci and rods to a Gram-negative bacterial community enriched with fusobacteria and filaments, and finally supplemented with spirilla and spirochetes. The initiation of microbiological changes coincided in time with the diagnosis of mild gingivitis. The reinstitution of oral hygiene and consequent reduction of visible dental plaque and gingival inflammation re-established the original sparse plaque microbiota.[7] The experimental gingivitis model continues to deliver valuable data, particularly when applied in conjunction with high molecular-throughput technologies.[8–12]

The experimental periodontitis model (cotton ligature-induced) in the beagle dog established by Swedish researchers led by Jan Lindhe was instrumental in establishing the relationship between longstanding dental plaque accumulation and irreversible periodontal tissue breakdown. Clinical and histopathological observations indicated that abstinence of oral hygiene and accumulation of dental plaque in the dogs led over time to a gradual conversion of subclinical to clinical gingivitis, and subsequently to periodontitis.[13–15] Matched histopathological observations in experimental periodontitis led by Swiss researcher Hubert Schroeder revealed the proximity of subgingival plaque to the pocket epithelium and established its role as an initiator of the cellular inflammatory events in the connective tissues beneath.[16–18]

Solid etiological theories for periodontal diseases started to emerge towards the last quarter of the previous century. Danish research supported the "non-specific" plaque hypothesis, which was led by dentist and microbiologist Else Theilade.[19] This hypothesis sets dental plaque mass in the center of the etiology of disease, claiming that all overgrown indigenous oral species contribute to the overall increased virulence properties of the plaque. Therefore, neither compositional differences in plaque were considered relevant to the disease, nor was there a clear distinction between pathogenic or non-pathogenic species. Hence, this theory directs preventive and treatment approaches towards suppressing the cumulative formation of dental plaque.

On the other side of the "non-specific" plaque hypothesis were the supporters of the "specific" plaque hypothesis, established by US microbiologist Walter Loesche.[20] The emergence of this hypothesis was met with prolific methodological progress in laboratory microbiology, such as the feasibility to cultivate anaerobic bacteria or detect non-cultivable organisms using genomic methods. According to this hypothesis, periodontal disease is established due to the overgrowth of specific indigenous plaque bacteria. Therefore, the theory supports that its treatment should be based on the targeted elimination of these bacteria with the use of suitable antimicrobials. The "specific" plaque hypothesis was consolidated by the seminal work of American researcher Sigmund Socransky and his co-workers, who classified subgingival bacteria in six color-coded distinctive microbial complexes, based on their association with periodontal health or periodontal disease.[21] The most recognizable complex globally is the "red complex", which consists of the Gram-negative anaerobes *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, which are found in elevated numbers and proportions in pockets from active periodontal lesions.

At this stage, it became apparent that periodontal diseases were not adhering to the classical traits of medical infections, in the sense that no sole exogenous pathogen is responsible as a causative agent for the disease. In contrast, members of the endogenous microbiota and their interplay with the host are decisively involved in the pathogenic outcome. A reconciliation of previously proposed hypotheses came in the late 1990s by UK microbiologist Philip Marsh, who proposed the "ecological" plaque hypothesis.[22,23] This hypothesis advocates that a homeostatic balance between the host and the microbiota prevails in health. Disease ensues when an imbalance occurs in the interaction between the two, driven by changes in their microenvironment. Under the newly established conditions, resident members of the oral microbiota that previously lived in homeostatic harmony with the host, begin to increase in proportions or virulence and act as opportunistic pathogens by instigating tissue-destructive inflammation.

A microbial imbalance is often referred to as "dysbiosis", and the synergizing microbiota as "dysbiotic microbial communities". The principles of the "ecological" plaque hypothesis were further framed in the "polymicrobial synergy and dysbiosis" concept established by US researchers George Hajishengallis and Richard Lamont.[24] This consolidated the notion that different bacterial members, or combinations thereof, within the community fulfill distinct roles that collectively shape and stabilize a disease-provoking microbiota that instigate chronic inflammation. The inflammation contains tissue-breakdown-derived nutrients that are readily available for well-adapted opportunistic pathogens that can also be perceived as "inflammophilic",[25] thus exacerbating further dysbiotic changes. The concept of the "keystone pathogen" hypothesis was formulated by the same researchers,[26] which gravitates around the potential role of *P. gingivalis* and its virulence factors in orchestrating inflammatory conditions in the periodontium that convert a symbiotic microbiota into a dysbiotic one. At this point, *P. gingivalis* was already considered as one of the most important periodontal pathogens. However, the main argument between the "keystone" hypothesis and earlier ones, which also focused on *P. gingivalis* (such as the "red complex" in the

frame of "specific" plaque hypothesis), is that even low numbers of *P. gingivalis* as a "keystone pathogen" could have a dramatic impact on the phenotypic profile of the subgingival biofilm. Therefore, according to the latter hypothesis, it is not a matter of abundance, but rather the role of *P. gingivalis* as the puppet-master orchestrating other members of the biofilm community.

In 2020, a new theory on the etiology of periodontitis was formulated by US and Australian researchers, which was coined the definition "Inflammation-Mediated-Polymicrobial-Emergence and Dysbiotic-Exacerbation" (IMPEDE) model.[27] In this model, inflammation is the hallmark of the dysbiotic events, which drives the transition from oral health to periodontitis. Indeed, periodontitis is a multifactorial disease, in which both the subgingival microbiota and the host immune response are central actors. However, there remains today a "chicken and egg" debate. In other words, are the compositional changes observed in the subgingival biofilm, as reflected in the 1990's "red complex" concept,[21] the course of the disease, or merely a consequence of the altered subgingival ecological conditions caused by inflammation? In that respect, the "keystone pathogen" hypothesis and the IMPEDE model might be looked upon as explanatory concepts for the complex interaction between the oral microbiota and host immunity in periodontitis, as viewed through the lens of the "oral microbiologist" and the "oral immunologist", respectively.

## 3 | ANAEROBIC MICROBIOLOGY

Anaerobic bacteria are among the major colonizers on mucosal surfaces of the human body, usually serving as beneficial or harmless commensals but many of them also being potential opportunistic pathogens. However, because of their slow growth, demanding growth conditions, and need for highly specialized workforce, knowledge of their presence and clinical significance remained unresolved for long time. Due to improvements in anaerobic microbiological techniques, the role of anaerobes in periodontal diseases burst onto the scene in the latter part of the 1970's. In a series of studies conducted by Jörgen Slots at the Royal Dental College, Copenhagen, Denmark, the composition of subgingival plaque collected from periodontally healthy, gingivitis, and periodontitis individuals was examined by using anaerobic culture and microscopy techniques.[28] Based on Gram-stain, cell morphology, and growth characteristics under different gaseous atmospheres, it was shown that subgingival bacterial communities were dominated by facultative Gram-positive cocci during health but switched to strictly anaerobic Gram-negative bacilli during periodontitis.

Since the early phases of periodontal microbiology relied merely on culture-based techniques, the purported dominance of Gram-negatives as suspected periodontal pathogens was somewhat biased. Oxygen tolerance and other growth characteristics within anaerobic taxa vary considerably, whereas the most sensitive ones fail to grow in pure culture with currently employed media or in anaerobic culture conditions even under extended incubation. They may instead require the co-existence of oxygen-consuming species and nutritional support in co-cultures with helper microorganisms, similar to conditions when growing in polymicrobial biofilms.[29] Indeed, UK microbiologist William Wade's research group has put praiseworthy efforts into developing methods to cultivate the very fastidious, difficult-to-culture oral bacteria.[29–31] By means of bacterial isolates, it is possible to describe novel species and to examine various characteristics connected to their potential virulence. Of special interest has been the phylum *Synergistetes* due to its consistent association with periodontal and peri-implant diseases.[31–35]

Open-ended culture-independent molecular techniques have implicated a wide variety of phylotypes within not only Gram-negative but also Gram-positive, mostly anaerobic taxa playing a role in periodontal disease.[35,36] In this context, compared to culture-based detection, an explanation for the observed emergence of Gram-positive taxa may be their incorrect interpretation as Gram-negatives due to the failure of Gram-staining to identify many Gram-positive anaerobes, such as *Filifactor alocis* and *Eubacterium*-like taxa.[36]

Over 50 years following the development of the experimental gingivitis model, such a study was conducted in the United Kingdom using 454-pyrosequencing and non-selective culture for characterizing the bacterial composition during the transition from health to gingivitis.[37] A shift in the community structure and an increased diversity were observed during the gingivitis-establishment period (eg, absence of oral hygiene). Alongside this, was an increase in relative abundance of species/phylotypes within Gram-negative, anaerobic or microaerophilic genera *Campylobacter*, *Fusobacterium*, *Lautropia*, *Leptotrichia*, *Porphyromonas*, *Selenomonas*, and *Tannerella*.

At approximately the same time, Dutch, Swiss and German research groups reported on fluorescence *in situ* hybridization (FISH) microscopy-visualized bacterial communities in subgingival biofilms.[38,39] *Porphyromonas* (*P. gingivalis*, *P. endodontalis*), *Prevotella* (*P. intermedia*), a Gram-positive anaerobic coccus, *Parvimonas micra*, and members of the *Synergistetes* phylum formed microcolonies in the top layer of biofilms, while spirochetes dominated the outer layers of the biofilm.[39] Schlafer et al[38] brought evidence of the involvement of the Gram-positive, strictly anaerobic rod *F. alocis* in periodontal disease; this asaccharolytic species was frequent in subgingival biofilms in patients suffering from chronic and aggressive periodontitis, but only occasionally detected in periodontitis-resistant older individuals. It is suggested that *F. alocis* is an important organism in the structural organization of the subgingival biofilm,[38] whereas Swedish researchers recently identified that it expresses a unique protein exotoxin.[40,41]

## 4 | SPECIFIC MICROORGANISMS AND THEIR VIRULENCE FACTORS

The advent of anaerobic microbiology and the development of biochemical and molecular microbiological assays have brought along significant discoveries at the individual species level. Whilst there

are numerous taxa to consider, this section will address further most well-studied, namely the black pigmenting anaerobes (ie, *P. gingivalis* and *Prevotella* spp.) and *Aggregatibacter actinomycetemcomitans*.

### 4.1 | Black pigmenting anaerobes

A hundred years ago, two scientific papers from the United States reported on bacteria that formed pigmented colonies on blood agar under anaerobic conditions. In 1921, Oliver and Wherry were the first to isolate these Gram-negative, non-motile rods from human samples and described the organism as *Bacterium melaninogenicum*. Some years later, in 1928, its characteristic growth and pigment production were further specified by Burdon: "this organism exhibits to a marked degree the habit of growing in very intimate mixture with other bacteria, and that strictly pure cultures are obtained with considerable difficulty … the colonies at first colorless, later become brown, then jet black".[42] After six decades, UK microbiologists Haroun Shah and David Collins reclassified these so-called 'black-pigmented anaerobic bacteroides' (BPB) to two novel genera, asaccharolytic species to *Porphyromonas*[43] and moderately saccharolytic species to *Prevotella*.[44] Their observed clinical relevance triggered a special symposium on black pigmenting bacteroides, organized by the Turkish Society of Microbiology, supported by the Federation of Microbiological Societies, which was held in Antalya, Turkey, in 1993. A topical issue was then published covering various aspects of BPB as important causative agents in a wide variety of human infections at different body sites (https://academic.oup.com/femspd/issue/6/2-3). In particular *P. gingivalis* but also *Prevotella intermedia/P. nigrescens* are clinically relevant species in the context of periodontal diseases. A long-line of research on various aspects of the pathogenicity of *P. gingivalis* has been the focus of many leading researchers from Europe and the United States, who put *P. gingivalis* on a pedestal as a 'keystone' pathogen and driving force for dysbiosis in subgingival biofilms, with the capacity to interact variably with the host's innate responses and persevere in the periodontal pocket.[45] This species is known to produce several secreted proteolytic enzymes.[46] The most well established and characterized among them are its cysteine proteinases, namely two Arginine-specific proteinases and a Lysine-specific proteinase, known as R and K gingipains respectively. UK and Polish researchers have made significant progress in the discovery and characterization of these bacterial enzymes.[47–51] Their action is known to deregulate the innate immune responses for the benefit of the species and its persistence and survival within the host. Hence, they are considered to be its most crucial virulence factor.[52]

Unlike the widely studied effects of *P. gingivalis*, our knowledge on the increasing number of oral *Prevotella* species interfering in dysbiotic biofilms is rather scarce. Notably, *Prevotella* is a highly diverse genus, including around 30 human species, which were originally isolated from the oral cavity,[53] with varying virulence and other properties; while some are commensals and protective for the host, other *Prevotella* species can act as pathobionts under inflammatory conditions. For example, within the phylogenetically closely related species of the *P. intermedia* group, the well-known black-pigmented, phenotypically similar *P. intermedia* and *P. nigrescens* play a role in periodontal diseases, whereas such a link is missing for the faintly pigmented *P. aurantiaca* and *P. pallens*.[54] Still, only limited information exists on the involvement of other pigmented or non-pigmented *Prevotella* species in dysbiotic oral biofilms.

### 4.2 | *Aggregatibacter actinomycetemcomitans*

*Aggregatibacter actinomycetemcomitans* is a Gram-negative bacterium with a central etiological role in periodontitis affecting young individuals, but has also been implicated in adult periodontitis, as well as severe non-oral infections.[55] The bacterium grows in both aerobic and anaerobic atmospheres and will develop star-like structures centrally when incubated on blood agar (Figure 1A).

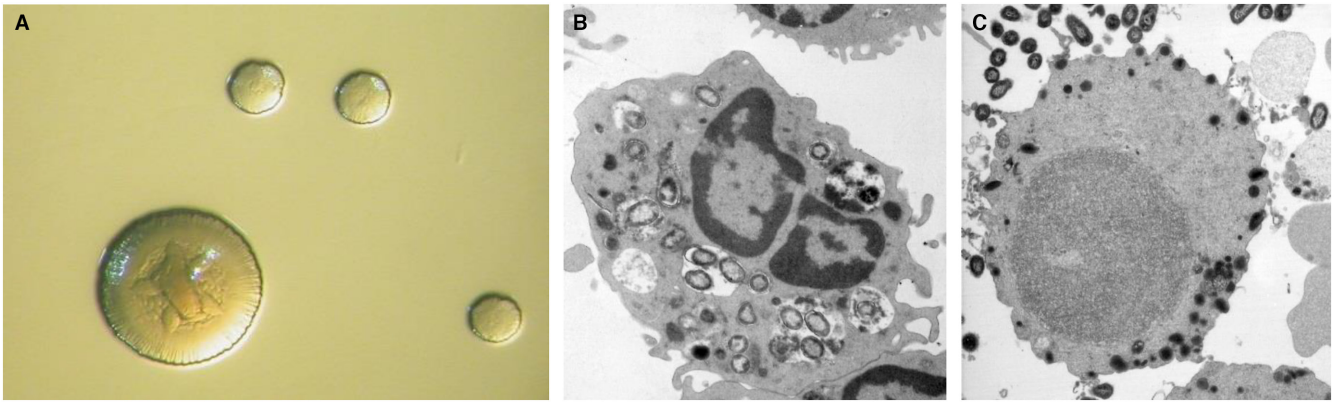


FIGURE 1 A, Microscopic picture of typical *Aggregatibacter actinomycetemcomitans*-colonies from a clinical sample growing on an agar surface. B, C, Transmission electron microscopic pictures of neutrophils exposed to *A. actinomycetemcomitans* under anaerobic conditions at 37°C during gently agitation. Reproduced with permission from Wiley from Johansson et al.[80] B, Neutrophils exposed for 60 min to a low leukotoxic serotype c strain (NCTC9710). C, Neutrophils exposed for 7 min to a highly leukotoxic serotype b strain of the JP2 genotype (HK1519).

Currently, it is classified in seven serotypes based on its immunodominant antigen, which is an O-polysaccharide of the lipopolysaccharide (LPS).[56,57] It has a complex dissemination pattern, acquired through transmission from the saliva of colonized individuals, and is suggested to initially colonize the oral mucosa early in life as a facultative intracellular pathogen,[58] and may translocate from the oral mucosa to the gingival crevices, where it competes with other bacteria within that niche.[59] Successful establishment and persistent colonization of *A. actinomycetemcomitans* in gingival crevices may lead to periodontal destruction in susceptible individuals.[60] Finnish researcher Sirkka Asikainen has shown in a series of studies that this species shows intrafamilial aggregation, with the child always fostering the same serotype as the parent,[61] which may partly explain the familial pattern of early onset or aggressive cases of periodontitis.[62] The pattern of interpersonal transmission of *A. actinomycetemcomitans* appears to be different to that of *P. gingivalis*, in the sense that the former is transmissable mainly from parents to children, whereas the latter is transmissable between adults.[63]

A substantial genetic diversity within this bacterium contributes substantially to the increased disease risk in colonized individuals.[64,65] The absolute numbers and relative proportions of *A. actinomycetemcomitans* in the subgingival biofilms of young individuals with periodontitis are greater than in those of older individuals.[64] Unique for this bacterium among the inhabitants of the oral microbiota is the expression of the two exotoxins, a leukotoxin (LtxA) and a cytolethal distending toxin (CDT),[66] studied thoroughly by Swedish researchers. The ability of *A. actinomycetemcomitans* to kill human immune cells was first reported in 1980 by US researchers, showing that the leukotoxicity varies substantially among isolates.[67,68] A later identified genotype of *A. actinomycetemcomitans* with a 530 base pair deletion in the promoter gene of the *ltxA* operon was shown to be highly associated with inducing periodontal tissue destruction.[69] Dissemination studies have traced the origin of this deletion in the Mediterranean part of Africa several thousand years ago,[70] whereas the strict vertical transmission pattern of *A. actinomycetemcomitans* resulted in a slow dissemination of this genotype, largely following the population trades.[71] Today, the JP2 genotype of *A. actinomycetemcomitans* can be detected sporadically in many parts of the world with the highest prevalence in North- and West-Africa as well as in some parts of South America.[72] Danish and Swedish researchers have documented the dramatically increased risk for an initiation of periodontal disease in individuals that harbor the JP2 genotype, consolidating on LtxA as an important etiological factor for periodontitis that affects adolescents.[73–75] They also identified that genotypes other than the JP2 clone can display an enhanced leukotoxicity and thus, an increased risk for the disease.[55,64,76] It was discovered that the shared features of all these highly virulent variants (JP2 and non-JP2) are their belonging to serotype b, a common arbitrary primed polymerase chain reaction (PCR) pattern, and an intact CagE gene.[77]

The research group at Umeå University, northern Sweden, has been studying for more than two decades the variety of mechanisms by which LtxA affects human immune cells,[78] including killing of leukocytes, inducing degranulation of neutrophils and protecting the bacteria from phagocytic killing (Figure 1B,C).[79,80] The rapid pro-inflammatory response in macrophages turns out to be an LtxA-induced inflammatory cell death, a phenomenon characterized as pyroptosis and, involved in the pathogenicity of several degenerative diseases.[81–83] The group also investigated in-depth the less studied CDT of *A. actinomycetemcomitans*, a genotoxin expressed by several non-oral Gram-negative pathogens. Apart from its capacity to cause cell growth arrest[84,85] the CDT of *A. actinomycetemcomitans* was also shown to regulate inflammatory and bone metabolic pathways of relevance to the pathogenesis of periodontitis.[86–89] However, the involvement of CDT in periodontal disease progression is not yet clinically confirmed.[90]

## 5 | BIOFILMS

The first ever microorganisms to be microscopically observed by Dutchman Antonie van Leeuwenhoek were bacteria of his own dental plaque scraped from the tooth surface. He described the lively and diverse-shaped structures seen under his primordial microscope as “animalcules”. It was not until the end of the 20th century, when we came to the realization that dental plaque posesses the properties of a microbial biofilm.[91–93] The term biofilm was introduced by US-based Canadian microbiologist William Costerton, to describe complex microbial communities attaching to and growing on surfaces in different ecosystems in nature, including the unique environment created by teeth in the oral cavity.[94] Allegedly, the ‘Eureka’ moment for Costerton came during a visit to Amsterdam, where he realized the importance of biofilms in disease, as well as the distinctive phenotypic properties of bacterial life within biofilms, such as antibiotic tolerance or slow growth rate.[95]

Early reports on dental biofilms in the microbial (rather than salivary pellicle) context came from the United Kingdom, where the bactericidal effect of chlorhexidine was tested on single species laboratory-grown biofilms of *Streptococcus sanguinis*.[96] This proved that the minimal inhibitory concentrations required for the elimination of biofilms are greater than those for planktonic bacterial cultures. Hence, the door was opened to investigations on the antimicrobial efficacy of various treatment modalities on dental biofilms.[97] Other UK studies focused on the ecological relationships within mixed oligo-species biofilms, revealing that coaggregation-mediated interactions between *Fusobacterium nucleatum* and other species facilitated the survival of obligate anaerobes in aerated environments.[98] Highly relevant experimental dental biofilm models were meticulously developed, including the multi-species Zurich subgingival biofilm model.[99] Within the model, it was possible to study the efficacy of commonly used antibiotics[100] or novel antimicrobial approaches,[101,102] the ecological interactions between species,[103–109] or the interactions between biofilms and host tissues[110] in complex bioreactor systems.[111,112]

Beyond the well-defined and controlled multi-species biofilms, it is possible to generate and maintain in culture natural "microcosm" biofilms from oral sources, which are heterogeneous and biodiverse microbial ecosystems of known and unknown species, as proposed by New Zealand researchers.[113] The origin of the sample (saliva/plaque and donor) is an important determinant for the development of the microcosms, whereas their bacterial composition can be retroactively determined by 16S rDNA sequencing.[114] Dutch researchers have made considerable progress in establishing reproducible subgingival microcosm biofilm communities,[115] in conjunction with clinically relevant periodontal questions, such as conferring the biogeographical associations of different sampled oral sites (saliva, tongue, tonsil, pocket) of periodontitis patients[116] or testing the effects of different interventions on gingivitis.[117]

The biofilm architecture and the spatial distribution of intact subgingival biofilms have been studied in detail by Dutch and Swiss researchers, who used a combination of fluorescence *in situ* hybridization (FISH) and confocal scanning electron microscopy (CLSM) to localize the most abundant phyla and species associated with periodontitis.[39] The biofilms were dominated by *Actinomyces* spp., *T. forsythia*, *F. nucleatum*, *Spirochaetes*, and *Synergistetes*. The latter were found at the outskirts of the biofilm layer, in possible contact to the juxtaposed epithelial layer and neutrophils in the periodontal pocket (Figure 2). Common periodontal pathogens colonize in a delayed fashion the biofilms and form microcolonies therein. These observations on the structure of subgingival biofilms utilizing FISH and CLSM complement the earlier landmark electron microscopy studies of Max Listgarten,[118] by deciphering the broader morphological diversity of the subgingival biofilm microbiota. The methodological basis for such studies has been the development of sensitive FISH and immunofluorescence assays by Swiss and German researchers, suitable for use in clinical dental plaque samples.[119,120]

Despite their close vicinity, supra- and subgingival biofilms differ from each other with regards to microenvironmental conditions such as redox potential, pH, and nutritional factors.[121] For fastidious anaerobic bacteria, synergistic interactions with oxygen-consuming organisms in subgingival biofilms are important, as it facilitates the conversion to an anaerobic microenvironment, favorable for their growth. Decreasing oxygen tension in deepening periodontal pockets offers highly reduced environments needed for strictly anaerobic periodontitis-associated taxa in dysbiotic biofilms. Inflammation also affects the microenvironment, including a shift towards alkaline pH, and increasing availability of host proteins and glycoproteins in gingival exudate, thus favoring the growth of proteolytic and asaccharolytic bacteria.[23] According to a consensus report of the Joint European Federation of Periodontology (EFP)/ European Organization for Caries Research (ORCA) workshop,[122] to further clarify functional roles of microbial populations in dental biofilms, studies on biofilm community structures and cell-cell communication by advanced imaging and gene expression analyses in both symbiotic and dysbiotic conditions, as well as randomized clinical trials exploring the microbiological endpoints are warranted.

## 6 | MOLECULAR TECHNOLOGIES FOR MICROBIAL DETECTION

The continuous development of molecular methods has been instrumental to the pioneering scientific discoveries that, led to the formulation of the different theories that describe the role of the oral microbiota in the etiology of periodontitis. Importantly, each theory was formulated based on analysis of data available in different research eras. Therefore, the methods used at different times had a significant impact on the questions that could be addressed by researchers. Consequently, without the massive technological improvement, our insight into the complex etiology of periodontitis would not have progressed with the same speed, as has been the case in the last two decades.

Microbial cultures and direct light or dark field microscopy were the methods available in the studies that founded the non-specific plaque hypothesis,[19] whereas the development of anaerobic culturing was the important technical improvement, which shifted the focus towards anaerobic bacteria as specific pathogens in periodontitis, thus formulating the specific plaque hypothesis.[20] The main challenge with culturing techniques at that time was the fact that a substantial proportion of the oral microbiota could not be cultured, which hampered the possibility to grasp the complexity of the subgingival community. This was clearly demonstrated by the landmark paper from 2001 by Paster and Dewhirst,[123] where culture independent molecular methods, cloning and sequencing, were used to determine the diversity of the subgingival plaque microbiota. Specifically, data revealed a hitherto unpreceded diversity comprised by as much as 700 different bacterial species, out of which 40% were unknown phylotypes, which had not previously been identified. In addition, a collaboration between American and Norwegian researchers, which also used cloning and sequencing, reinforced this complexity, by showing that in oral health, the microbiota found at different oral sites was composed of as many as 50 predominant species.[124] The transition from culture-based identification to molecular techniques was a critical step towards revealing the complexity of the subgingival microbiota both in health and periodontitis, which fertilized the transition from the non-specific and specific plaque hypotheses towards the ecological plaque hypothesis. In addition, the realization that a substantial proportion of the oral microbiota could not be cultured by means of standardized procedures, led to a whole new era, in which more sophisticated approaches were developed in the quest to culture the unculturables.[125] This endeavor is still ongoing with significant contributions made from both European and American researchers,[29,30,126,127] which, in combination with whole genome sequencing,[128–131] has illuminated hitherto unknown details about the uncultured part of the oral microbiota, and their potential aetiological role in periodontitis.

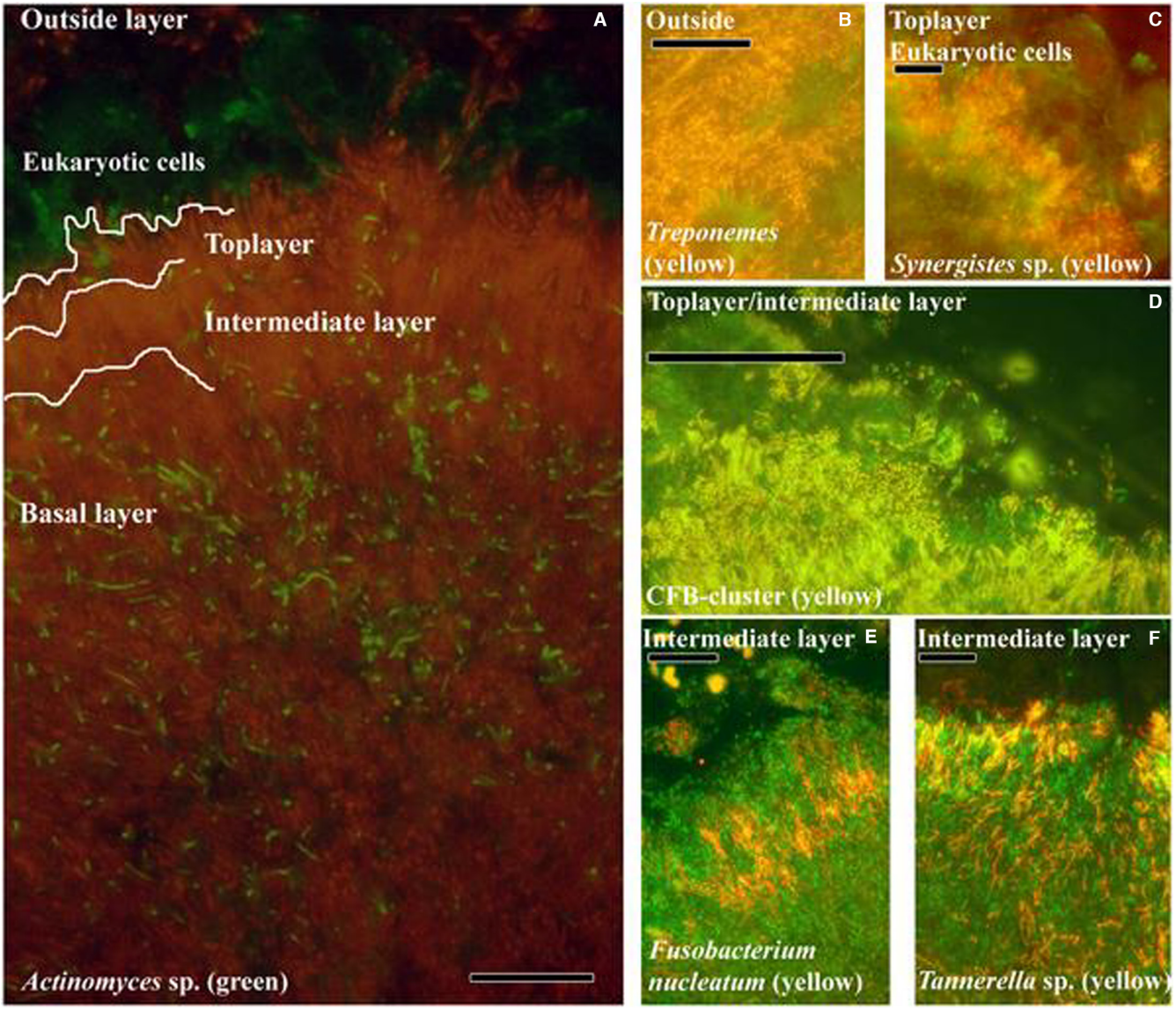


FIGURE 2 A, Overview of the subgingival biofilm with *Actinomyces* sp. (green bacteria), bacteria (red) and eukaryotic cells (large green cells on top). B, *Spirochaetes* (yellow) outside the biofilm. C, Detail of *Synergistetes* (yellow) in the top layer in close proximity to eukaryotic cells (green). D, CFB-cluster (yellow) in the top and intermediate layer. E, *Fusobacterium nucleatum* in the intermediate layer. F, *Tannerella* sp. (yellow) in the intermediate layer. Each panel is double-stained with probe EUB338 labeled with fluorescein isothiocyanate (FITC) or Cy3. The yellow color results from the simultaneous staining with FITC and Cy3 labeled probes. Bars are 10 μm. Reproduced under the terms of the Creative Commons Attribution License, from Zijnge et al[39]

In periodontal microbiology, there is a time prior to and following the 16S rDNA gene was identified as a tool to be used for taxonomic classification by Carl Woese in 1990,[132] which dramatically changed the possibilities to identify the unculturable part of a microbial community.[133] Researchers have used the 16S gene sequence in different ways. The first approach was to design primers for PCR-based identification of specific bacterial species in oral samples, as employed in the 1990's, confirming the presence of specific bacterial species in the subgingival environment in periodontitis.[134,135] Subsequently, quantitative PCR (qPCR) was developed, and in the early 2000s it was used to quantify proposed periodontal pathogens in clinical samples.[136–138] Notably, this approach was later commercialized into clinical screening, and is still used today, as evaluated very recently by Dutch researchers.[139] Swiss researchers demonstrated that qPCR, FISH, and conventional microbial cultures show convergent trends for species-specific bacterial quantification.[140]

When it comes to the development of molecular methods dedicated with the specific aim of studying the oral microbiota, the Forsyth Institute, Boston, USA, has been at the forefront. Starting in 1994 with the DNA-DNA checkerboard technique developed by Sigmund Socransky, which enabled the simultaneous identification of 43 bacterial species within the same sample using whole genomic probes.[141] the DNA-DNA checkerboard was the method used to conduct the study, leading to the pioneering red complex theory in 1998.[21] Next, in 2009 the Human Oral Microbe Identification Microarray (HOMIM) was developed by Bruce Paster,[142] which by

means of small DNA probes enabled the identification of more than 300 different phylotypes in the same sample. HOMIM was used in multiple studies to characterize the microbiota of periodontitis patients.[143–145] Finally, in 2016, HOMIM was replaced by the Human Oral Microbe Identification using the next generation sequencing (HOMINGS) technique. In HOMINGS, next generation sequencing (NGS) was used in combination with reference-based identification by means of the DNA probe sequences known from HOMIM, which enabled the quantification of approx. 500 different phylotypes.[146] Importantly, the continuous development of dedicated oral molecular methods fueled the possibility of more complex species-level analysis of the subgingival microbiota. This led to the identification of multiple new organisms, which among others include *F. alocis* that are now considered important periodontal pathogens.[41,147,148] Consequently, these methods continuously supported the transition from the specific plaque hypothesis towards the ecological plaque hypothesis, with more focus on the bacterial community, rather than presence of specific organisms.

The development of the Human Oral Microbe Database (HOMD) in 2010[149] and the expanded HOMD in 2018,[150] provided researchers with an invaluable reference database for taxonomic classification of 16S-based data. The true value of HOMD became clearly evident after the development of NGS techniques, which revolutionized the possibility to perform high-throughput characterization of the subgingival microbiota in large numbers of clinical samples. Since 2010, researchers worldwide have used NGS extensively to study the periodontal microbiota,[151–155] revealing that, even in health, it represents a distinct ecological niche.[156]

While 16S-based analysis revolutionized our knowledge of the composition of the subgingival microbiota in periodontitis versus periodontal health, this technique merely delivers taxonomic information. In other words, 16S provided researchers with the possibility to ask the question, "who is there?". The development of other OMICS techniques has dramatically changed this situation, and using techniques such as metagenomics, metatranscriptomics, metaproteomics, and metabolomics, we are now able to ask the question, what are you doing? Metaphorically speaking, we can study the phenotypic profile rather than the genotypic profile of the subgingival biofilm. Metagenomics,[157–159] metatranscriptomics,[160–163] metaproteomics,[164–167] and metabolomics[165,168–170] have all been employed in periodontology, providing valuable insights into the complex microbial networks encountered in the subgingival environment. Interestingly, metatranscriptomics was used in 2012 to demonstrate the effect of *P. gingivalis* and *A. actinomycetemcomitans* on gene expression profiles of a multispecies biofilm comprised of oral commensals.[171] Also, in 2014 the same researchers used metatranscriptomics to show that *P. gingivalis* induces expression of transportases and cell death in a *Streptococcus mitis* biofilm model,[172] in concert with the keystone pathogen hypothesis. This is an example of how the development and implementation of a new molecular method (metatranscriptomics) enables new analytical possibilities that can pave the way for paradigm shifts in understanding a disease. In this context, European researchers have recently combined metagenomics and metatranscriptomics data obtained from the same sample material, which allows for the characterization of the bacterial activity (eg, expressed by log10(RNA/DNA)).[173,174] Metaproteomics has also proven to be a popular approach in characterizing proteomic interactions and inter-species relationships within polymicrobial biofilm communities.[104–106,109] Combined layers of proteomic and metagenomic data have also been applied in studying the "interactome" of the human host proteome and the microbiome in inflamed gingival tissue.[175] Only the future will tell, if these approaches will advance our understanding of the aetiological role of oral bacteria in periodontitis. Nevertheless, history has shown us that the continuous development of molecular tools is paramount in providing researchers with the data needed to constantly progress and ultimately challenge any current paradigm describing the aetiological role of oral bacteria in periodontitis.

## 7 | CLINICAL MICROBIOLOGY—PERIODONTAL DIAGNOSTICS AND TREATMENT

### 7.1 | Specific bacteria or taxonomic groups in periodontal diagnosis

Periodontal research has largely been concerned with the composition of subgingival biofilms at sites of advanced periodontal tissue destruction. Gradually, increasing knowledge of the triggers of periodontal infection and roles of specific bacterial taxa, such as *A. actinomycetemcomitans*, *C. rectus*, *P. gingivalis*, *P. intermedia/P. intermedia*, *T. forsythia*, and *T. denticola*, generated interest in identifying the occurrence of the major periodontal pathogens in clinical samples from deepened pockets.[176–179] These fundamental findings led to the establishment of oral microbiology testing to guide clinicians, when considering the need for adjunctive antimicrobial agents in the treatment of patients suffering from advanced periodontitis. First, anaerobic culture techniques formed the gold standard for detecting periodontal pathogens until DNA-based techniques replaced their detection by culture and identification by biochemical methods. In oral microbiology service laboratories, the DNA-DNA checkerboard and qualitative or quantitative PCR were validated and used for selected target organisms.[136,139,180,181] Microbiological testing was meant to support the clinician in the selection of an appropriate treatment option but also to monitor the treatment outcome from a microbiological standpoint.[182] A Dutch research group estimated the outcome of molecular open-ended approaches instead of targeted identification of classical periodontal pathogens from diseased sites for clinical decision-making.[183] Based on the literature, they underlined the presence of a multitude of potential non-culturable and fastidious pathogens in subgingival biofilms other than those identified by routine analysis. This may hamper the value of acquired microbial information to assist in providing optimal therapy. According to a Swiss study by Eick et al,[184] however, the detection of the red complex (*P. gingivalis*, *T. forsythia*, *T. denticola*)

and *A. actinomycetemcomitans* in subgingival samples proved to be indicative of microbial dysbiosis, and to offer relevant information for a clinician, when considering the additional use of systemic antibiotics in the treatment of advanced periodontitis. Indeed, knowledge of periodontitis-associated bacterial quantities in diseased pockets is believed to broaden the periodontal diagnosis and to guide treatment planning as well as to screen treatment outcomes. For such purposes, commercial tests had been developed to simplify microbiological testing in clinical settings. Nevertheless, they have exhibited variable performances in detecting and quantifying the target organisms in subgingival samples, thus casting doubt upon their reliability.[139]

The current notion of periodontal microbiology is that research efforts should focus principally on recognizing the overall shifts in the microbiome, rather than changes in the levels of individual bacterial species. A French group aimed to determine the genera present at higher prevalence in at least 95% of subgingival samples in favor of periodontal health or disease.[185] For periodontal disease with deep pockets, *Treponema*, *Campylobacter*, *Eubacterium*, and *Tannerella* were the genera utilized for extrapolating the dysbiosis ratio of periodontitis. The species *T. denticola* and *T. forsythia*, and *C. rectus* and *E. nodatum*, which are typical members of the 'red' and 'orange' bacterial complexes, respectively,[21] were notable during subgingival dysbiosis. In contrast, *Porphyromonas* was not useful in deducing the dysbiosis ratio, since this genus was found in both health and disease, although a significant increase in its abundance was observed in disease (from 3.34% to 13%).[185] Recently, a machine learning approach was used to overcome statistical shortcomings that were observed in the latter study, such as the lacking of an assessment of the diagnostic accuracy of the ratio. A subgingival microbial dysbiosis index (SMDI) was developed and found to be reproducible and capable of identifying patients and sites at risk for periodontitis.[186] Discriminating bacterial taxa for periodontal dysbiosis were *T. denticola*, *T. forsythia*, *Mogibacterium timidum*, and members of the genus *Fretibacterium*, whereas typical oral commensals *Actinomyces naeslundii* and *S. sanguinis* were linked to periodontal health. Based on three genera, *Treponema*, *Fretibacterium*, and *Actinomyces*, the authors introduced a simplified index, improving its clinical utility.[186] In the context of genus-level analyses, it is noteworthy that there can be drastic differences in virulence between the species within a genus; for example, *T. forsythia* versus a periodontal health-associated *Tannerella serpentiformis*[30] and *P. gingivalis* versus *P. catoniae*, the latter being a common and harmless colonizer in infants' mouths.[187] Furthermore, the virulence of a pathogenic species like *P. gingivalis* varies at the strain level.[188,189]

## 7.2 | Combination of microbial with host markers for diagnosis

The etiopathogenesis of periodontitis has an infection-induced inflammatory character. Initiation and progression of the inflammatory process is coupled to the biomass and the virulence of biofilms, whereas the severity and duration of inflammation determine the extent of tissue destruction.[190] This means that the microbiological and immunological causal components of periodontitis are overlapping and highly integrated to the microenvironment, while evolving in a non-linear episodic character.

With the increased knowledge of both microbial and host components, researchers naturally aimed to define new diagnostic biomarkers of periodontitis and also to describe actionable therapeutic targets. This interest boosted a trend for biomarker studies in periodontology during the last three decades, considering a combination of factors rather than single molecules as a golden key to diagnose the initiation, monitor the remission or detect the recurrence of periodontitis at the individual level or public trends.[191,192] A critical step for the definition of biomarkers with high diagnostic accuracy was the implementation of time and interplay between microbial-immune-tissue degradation components into the classic episodic periodontal disease pathogenesis model.[193] This approach was first consolidated by Finnish researchers, elaborating that the shifts in bacterial burden, inflammatory response, and tissue destruction do not occur simultaneously, but consecutively.[194] According to this hypothesis, a periodontitis-associated species may not always be detected at high levels in oral samples, as it may undergo suppression due to the effect of high levels of anti-bacterial components within the inflammatory response. Similarly, a host-response marker, which plays a significant role in inflammation, can be downregulated at a specific time due to the decreased bacterial burden. Thus, the hypothesis supports the idea that the microbial and host-components of periodontitis should be evaluated together, in a cumulative manner, in order to deduce suitable biomarker(s) with high accuracy, sensitivity, and specificity (Figure 3). The success of the cumulative use of host- and bacterial biomarkers in detection of periodontitis over the fixed thresholds of single markers has been demonstrated in various independent studies.[195–197] Yet, the successful outcomes observed in those studies are usually dependent on participant recruitment criteria; therefore, the validation of the diagnostic power of candidate biomarkers in further independent populations is extremely important.

## 7.3 | Susceptibility of periodontal species to antimicrobials

Due to awareness of specific bacteria involved in periodontitis, researchers and clinicians became conscious of the potential of adding systemic antimicrobials in the therapeutic armamentarium to treat periodontitis, especially those with rapid progression in young individuals. Intensive research was directed to the properties of potential antimicrobial agents in studies conducted during the 1980s and 1990s. The first studies in Europe were published by the groups of Jan Lindhe,[198] who used metronidazole. In a split-mouth design, it was shown that systemic metronidazole improves periodontal conditions, but the major effect was related to the mechanical disruption of the biofilm. Metronidazole was shown

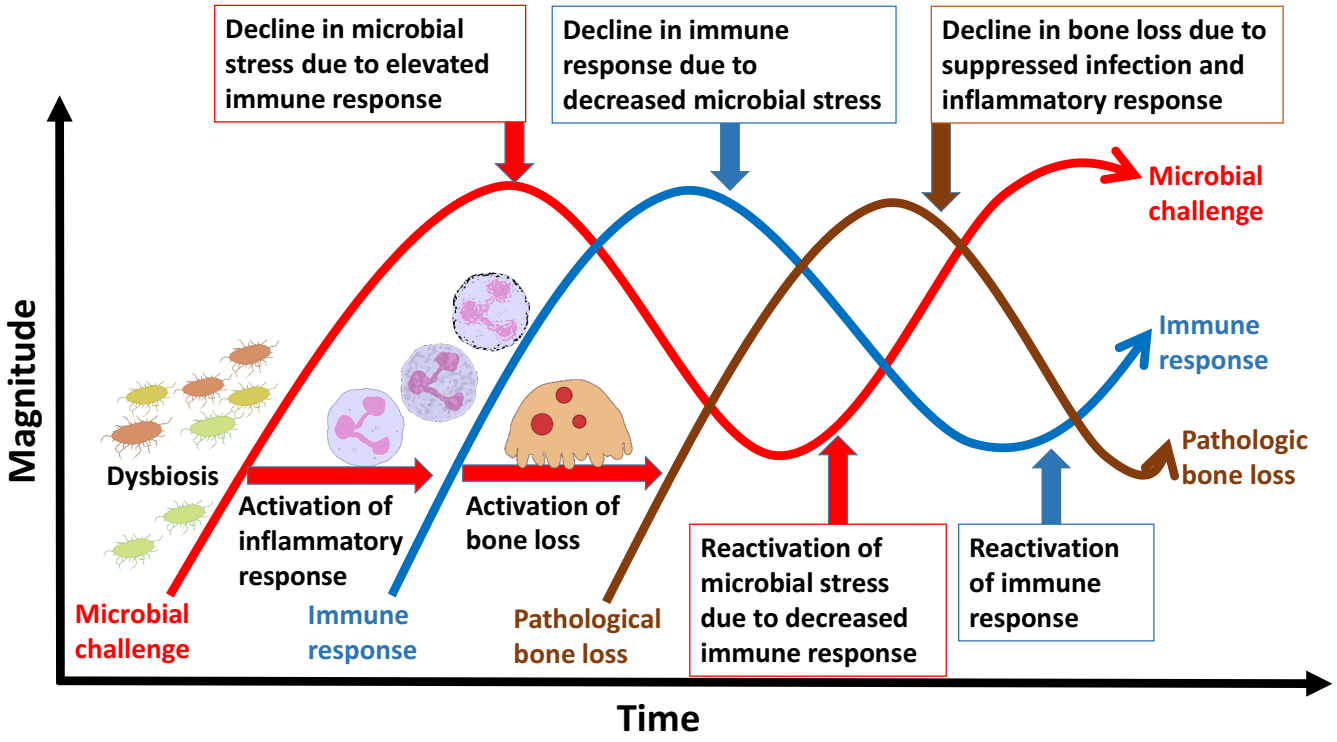


FIGURE 3 Implementation of the sequential and inter-dependent changes to the episodic periodontitis pathogenesis model. The current figure is modified from the original hypothesis, reproduced under the terms of the Creative Commons Attribution License from Gursoy et al[194]

to be particularly effective against anaerobic bacteria, and since those are dominant in deepened pockets, this drug appears to be reasonable choice. Yet, it is not as efficient in eliminating facultative pathogens like *A. actinomycetemcomitans*.[199] This is expected, since aerobic and facultative bacteria are intrinsically resistant to metronidazole. The synergistic effect of metronidazole and its hydroxymetabolite with amoxicillin against this facultative, capnophilic species was demonstrated by Dutch researchers.[200,201] The synergistic activity was verified by an enhanced uptake of metronidazole in the presence of amoxicillin. Clinically, an adjunctive application of metronidazole with amoxicillin was able to eradicate *A. actinomycetemcomitans* and improved the periodontal treatment outcome.[202]

This novel treatment option was indeed welcome, since *A. actinomycetemcomitans*, if present in subgingival biofilms, is widely distributed also in other oral surfaces; therefore, its eradication by subgingival scaling alone was not successful and did not result in the expected clinical outcome.[203] The observations gradually led to the combined use of these two antimicrobial drugs as an adjunctive treatment of periodontitis, including both its so-called aggressive and chronic forms with or without the detection of *A. actinomycetemcomitans*.[204–207] This combination is the most widely used adjunctive antibiotic regimen in severe cases of periodontitis independent of the prevalent bacterial species.[208,209] In the systemic use of such a broad-spectrum combination of antimicrobials, the potential side effects should be carefully considered. In line with World Health Organization (WHO) and European Union (EU) recommendations to prevent the development of bacterial resistance, the prescription of metronidazole and amoxicillin in periodontal therapy without microbial diagnosis was heavily criticized by Scandinavian researchers. As underlined in their letter to the Editor of *Journal of Periodontology*, the tools in the fight against resistant strains include the avoidance of an unnecessary use of any antibiotic and broad-spectrum or combination antibiotics, in particular. Therefore, microbial testing was seen as necessary to legitimize the adjunctive use of combined metronidazole and amoxicillin.[210] In contrast to this is a recent systematic review from the United Kingdom, stating that evidence is lacking to support a baseline detection of periodontitis-associated species as a criterion for adjunctive antibiotics.[211] Of note, this conclusion was based on limited microbial data and specific antimicrobials available for analyses.

The rationale of using antibiotics is to inhibit bacterial growth by targeting special structures. The in vitro activity of antibiotics against bacteria being associated with periodontal disease has been measured by several European groups. A comparison between Spain and the Netherlands found a higher proportion of resistant strains in Spain and underlined the dependence of increased antibiotic resistance on antibiotic consumption in the respective countries.[212,213] In Spain, the percentage of beta-lactamase producing strains was high, with 54% of the isolated *Prevotella* strains being positive in 2007.[214] In the Netherlands, the commonly used antibiotics in dentistry proved to be active against oral species tested; in 2012, none of the tested 50 *P. gingivalis* strains exerted any resistance.[215] In Portugal, where resistant strains were screened for

the presence of resistance genes, 55% of black-pigmented strains (*P. intermedia*, *P. nigrescens*, *P. gingivalis*) harbored either an *ermF* gene or a *tetQ* gene or both.[216]

The use of quinolones was has been repeatedly discussed in the context of periodontal treatment. Moxifloxacin was shown to be active against *A. actinomycetemcomitans*,[217] but also against anaerobes,[218] with in vitro documented activity on intracellular pathogens[219] and within biofilms.[220] Yet, it is shown to develop resistances in vitro[221] and in vivo.[222] Hence, due to its toxicity and the rapid development of resistance against quinolones, moxifloxacin has not been introduced in periodontal therapy, and according to a decision made by European Medicines Agency and European Commission in 2019, the use of fluoroquinolones should be limited mainly to patients in hospital care, with only very few outpatient indications (EMA/175398/2019). Therefore, they should not be used in the context of periodontal treatment.

Currently, azithromycin can be used as an adjunctive medication, for example, in treating advanced periodontitis of penicillin-allergic patients. Another benefit is due to a 3-day course, one tablet per day which may improve patient compliance. As shown in a multispecies biofilm model, the combination of amoxicillin and metronidazole resulted in the strongest reduction in total bacterial numbers, but azithromycin also reduced bacterial counts significantly.[100] To draw clear conclusions, however, comparable data on their efficacy are very limited so far.[223,224]

A clinically relevant question is what kind of patient could gain benefit from adjunctive systemic antimicrobial use. In a Swiss randomized clinical trial where patients received non-surgical periodontal treatment and the combination of metronidazole and amoxicillin (test group) or placebo (controls), an improved clinical outcome was shown in the test group irrespective of their baseline detection of *A. actinomycetemcomitans*, sex, age, or smoking status.[225] Recently, a German group aimed to identify thresholds for recognizing patients potentially benefitting from adjunctive antimicrobials; the patient's age (<55 years) and severity of periodontal disease (the baseline proportion of pockets ≥5 mm exceeding 35% and mean attachment level >5 mm) may guide the clinician to select such a treatment option.[226] One potential patient group could be smokers whose periodontal treatment outcome is weaker than that of non-smokers. A comparison between periodontitis patients treated with or without the combination of metronidazole and amoxicillin revealed a significant microbial shift in the test group towards reduced amounts of genera involved in periodontitis and an increase of commensals following adjunctive antimicrobial therapy.[227] Moreover, studies in several Central and North European countries (Germany, Sweden, Switzerland) do not show an increase in resistances in periodontitis-associated species when using the combination of amoxicillin and metronidazole[222,228] or metronidazole alone[229] as adjunctive treatments.

Another interesting question is whether metronidazole alone, ie, a narrow-spectrum medication targeted to strict anaerobes, is sufficient as an adjunctive antimicrobial. Compared to a combined regimen of two medicines, this could be expected to have fewer side effects and to limit unwanted effects on the aerobic and facultative residents of the oral microbiome. An adjunctive metronidazole treatment has been shown to result in reductions of *P. gingivalis* and *T. forsythia*, persisting up to 12 months after treatment.[230] In a recent clinical study dealing with *A. actinomycetemcomitans*-negative individuals,[231] better clinical outcomes were demonstrated for patients adjunctively treated with amoxicillin and metronidazole than for those treated with metronidazole alone. Similarly, the combinatory regimen was more effective than metronidazole against strict anaerobes *P. gingivalis*, *T. forsythia*, and *T. denticola*. In comparison to subgingival instrumentation alone, the adjunctive use of systemic antimicrobials, especially the combination of metronidazole and amoxicillin, has been shown to result in significantly improved probing pocket depth, clinical attachment, and bleeding on probing values up to 6 and 12 months after treatment.[209] Despite these observed favorable clinical effects when adjunctive systemic antimicrobials are used, the counter case for adjunctive antibiotic use is drug resistance, which is a serious health and socio-economic problem globally.[232] Therefore, in the EFP S3 clinical practice guidelines, created to support clinicians in their decision-making in the treatment of stage I-III periodontitis, the routine use of systemic antibiotics as adjunct to subgingival debridement is not recommended.[233] However, their adjunctive use may be considered for specific patient categories, for example, generalized periodontitis stage III in young adults.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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