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REVIEW ARTICLE

Microbial diagnostics in periodontal diseases

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Abstract

Microbial analytical methods have been instrumental in elucidating the complex microbial etiology of periodontal diseases, by shaping our understanding of subgingival community dynamics. Certain pathobionts can orchestrate the establishment of dysbiotic communities that can subvert the host immune system, triggering inflammation and tissue destruction. Yet, diagnosis and management of periodontal conditions still rely on clinical and radiographic examinations, overlooking the well-established microbial etiology. This review summarizes the chronological emergence of periodontal etiological models and the co-evolution with technological advances in microbial detection. We additionally review the microbial analytical approaches currently accessible to clinicians, highlighting their value in broadening the periodontal assessment. The epidemiological importance of obtaining culture-based antimicrobial susceptibility profiles of periodontal taxa for antibiotic resistance surveillance is also underscored, together with clinically relevant analytical approaches to guide antibiotherapy choices, when necessary. Furthermore, the importance of 16S-based community and shotgun metagenomic profiling is discussed in outlining dysbiotic microbial signatures. Because dysbiosis precedes periodontal damage, biomarker identification offers early diagnostic possibilities to forestall disease relapses during maintenance. Altogether, this review highlights the underutilized potential of clinical microbiology in periodontology, spotlighting the clinical areas most conductive to its diagnostic implementation for enhancing prevention, treatment predictability, and addressing global antibiotic resistance.

KEYWORDS
clinical microbiology, diagnostics, dysbiosis, metaproteomics, next-generation sequencing, oral microbial communities, oral microbiology

1 | BEYOND KOCH'S POSTULATES

The ability to detect microorganisms and correlate their presence with specific symptoms has shaped our understanding of infectious diseases. This understanding owes much to the pioneering work of historical figures such as Louis Pasteur (1822–1895), or to his contemporary, Robert Koch (1843–1910), whose postulates still provide a systematic framework for establishing a causal relationship between a microbe and a disease. According to these postulates, a microorganism should be present in every case of

the disease, isolated from the host, grown in pure culture, and cause the same disease when introduced into a healthy host. Corollaries to these postulates entail that the sole detection of a pathogen holds a diagnostic value, and as a result, treatment success hinges on the effective eradication of the pathogen, while prevention revolves around avoiding exposure to it. To this day, the identification of causative pathogens remains the cornerstone of infectiology, and modern microbial detection methods have enabled us to fulfill these postulates with unprecedented precision. For instance, high-throughput sequencing of bacterial 16S rRNA genes has proven successful in detecting pathogens that were previously missed by traditional culture techniques or broad-range PCR amplification, thus contributing to informed therapeutic decisions.[1]

Whereas Koch's postulates have undeniably laid the foundations of modern clinical microbiology, they appear limited to the description of purely pathogenic relationships between hosts and microorganisms. The human body, however, hosts several microbial ecosystems that evolve in homeostasis with their host, and thus, fail to abide by Koch's postulates. Typically, the oral cavity is "homeostatically colonized" by one of the richest and most complex microbiota, primarily dominated by the domain Bacteria.[2] The 774 species-level taxa currently identified within the oral microbiota exist in a mutually beneficial equilibrium, contributing to the regulation of our immune system and preventing colonization by exogenous pathogens. However, ecological alterations that disrupt this homeostasis may lead to shifts in microbial community composition and trigger aberrant inflammatory host responses, a state known as dysbiosis.[3] In a dysbiotic state, the sole detection of one pathogenic taxon holds no diagnostic value, and treatment and prevention strategies rely on re-equilibrating and maintaining homeostatic factors. Indeed, Socransky had foreseen this at the early stages of contemporary research in periodontal microbiology, and in 1979, he proposed a modification of Koch's postulates to better fit the context of periodontal diseases.[4] Although diverging from Koch's original purpose, the application of microbial analytical approaches in the oral ecosystem aims at detecting ecological shifts in oral microbial communities and assessing the expressed microbial effectors that underlie the specific disease under scrutiny. Thus far, the application of microbial analytical approaches has been mostly driven by the research purpose of etiological discoveries, with few efforts invested in translating this knowledge to tangible clinical applications.[5,6] Although the potential of clinical microbiology approaches remains underexploited, their implementation in oral healthcare may prove highly valuable to assess early risks for oral diseases, complement clinical observations to support diagnosis and treatment planning, and finally, provide auxiliary information for prognosis and monitoring of outcomes. This review aims to summarize how microbial analytical methods have shaped our understanding of periodontal diseases and spotlight those clinical microbiology approaches that may relevantly bridge to patient-oriented applications.

2 | CO-EVOLUTION OF MICROBIAL DETECTION WITH PERIODONTITIS ETIOLOGICAL MODELS

2.1 | From culture to DNA probes

The first scientific model that attempted to explain the etiology of periodontitis closely followed the establishment of the "Germ Theory" by L. Pasteur and R. Koch. Relying on the theory's fundamental principle that attributes infectious diseases to microorganisms, Willoughby Dayton Miller (1853–1907), an American dentist and student of Robert Koch, extended this principle to the endogenous nature of oral diseases. His pioneering culture and light microscopy observations, albeit rudimentary, led him to formulate his "chemo-parasitic" theory, which surmises that dental plaque bacteria can challenge the integrity of gingival and dental tissues.[7] The further refinement of basic microbial methodologies, together with the development of novel detection techniques throughout the 20th century, have gradually enhanced our understanding of the role of bacteria in the etiology of periodontitis. As such, the technological improvements to microbial methodologies have rendered it possible to address more complex scientific questions and resulted in more accurate discoveries. Figure 1 didactically links the emergence of key microbial hypotheses in periodontology, with the microbial techniques that led to their formulation.

Until the 1970s, the prevailing theory suggested that periodontal inflammation resulted from the overall accumulation of plaque bacteria. In this view, the inflammatory transition ensued from an unspecific overgrowth of indigenous oral bacteria, with little relevance attributed to taxonomic composition or species pathogenicity. This standpoint gained support from the still famous experimental gingivitis model, which demonstrated that plaque accumulation through hygiene abstinence causes reversible gingivitis.[8] This theory became known as the "non-specific plaque hypothesis."[9]

The "non-specific plaque hypothesis" was readily challenged by revolutionary advances in anaerobic culturing. For example, it became achievable to generate an anaerobic atmosphere with controlled carbon dioxide partial pressure by injecting within culture jars a mixture of nitrogen, hydrogen, and carbon dioxide. Filtration of this gas mixture through a palladium catalyst additionally ensured the absence of oxygen traces.[10] Concurrently, improved culture media formulations that included cysteine, hemin, menadione, or defibrinated sheep blood allowed the isolation of strictly anaerobic and fastidious species such as *Porphyromonas* spp. or *Prevotella* spp.[11]

These breakthroughs in culturing, together with microscopy observations, resulted in the detection of specific taxa enriched in disease-associated sites, which led to their classification as periodontal pathogens (or periodontopathic communities).[12] Figure 2 shows representative dark-field photomicrographs from deep periodontal pockets. The pathogenic character of these taxa was strongly supported by the discovery that these periodontal species express potent virulence factors.[13–15] For instance, *P.gingivalis* secretes

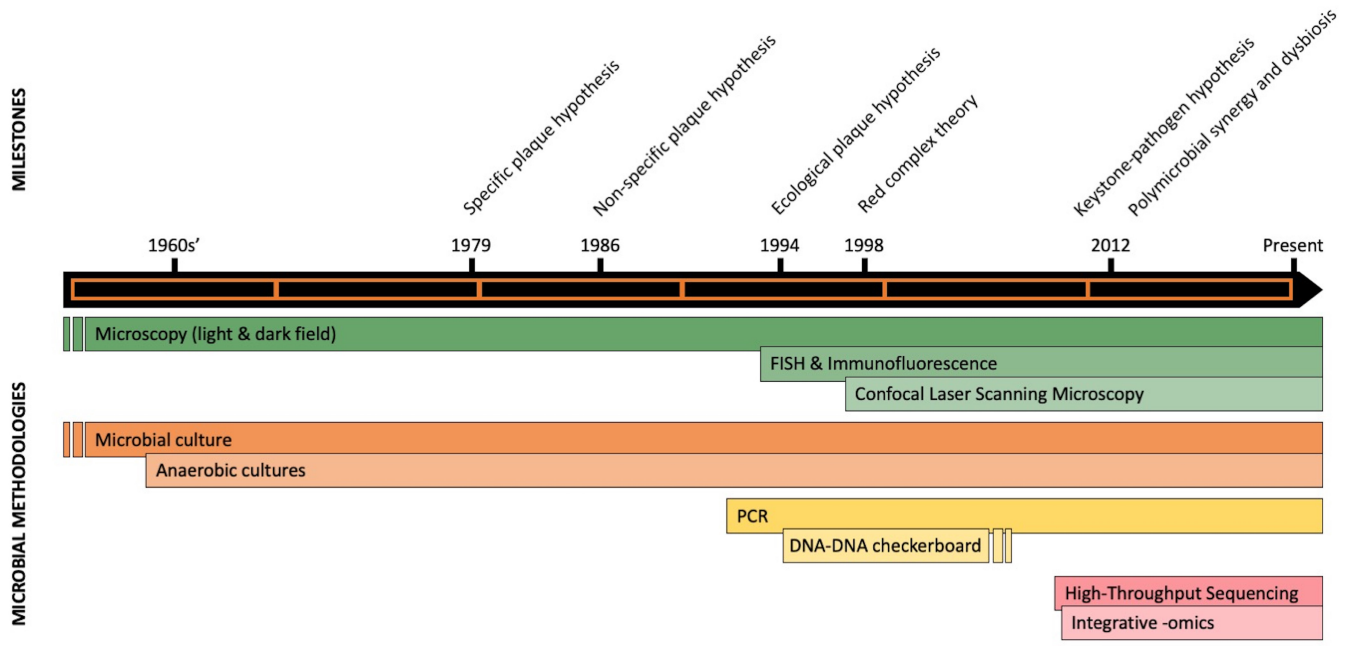

FIGURE 1 Evolution of microbial hypotheses in periodontology. This timeline overlaps the chronological emergence of key microbial hypotheses proposed to underlie the etiology of periodontitis, with the concurrent advances in microbial methodologies that shaped these theories. This timeline results from the compilation of several scientific references. "Specific plaque hypothesis,"[20,189] "non-specific plaque hypothesis,"[9,190] "red complex theory,"[22] "ecological plaque hypothesis,"[45,46] "keystone-pathogen hypothesis,"[98] "polymicrobial synergy and dysbiosis model,"[101] microbial methodologies.[191–200]

several gingipains, notably two cysteine proteases with arginine and lysine peptide bond specificity, shown to dysregulate host immune responses and thereby contribute to the species' survival and persistence.[14,16] *Aggregatibacter actinomycetemcomitans* releases a pore-forming leukotoxin that kills neutrophils.[15,17] *Treponema denticola* expresses chymotrypsin- and trypsin-like proteases that confer to the taxon highly invasive and immunomodulatory properties by degrading host tissues and impeding neutrophil motility.[13,18] Furthermore, these periodontopathic communities were detected at increased rates in distinct types of periodontal affections. Typically, "adult periodontitis" was associated with gram-negative rods, including *Fusobacterium* spp. and *Prevotella* spp., as well as high microscopical counts of *Treponema* spp.[19] "Early-onset periodontitis" was distinguished by high detection rates of *Porphyromonas* spp. and *Prevotella intermedia*, while the "localized juvenile periodontitis," a distinct form of early-onset periodontitis, was characterized by the detection of *A. actinomycetemcomitans*.[19] In contrast, higher proportions of *Streptococcus sanguinis*, *Actinomyces viscosus*, *A. odontolyticus*, or *S. mutans* were rather associated with healthy, or successfully treated sites.[19] Altogether, these findings suggested that specific microbial communities were underlying the etiology of distinct periodontal affections, and culminated in the "specific plaque hypothesis" in 1979.[20] The "specific plaque hypothesis" entailed two important clinical corollaries; first, that detection of these periodontopathogens in elevated proportions holds diagnostic value, and second, that some form of antimicrobial chemotherapy should be used as adjunct to mechanical debridement.[20] The "specific plaque hypothesis" described periodontitis as deriving from a conventional host–pathogen infective interaction. This appreciation was influenced by the limitations of the early analytical methods underlying this hypothesis that overlooked low-abundant taxa, and a substantial part of the subgingival microbiota. Importantly, however, this understanding prompted the exploration of additional putative periodontopathogens using culture-independent techniques as these became increasingly accurate and accessible over time. Interestingly, these endeavors led to the models prevailing today, which may be perceived as refinements of the "specific plaque hypothesis" that integrate the notions of *synergistic polymicrobial interactions* and *community dysbiosis*, both further elaborated in this section.

The next years of periodontal microbiology research would witness the advent of molecular approaches to study the ecology of microbial communities, among which one pivotal breakthrough was Socransky's DNA–DNA checkerboard in the 1990s. In a nutshell, this approach was a modification of the classical Southern blot technique that enabled the detection of up to 40 different bacterial species in a maximum of 28 clinical samples, all performed on one single-nylon membrane.[21] Based on the frequency of detection of the investigated species, Socransky statistically associated co-occurring clusters of these species with the severity of periodontal lesions.[22] In doing so, this seminal work proposed the classification of subgingival bacterial species into five color complexes, among which the notorious red complex characterized by *P. gingivalis*, *Tannerella forsythia*, and *T. denticola*, was strikingly correlated with deep and active periodontal lesions.[22] Methodologically, identification of the bacterial species in the DNA–DNA checkerboard relied on the use of whole-genomic probes, which, in essence, were a purified collection

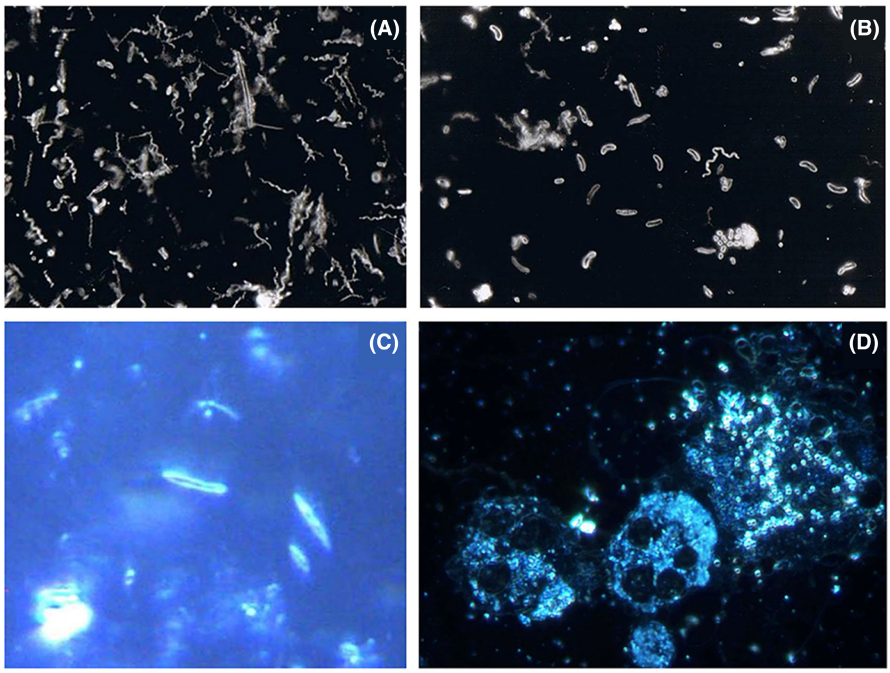

FIGURE 2 Darkfield photomicrographs showcasing the landscape of a deep periodontal pocket. A subgingival biofilm sample, collected using paper points and transported in an anaerobic transport medium, was eluted and a fraction of the resulting suspension was spread on a glass slide. Microscopy examination unveiled distinctive bacterial "morphotypes" prevalent in deep periodontal pockets. Panel (A) shows a substantial presence of motile spirochetes, while panel (B) exhibits curved motile rods, primarily indicative of *Campylobacter* spp. and *Selenomonas* spp. within the pocket environment. Panel (C) displays fusiform bacteria. In panel (D), neutrophils are discernible along with a substantial number of coccoid bacteria in their vicinity, likely indicative of localized host–microbe interactions. As frontline defenders against the periodontal biofilm, neutrophils are anticipated findings. Notably, an elevated count of neutrophils may signify rapid ongoing tissue damage. All panels were observed at a magnification of 1000×. (*Source*: the photomicrographs were compiled and reproduced from Claesson et al. 2022[161] in agreement with the terms of a Creative Commons CC-BY license with Frontiers Media SA).

of DNA extracts from pure cultures.[21,23] The undeniable advantages of this approach lay in its ability to readily detect species-level taxa in several clinical samples simultaneously, with no need to culture samples. However, prior culture of the pure species was indispensable to the generation of the probes. In other words, the technique was essentially detecting culturable taxa, albeit with greater sensitivity and higher throughput.

2.2 | The 16S rRNA gene for taxonomy identification

Fairly in parallel to the DNA–DNA checkerboard, in 1990, the field of periodontal microbiology was revolutionized by the emergence of the 16S ribosomal RNA gene as a new tool for the systematic classification of bacterial taxonomy.[24] The gene bears several distinctive features that render it uniquely suitable for taxonomic identification, particularly within complex polymicrobial communities.[25,26] The 1500 bp average sequence of the gene encodes the RNA strand that composes the ribonucleoprotein complex of the small ribosomal subunit required for protein translation. This function is indispensable to life, and the gene is therefore ubiquitous in all prokaryotes. Additional features rely on its sequence, which combines slow-evolving regions along with nine fast-evolving regions. The slowly evolving regions guide the rRNA self-hybridization into its 3D folding. Because the folding is essential for the rRNA to reach its functional structure, sequences of these slowly evolving regions must be conserved.[27] In contrast, the fast-evolving regions are "variable" in that their sequence differs among distinct taxa, thereby constituting valuable targets for taxonomic assignment.[28] These variable regions are numbered from V1 to V9, and taxonomic assignment is commonly achieved by spanning a compilation of variable regions.[29] Oral microbiology studies typically target regions V1 to V2, V3 to V4, or V4 alone to generate community profiles that confidently reach the genus level.[30,31] It is noteworthy that regions V1 to V2—sometimes including V3—are longer and display higher variability.[32] These features endow these regions with higher discrimination potential and have been shown to possibly distinguish between species of the genus *Streptococcus*, acknowledged for their similarity.[30,33]

These features enable the 16S sequence to be exploited either in a closed-ended manner (understand taxa-targeted) or in an open-ended manner (untargeted to specific taxa). Researchers have initially used the 16S in a closed-ended manner, mostly by designing PCR primers that anneal within the taxa-specific variable regions. An amplification using such primers was testimony of the presence of these specific taxa. This approach was originally used to confirm the occurrence of previously identified bacteria in the subgingival ecosystem, yet with PCR-like sensitivity. Besides bacterial presence,

it was soon possible to quantify bacterial abundance with the advent of quantitative PCR (qPCR) in the early 2000s.[34–36] Although these molecular approaches were culture independent, their closed-ended nature impeded the discovery of new taxa. Their application, however, drastically facilitated the identification of fastidious periodontal pathogens in pockets and consolidated the "specific plaque hypothesis."

2.3 | Ecological perspective on periodontal diseases

The real turning point emerged with the use of the 16S in an open-ended manner. This entailed amplifying any 16S variable regions using PCR primers in the flanking conserved regions and sequencing the PCR products.[37] With this approach, any 16S sequence from virtually all bacteria present in a sample can be detected irrespective of their prior characterization. In its original application, the PCR amplicons were cloned to maximize the likelihood of multiplying and sequencing individual bacterial sequences.[38] Taxonomy was then assigned by comparison with existing 16S databases, and yet-unknown sequences were assigned a "phylotype" based on similarities with known taxa (an approach conserved to this day).[39] This approach unveiled an unprecedented diversity of the subgingival microbiota comprising around 400 species-level taxa, of which 215 were novel, likely species-level, phylotypes.[40] The realization of this microbial "dark matter," hidden in artificial culture, spurred considerable efforts to devise innovative culture strategies to characterize their physiological and pathological properties.[41,42] This involved simulating the ecological niche of these taxa in vitro, typically by diluting the culture media with saliva for oligotrophic taxa, or supplementing it with compounds naturally present in their polymicrobial ecosystem (e.g., acyl-homoserine lactones, siderophores, spent medium from "helper" species).[42–44]

The revelation of this previously unrecognized microbial diversity not only introduced a myriad of unknown taxa but also drew attention to the presence of typical periodontopathogens in healthy sites. With this, the key concept of ecological balance within microbial communities emerged, bridging the apparent dichotomy between the "non-specific" and "specific" plaque hypotheses. This is the framework of the "ecological plaque hypothesis," which posits that the microbiota and the host coexist in a homeostatic equilibrium during health.[45,46] Yet, ecological alterations may generate a microenvironment conducive to the expansion of inflammophilic periodontal pathogens that now increase in proportions or virulence.[47] These taxa elicit aberrant inflammatory responses that create a microenvironment bearing higher peptide content, higher pH, and reduced redox potential.[48] Within this altered ecosystem, these periodontopathogens thrive and instigate a self-perpetuating cycle that fuels sustained inflammation, which in turn supports further selection of pathogenic taxa.[49] This cycle gradually leads to a dysbiotic state, marked by shifts in the composition and metabolic function of the subgingival microbiota that are no longer compatible with health. These alterations may be detected clinically as early as the gingivitis stage.[50–52] Periodontitis, therefore, results from the induction of a self-destructing inflammatory response driven by microbial dysbiosis.[53,54]

2.4 | High-throughput technologies to tackle the subgingival diversity

The need to better grasp this subgingival microbial diversity prompted the development of microbial identification methods able to address such complex composition. The original "cloning and sequencing" was hardly affordable on large scales and was displaying limited throughput. Yet, relying on the 16S sequences identified in the subgingival microbiota, a microarray method was developed that allowed the detection of over 300 species-level taxa using predefined DNA probes; namely the human oral microbe identification microarray (HOMIM).[55] The application of HOMIM proved especially valuable in comparing the distribution of these 300 species across different periodontal conditions, although its closed-ended nature prevented further exploration of new taxa.[56–58] One important achievement of these efforts to compile 16S sequences of oral taxa was the creation of a dedicated repository—the Human Oral Microbiome Database (HOMD).[59] With the concurrent development of next-generation sequencing (NGS), that is, high-throughput sequencing technologies, HOMD became a reference database for taxonomic assignment.[2,60] To this day, sequencing and mapping the 16S rRNA gene remain instrumental for the identification and phylogenetic classification of bacterial taxa in an open-ended manner.[30,61] The method, however, is bound to inherent limitations as the 16S sequence is poorly informative of other genomic regions,[62,63] which yet underpin bacterial pathogenicity in periodontitis.

The ever-increasing sensitivity and base call accuracy of NGS technologies allow the direct sequencing of microbial genomic fragments present in a sample without prior PCR amplification.[64] This approach is termed shotgun metagenomic sequencing. By sequencing virtually every bacterial genomic fragment, complex communities can be profiled with taxonomic resolution reaching the species or strain level.[65,66] More importantly, sequence reads may be employed to re-assemble whole genomes or predict the functional potential of microbial communities.[67] These developments led to the identification of new disease-associated taxa. Typically, yet-uncharacterized species of *Desulfobulbus*, *Dialister*, or *Anaeroglobus* were recognized, and the presence in deep pockets of asaccharolytic gram positives such as *Peptostreptococcus stomatis* or *Filifactor alocis* was described.[5,68–72]

While the level of taxonomic characterization provided by metagenomics offers indispensable insights into microbial communities, full elucidation of periodontitis etiopathogenesis ultimately requires knowledge of the actively expressed effectors. Integration of metagenomic data with metatranscriptomics or metaproteomics becomes imperative in this pursuit.[73,74] An effective approach to delineate the functional roles of specific

subgingival bacteria within the periodontal niche involves metatranscriptomics, which includes analyzing the entire set of active gene transcripts in the microbial community. Metatranscriptomic investigations of subgingival plaque have unveiled disease-specific microbial transcripts, highlighting a conserved core metatranscriptome associated with periodontitis.[75] Despite significant variabilities between sites and patients, this core metatranscriptome predominantly involves metabolic and biosynthetic pathways, including iron acquisition, lipopolysaccharide synthesis, and the production of short-chain fatty acids.[73,75,76] Interestingly, evidence shows that bacteria usually associated with health may exhibit heightened activity during periodontitis, typically transcribing putative virulence factors associated with cobalamin synthesis, proteolysis, or potassium transport.[73] Furthermore, treatment does not appear to restore the subgingival microbial communities to a "healthy" microbiota; instead, it tends to diminish the existing bacterial activity, particularly at actively progressing diseased sites.[77] Certainly, bacteria are acknowledged for their ability to adapt to distinctive physicochemical conditions through the modulation of protein synthesis, metabolism, and the secretion of small biomolecules. Thus, a comprehensive understanding of subgingival microbiome functional diversity necessitates integrating DNA- or RNA-based information with high-throughput measurements of microbial metabolic products and proteins, namely through metabolomics and metaproteomics.[78–82]

In this context, metaproteomics emerges as a powerful tool, offering a more comprehensive and real-time snapshot of the subgingival microbiota compared to DNA or mRNA transcripts.[83] Mass spectrometry and bioinformatics are the current tools for generating metaproteomic data from polymicrobial communities. Metaproteomics has been effectively employed in various studies to characterize in vitro grown, mono- or multispecies subgingival biofilms (recently reviewed in Bostanci et al. 2021[82]). Focusing on the proteins expressed or secreted by specific periodontal pathogens, the "metaproteome" can shed light on how the cellular units of the subgingival microbiota interact with each other and compete for nutrients and resources, that is, "which organism is doing what."[84–89] Although metaproteomics has been in existence for nearly 20 years, their application to oral microbial communities is still considered to be in its early stages, and specific explorations of the metaproteomic profiles of communities at different stages of the disease are still warranted. The complexity and heterogeneity of subgingival microbiota pose significant challenges in accurately identifying and quantifying proteins in the metaproteome, making it a more daunting task compared to conventional proteomics of single organisms.

2.5 | The current ecological framework: polymicrobial interactions orchestrate dysbiosis

Whereas the extensive taxonomic and metagenomic profiling of subgingival communities reinforced the concept of "ecological plaque hypothesis," it also highlighted the need to better understand the functional interactions driving dysbiosis.[90] Attention was naturally turned first to known inflammophilic periodontal pathogens such as *P. gingivalis*.[91] The taxon demonstrates the ability to inhibit the secretion of IL-8, thereby potentially hampering neutrophil chemotaxis.[92–94] This inhibition may facilitate the establishment of *P. gingivalis* in its niche and foster the proliferation of other taxa.[95,96] Moreover, the gingipains released by *P. gingivalis* were found to act as convertases on the complement, generating C5a moieties that initiate a subversive cross-talk between the C5a receptor and the Toll-like receptor 2. This interaction hinders the killing activity of neutrophils and is essential for the persistence of *P. gingivalis* in the subgingival ecosystem.[95] In essence, despite its low abundance within a microbial community, *P. gingivalis* exerts a community-wide impact that is able to tilt the balance toward dysbiosis. This prompted the concept of "keystone pathogen," drawing an analogy to the marine ecological definition of "keystone species," precisely referring to species that disproportionately affect their communities despite their low abundance.[97,98] Two notions unfolded from this "keystone pathogen" concept; first, that *P. gingivalis* requires physiological and metabolic support from its fellow consortium members to exert its pathogenicity, and second, that other members also possess the ability to heighten the overall community pathogenicity.[3,49,99,100] These notions reconciled the "ecological plaque hypothesis" with the concept of "keystone pathogen" in the model of "polymicrobial synergy and dysbiosis".[101]

Metatranscriptomics and, to a greater extent, metaproteomics emerged as analytical tools of choice to investigate the effector pathways exploited by putative periodontopathogens to enhance the pathogenicity of their whole community.[82] One such example is the species *Anaeroglobus geminatus*, a strictly anaerobic gram-negative coccus of the Veillonellaceae family showing close relation to the genus *Megasphaera*.[102] The species was shown to display higher rates of subgingival colonization in cases of chronic and aggressive periodontitis, although a potential role of *A. geminatus* in influencing the composition and dynamics of polymicrobial communities remained unexplored.[103] The application of an LC-MS/MS label-free proteomic approach in an in vitro polymicrobial subgingival biofilm model revealed the ability of *A. geminatus* to instigate the proliferation of other periodontopathogens, such as *P. intermedia*, and to up-regulate virulence properties across the entire community.[70] Similarly, mounting evidence also points to the newly discovered *F. alocis* as another potential orchestrator of microbial community dynamics. Epidemiologically, the taxon appears to co-occur with *A. actinomycetemcomitans*, and to be associated with increased attachment loss.[57] Investigation into its pathogenic potential unveils a repertoire of virulence factors, notably the taxon expresses a membrane protein that binds the C3 moiety, thereby impeding a pivotal step in complement activation.[104] *F. alocis* is also notorious for its secretion of extracellular vesicles that interact with Toll-like receptor 2, promoting osteoclastogenesis.[105,106] A proteomic exploration of these vesicles delineated strain-specific differences in excretion, establishing a connection between this secretory activity and distinct virulence profiles among strains.[88] Additionally, the recent

identification of FtxA, an unknown protein belonging to the RTX superfamily of exotoxins with cytolytic activity, further underscores the multifaceted virulence strategies employed by *F. alocis*.[71,89]

These findings highlight how low-abundant members of the subgingival microbiota act as drivers of dysbiosis and orchestrate pathogenicity.[3] Their adeptness at exploiting synergies with fellow community members and manipulating host immune responses underscores the complexity of microbial interactions in the context of periodontal health and disease.

3 | CLINICAL MICROBIOLOGY FOR PERIODONTAL DIAGNOSTICS

The era of the "specific plaque hypothesis," which posited that defined microbial clusters were the primary instigators of periodontal diseases, provided the ideal ground for targeted microbial detection. Within this framework, the identification of specific taxa was holding diagnostic value; a concept fairly close to the "classical" medical approach geared toward detecting exogenous pathogens in mono-infections. It is now clear that these concepts of microbial detection for diagnostic purposes cannot be seamlessly transposed to periodontology; instead, they require tailored translation to account for the polymicrobial and ecological dynamics inherent to the etiology of periodontal diseases. The next subsections offer a description of the clinical microbiology analytics either currently accessible in periodontology or with prospective clinical applications.

3.1 | Bacterial culture and antimicrobial susceptibility testing

In spite of the most recent advances in microbial genomics, bacterial culturing remains the mainstay to provide a phenotypical description of a taxon. As outlined in previous sections, such functional, biochemical, and enzymatic properties are crucial to complement genomic data and to achieve the complete characterization of a taxon.[107] Besides its research applications, bacterial culturing also finds indispensable clinical use, notably in the acquisition of antimicrobial susceptibility testing (AST) data for clinical isolates.[108]

AST involves dispersing a previously isolated clinical bacterial strain onto a culture agar, exposing it to various antibiotic concentrations through diffusion from discs or gradient strips. The strain's ability to grow in the presence of increasing antibiotic concentrations determines minimal inhibitory concentration (MIC) values. Figure 3 illustrates an example of MIC determination for vancomycin using two oral clinical isolates of *Enterococcus faecalis*.[108] International institutions such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the Clinical Laboratory Standards Institute (CLSI) issue guidelines regulating AST procedures to ensure standardized and comparable MIC values across laboratories. Ultimately, the compilation of thousands of MICs, coupled with parameters such as prevalence of resistance, pharmacokinetics, or pharmacodynamics, serves to establish clinical breakpoints and provide guidance for prescription. In brief, if an isolate's MIC falls below the clinical breakpoint of that species for a given antibiotic, it is deemed "susceptible" and treatment with this antibiotic is likely to be successful. Conversely, if the MIC surpasses the clinical breakpoint of the species, the isolate is deemed "resistant" and use of this antibiotic is unadvised.[109]

AST stands as the primary tool for unequivocally confirming phenotypical antibiotic resistance, and although its application in periodontology faces the challenges imposed by the 30% of yet-unculturable subgingival taxa, AST remains instrumental in acquiring crucial epidemiological data. In a comparative study, ASTs were conducted using first-resort antibiotics (amoxicillin, amoxicillin, clavulanate, penicillin G, clindamycin, tetracycline, ciprofloxacin, and azithromycin) on a spectrum of typical periodontal taxa collected from Spain and the Netherlands, including *P. intermedia*, *Parvimonas micra*, *A. actinomycetemcomitans*, *Fusobacterium nucleatum*, and *P. gingivalis*.[110] Their outcomes revealed an increased prevalence of resistant taxa in Spain, notably in isolates of *A. actinomycetemcomitans*, *F. nucleatum*, and *P. intermedia* to amoxicillin.[110] These observations were complemented and extended by another group, which demonstrated that 17%–26% of "Spanish" periodontal *Prevotella* spp. isolates were resistant to amoxicillin.[111] This resistance could be in part attributed to the detection of beta-lactamase production in 54% of these *Prevotella* spp. isolates. In contrast, a study that investigated the AST profiles of 247 periodontal isolates to commonly prescribed antibiotics in the Netherlands reported only few isolates of *A. actinomycetemcomitans*, *F. nucleatum*, and *P. intermedia* to show resistance to amoxicillin.[112] These findings align with a recent longitudinal German study spanning 8 years (2008–2015) that assessed antibiotic resistance in typical periodontal taxa, including *P. intermedia*, *F. nucleatum*, *P. gingivalis*, and *T. forsythia*. This comprehensive investigation, which tested between 2196 and 3881 isolates every year, observed a significant increase in the number of resistant taxa over the years.[113] Although implementation of ASTs in periodontology on an individual basis is likely impracticable, the compilation of such epidemiological data finds paramount microbiological relevance both for resistance surveillance and to provide prescription guidance.

3.2 | qPCR for the clinical identification of signature microbial profiles

Quantitative PCR is a molecular technique used to quantify the amount of a specific DNA sequence in a given sample.[114] It involves the amplification of the target DNA, most commonly coupled with fluorescent-intercalating agents, allowing the real-time monitoring of the amplification process and providing accurate quantification of the initial DNA concentration in the sample.[115] Typically, qPCR can be employed to detect and quantify selected species using primers that anneal into the taxa-specific variable regions of the 16S rRNA gene.[116] It is further possible to transform the amount of DNA

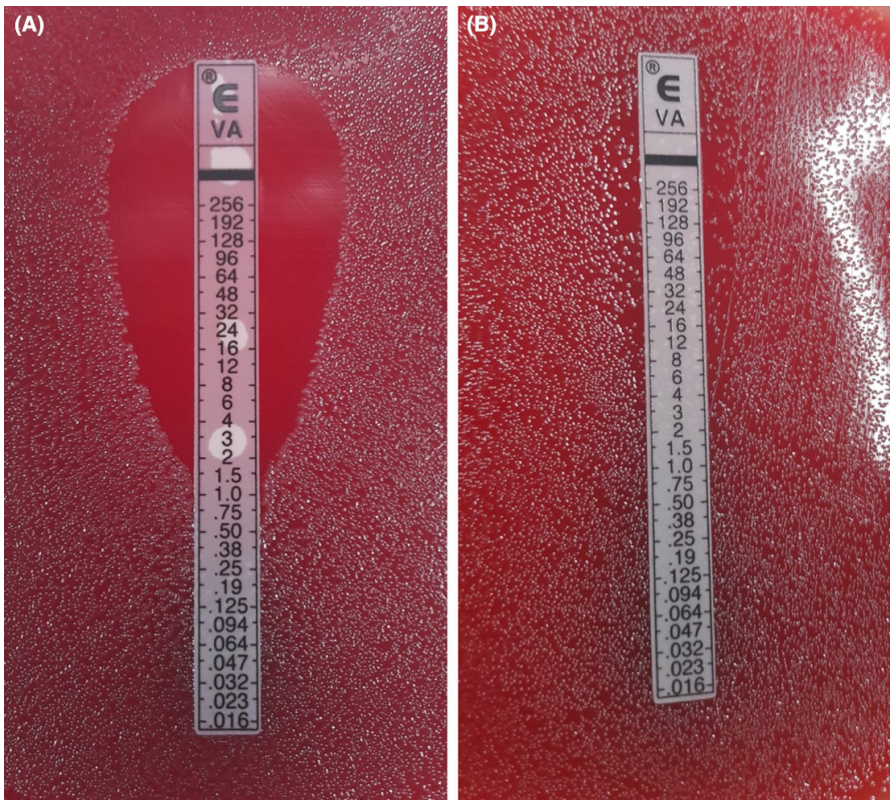

FIGURE 3 Antimicrobial susceptibility testing using Etest gradient strips. Photographs show two distinct *Enterococcus faecalis* oral isolates plated onto Mueller–Hinton agars (supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD), onto which vancomycin gradient strips were deposited. Strips are labeled with the vancomycin concentration, in micrograms per milliliter (μg/mL). Panel (A) shows a vancomycin-susceptible *E. faecalis* isolate, which MIC can be read at the intersection of the lower part of the ellipse-shaped inhibition zone with the strip (1.5 μg/mL). This typical shape results from the diffusion of increasing vancomycin concentrations along the strip. In contrast, panel (B) shows a vancomycin-resistant *E. faecalis* isolate, recognizable by the irregular ellipse-shaped inhibition zone, lacking a clear intersection point and displaying colonies that invade the area. The clinical breakpoint for the species is defined at 4 μg/mL.

detected into the number of cells of each species investigated using their genomic weight.[117,118]

The growing recognition of the role of subgingival communities in periodontitis has catalyzed the development of validated clinical oral microbiology laboratories that offer qPCR services to periodontists. To date, such qPCR tests are rather broadly accessible and include, for instance, the IAI PadoTest® ("Institut für Angewandte Immunologie"—mostly Switzerland and Germany but available across Europe),[119] PerioPOC® (distributors all over the world),[120] or MyPerioPath®[121] and HR5®[122] (exercising in the United States). These qPCR services typically encompass the quantification of a panel of periodontal taxa, ranging from 5 to 11 species, often classified to mirror Socransky's complexes. Figure 4 showcases an example of qPCR test results generated using the services of such laboratories.[123,124] Whereas companies tend to advertise these tests beyond their scientifically established value, they may find clinical relevance provided that the periodontist understands the significance of the findings and is able to translate them into informed therapeutic decisions. The interest in qPCR microbiological analytics in periodontology is multifaceted.

Originally, these efforts were geared toward the identification of predefined periodontopathogens, which detection was meant to guide the clinician when considering the need for an antibiotic treatment adjunctive to mechanical debridement.[125] To date, there is rather little evidence to support this approach.[126,127] A retrospective study evaluated whether implementing an adjunctive antibiotherapy, informed by microbiological analyses, positively impacted treatment outcomes.[128] Their findings supported that the incorporation of antibiotherapy, amoxicillin/metronidazole, may enhance treatment outcomes specifically when detecting typical periodontopathogens, such as members of the "red complex."[128] The use of adjunct antibiotics may also find a rational in cases where *A. actinomycetemcomitans* is specifically detected regardless of other periodontopathogens. As *A. actinomycetemcomitans* colonization is not limited to periodontal pockets but also invades other oral mucosae, mechanical debridement alone is unlikely to eliminate the taxon.[129,130] In instances of *A. actinomycetemcomitans* detection, adjunct amoxicillin or moxifloxacin was shown as possible alternative to suppress the taxon, although this approach weakly correlated with clinical improvement.[129,131,132] Importantly, however, based on the few bodies of evidence currently existing, current guidelines for antibiotic prescription do not incorporate baseline detection of specific periodontopathogens, relying exclusively on the severity of periodontitis

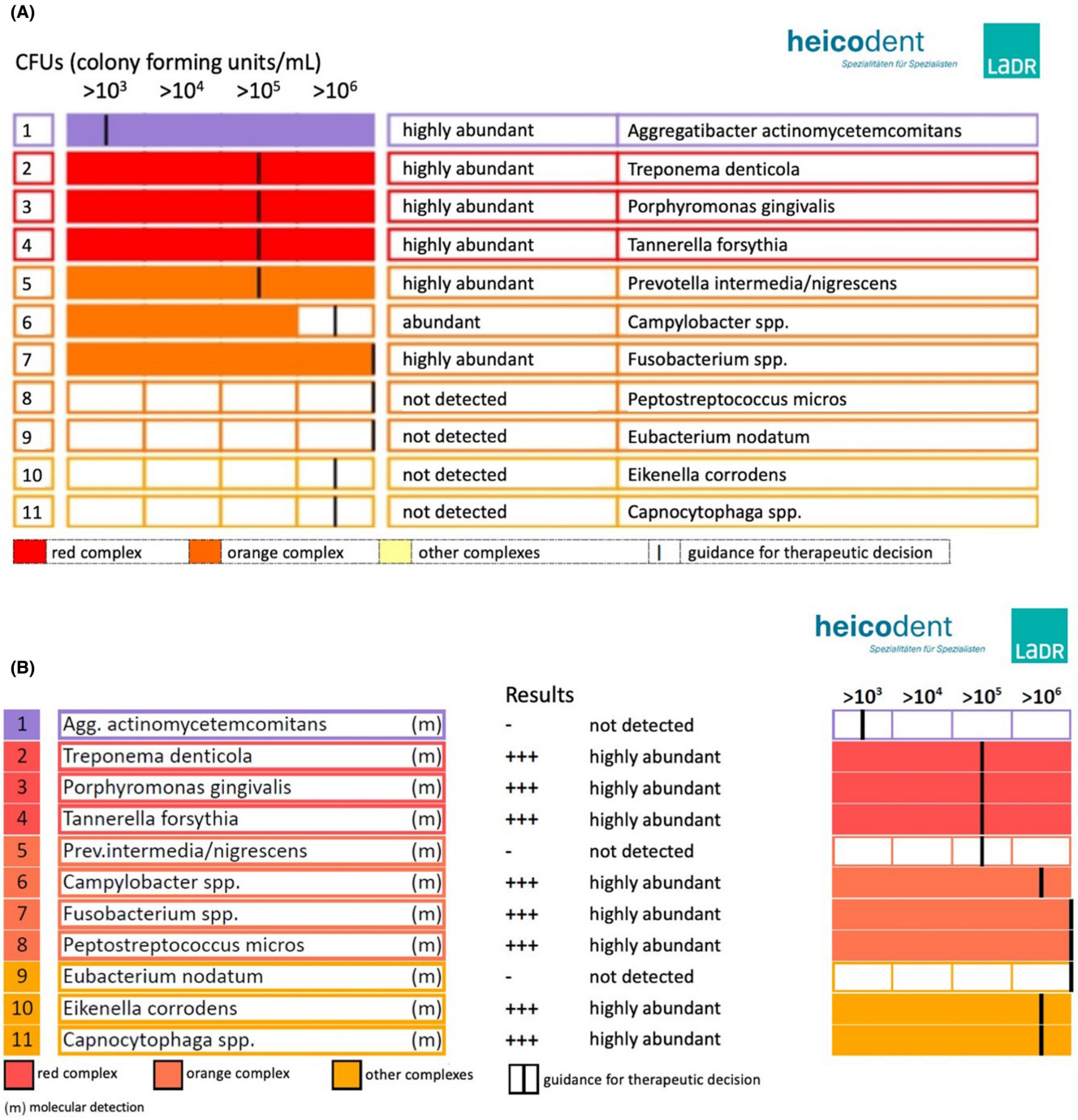

FIGURE 4 Characteristic clinical test results of subgingival qPCR assays. The figure provides an example of typical test results performed by a periodontist. On both panels, 11 taxa were investigated and their quantification is didactically displayed as horizontal bars. Increments on these bars are representative of equivalent bacterial colony-forming units/mL. In (A), samples originate from a female in her 60s displaying a "highly inflamed periodontium," halitosis, and abundant subgingival calculus on initial examination. The patient was diagnosed with Stage IV, Grade B generalized periodontitis. Reportedly, the reason for conducting the microbiological test was to gather data as to whether to complement the scaling and root planing with an adjunctive antibiotherapy. The clinician opted for an antibiotherapy using amoxicillin and metronidazole. In (B), samples originate from a female in her 40s with good oral hygiene and diligently attending her hygienist recalls. The patient presented with few supragingival biofilm deposits and minor subgingival calculus on initial examination. The patient was diagnosed with Stage II, Grade B localized periodontitis. Reportedly, the periodontist assessed that the severity and localization of periodontal lesions were incommensurate with the low amount of biofilm deposits, hence the reason for seeking therapeutic guidance from microbiological tests. The periodontist implemented an amoxicillin/metronidazole antibiotherapy adjunctive to scaling and root planing. Heicodent is a Swiss-based company that delegates its qPCR analyses to LADR laboratories in Germany. Websites of the companies: https://heicodent.ch/ and https://www.ladr.de/startseite (URL accessed on December 12, 2023). Test results are courtesy of Dr. P.-J. Loup (Lecturer, University Clinics of Dental Medicine, Faculty of Medicine, University of Geneva, Switzerland). This figure provides an example of clinically applied qPCR analytics alongside their clinical context and authors remain neutral with regard to the adopted clinical course.

as assessed by clinical parameters.[133,134] This is explained by the important microbial diversity within the periodontal niche, comprising myriad anaerobes, as well as aerobes, gram-negatives as well as gram-positives, which preclude the tailored selection of an antibiotherapy. Whereas such approach aims at clinical efficiency, it may be pointed out that the complete dissociation of microbiological characterization from antibiotic prescription may impede outcome monitoring, hinder future optimization of antibiotherapy, and compromise the surveillance of emerging resistances.[135,136]

Microbial qPCR detection holds other clinical interests besides antibiotic guidance. The latest ecological findings have re-oriented the focus of microbial detection on the recognition of taxonomic shifts driving dysbiosis.[137] Although these shifts are typically identified at the community level by open-ended sequencing approaches, several analyses of subgingival sequencing datasets have sought to pinpoint a handful of "signature" taxa indicative of a dysbiotic transition.[138,139] These taxa can be further targeted through qPCR to serve as early indicators of ecological imbalance. In a notable example, French researchers utilized deposited 16S rRNA datasets from health- and periodontitis-associated subgingival biofilms to identify genus-level taxa differentially abundant in health or periodontitis.[138] Among these differentially abundant taxa, those that were present in at least 95% of the samples of their respective group were used to define either health- or periodontitis-associated microbial markers. In doing so, the authors singled out the genera *Treponema*, *Campylobacter*, *Tannerella*, and *Eubacterium* as markers indicative of periodontitis-related biofilms, while *Veillonella*, *Neisseria*, *Rothia*, *Corynebacterium*, and *Actinomyces* served as testimony of health-associated biofilms. Interestingly, because the genus *Porphyromonas* was present in both health and periodontitis biofilms, the taxon was excluded from periodontitis markers, although it was detected in increased abundances in diseased sites.[138]

Employing a comparable strategy, machine learning algorithms were used to re-analyze a collection of 16S datasets from health and periodontitis biofilms, seeking to identify species-level taxa that differentiate both conditions.[139] The authors found more than 200 species differentially abundant in health and disease, which were further narrowed down to 49 species informatically determined to discriminate between conditions with the highest accuracy. The algorithm was shown to reproducibly and accurately classify unknown samples into either health or periodontitis associated, based on the abundance of the species within. This innovative approach culminated in the development of a "subgingival microbial dysbiosis index" (SMDI), providing a quantitative measure for determining the dysbiotic state of a subgingival microbial sample.[139] Among the species that were highly discriminating for periodontitis, one finds *T. denticola*, *T. forsythia*, *Mogibacterium timidum*, *F. alocis*, and several species of the genus *Fretibacterium*, while typical commensal taxa such as *S. sanguinis* and *Actinomyces naeslundii* ranked among top health-associated species.[139]

These studies exemplify how sequencing data can be pragmatically analyzed to identify signature taxa that can further be targeted by qPCR to serve as dysbiosis markers. This approach enables the translation of academic knowledge into clinically applicable tools with readily interpretable outcomes.

3.3 | Clinical applications of shotgun metagenomic sequencing

Conceptually, shotgun metagenomic sequencing exploits every fragment of bacterial genome in a sample, and generates a collection of "reads" representative of the aggregate genomes of the entire community.[140] The interpretation of these reads may be either achieved by computing them individually against annotated databases, termed read-based analysis (or "mapping"), or by re-assembling them into whole genomes, termed assembly-based analysis.[67] Read-based analysis is less computationally demanding and more easily deals with highly complex communities. In effect, the approach relies on the fraction of reads that effectively map against reference genomes to profile taxonomy, fairly often to the species level, and to generate an aggregate picture of the community's predicted metabolism.[141] However, as efficiency of this approach heavily depends on the comprehensiveness of the selected database, it often "struggles" to link gene functions with specific taxa. Conversely, an assembly-based analysis imposes a much heavier computational burden and requires a much deeper sequencing depth to deal with polymicrobial communities, often resolving only a fraction of the community genomes. Yet, for taxa with sufficient coverage, whole genomes can be successfully re-assembled even for entirely novel microorganisms with no sequenced relatives.[142] By re-assembling nearly full genomes, shotgun additionally allows linking genes' function to their phylogeny even for novel diversity.[143] Databases available to map shotgun sequencing reads also include dedicate libraries for genes with known functions in virulence[144] or antibiotic resistance,[145–147] which may find particular clinical relevance.[148]

Surpassing mere taxonomic identification, the ability of shotgun metagenomic sequencing to inform on the function of the genomes present has found insightful clinical interests. The application of shotgun to subgingival communities was shown to discriminate between health and disease solely based on the presence of predicted gene functions. For instance, genes associated with "invasion and intracellular resistance," "proteolysis," "toxins," or "antibiotic-resistance" were shown to be potent discriminators of periodontitis-affected sites.[149] Another notable longitudinal study monitored the metagenome of subgingival communities before and after mechanical debridement and attempted to identify microbial indicators useful for diagnosis or prognosis.[150] The authors determined that "healing" gingival sulci displayed shifts in abundance both at the taxonomic and functional levels. Analyses compiling taxonomic profiling, co-occurrence networks, and genes' functions allowed inferring the clinical state of samples with an accuracy above 80%, and more importantly, allowed predicting their clinical trajectory as assessed on subsequent visits.[150]

The utility of shotgun metagenomic sequencing in reconstructing complete genomes is especially compelling for

genotyping intraspecies genetic diversity. This holds particular clinical relevance because different strains of the same species may comprise both harmless and highly virulent variants.[151] These genetic singularities are exemplified by variations in periodontal pathogenicity among distinct strains of *P. gingivalis*,[152,153] or within the stark differences in virulence observed among strains of *A. actinomycetemcomitans*.[154] The taxon is classically subtyped into seven different serotypes, among which serotype b is associated with higher leukotoxic activity, that is, increased expression of the leukotoxin LtxA.[155] Investigations of these variants led to the identification of a 530 bp deletion within the promoter of the *ltxA* toxin-encoding gene, resulting in a loss of transcriptional regulation.[156] This genotype, referred to as JP2, is associated with a significantly elevated risk of periodontitis initiation and more severe progression, particularly in younger individuals. Additional genotypes were further identified with either an 886 bp insertion or a 640 bp deletion within the *ltxA* promoter.[157,158] Importantly, all three genotypes induce increased expression of LtxA, leading to enhanced virulence. These findings highlight the potential value of shotgun metagenomic sequencing as a strategy to monitor virulent genotypes of *A. actinomycetemcomitans*, serving as a major risk determinant for periodontal disease, especially in young populations.[159]

The clinical insights offered by shotgun metagenomic sequencing have encouraged companies to develop streamlined commercial applications. One such example is the PadoBiom® kit of the company IAI (Switzerland).[160] Although the detailed methodology and bioinformatic pipelines remain undisclosed, the company offers an array of outcomes that include:

- Evaluation of the community diversity.
- Assessment of the dysbiotic state.
- Determination of the abundance of members of the "red complex" alongside *A. actinomycetemcomitans* and *F. alocis*.
- Genotyping of *A. actinomycetemcomitans* serological subtypes a, b, c, d, and e, including identification of the JP2 genotype.
- Detection of antibiotic resistance genes to five commonly prescribed molecule classes, that is, beta-lactams, nitroimidazoles, tetracycline, quinolones, and macrolides.

Based on these microbiological parameters, coupled with periodontal probing, such a utility offers to guide the patient management into continued check-ups, the implementation of prophylaxis measures, or scaling and root planing, possibly with adjunct antibiotics.

4 | OUTLOOK ON MICROBIAL DIAGNOSTICS IN PERIODONTOLOGY

The gradual technological development of microbial analytical methods has been instrumental in exploring the microbial etiology of periodontal diseases. While these approaches have provided us with experimental tools for etiological discoveries and concept validation, they admittedly met little recognition as a global standard in routine clinical practice. Current periodontal treatment guidelines predominantly hinge on clinical parameters and empirical evidence to advise on treatment courses, prevention strategies, diagnosis, monitoring, or antibiotic prescription, overlooking the underlying microbial factors. This oversight of the microbial etiology may seem somewhat scientifically counterintuitive as mounting evidence supports the ability of microbial diagnostics to relevantly broaden the periodontal diagnosis and inform on both patients' risk and monitoring.[161,162]

One clinical situation where the implementation of microbial diagnostics may seem particularly relevant is in improving decision-making as to the use of systemic antibiotics. Currently, the decision to use an adjunct antibiotherapy is contemplated for patients presenting generalized stage III or IV periodontitis, as well as those with "molar-incisor" patterns.[134] Whereas such clinically based approach may inform on "when" to prescribe, it sorely lacks insights into "what" to prescribe. Prior taxonomic identification was shown to be of little help, except perhaps in cases of *A. actinomycetemcomitans* detection, and thus the polymicrobial nature of periodontitis continues to pose a dilemma as to the selection of an antibiotic molecule.[132,163] An alternative strategy to devise an efficient antibiotherapy may rely on the detection of antibiotic resistance genes (ARG) present within the periodontal metagenome, irrespective of their taxonomic assignment. Evidence supports the presence of a reservoir of latent ARGs within the periodontal metagenome, readily expressed upon antibiotic introduction, compromising treatment efficacy and prompting systemic dissemination.[164,165] Detecting these ARGs, or a panel of the most epidemiologically prevalent ones, can guide decisions on antibiotic selection, helping to mitigate resistance risks and enhance treatment success.[166] On a practical level, these ARGs may easily be detected by qPCR approaches prior to antibiotic selection. Notably, there currently exist point-of-care solutions that have streamlined the entire qPCR workflow on ready-to-use disks requiring only a 200 µL droplet of saliva, yielding results within a remarkably short 3-hour interval (from sample to results).[136,167] This rapid turnaround time may perfectly align with the routine pace of a dental practice, possibly envisioned as an interval between discharging the patient and confirming the prescription. Beyond aiding antibiotic selection, the screening of ARGs represents a cutting-edge advantage for surveilling emerging resistances and refining future antibiotic prescription guidelines.[168]

Another area where microbial diagnostics may relevantly bridge with clinical interests is in enabling the identification of a "disease-provoking microbiota" before periodontitis can be declared. As a dysbiosis-induced inflammatory disease, periodontitis chronologically exhibits a "dysbiotic onset" that precedes the "inflammatory onset" of the disease. Whereas host-dependent components influence the duration and severity of inflammation and tissue damage,[169] the inflammatory process itself is driven by the pathogenicity of the dysbiotic polymicrobial communities.[170] As causal factors, these dysbiotic shifts are inherently preceding

episodes of inflammatory flares, and hence their detection may predict upcoming periodontal tissue destruction.[162,171] As outlined in this review, there is mounting evidence that indicates the feasibility of translating complex sequencing data into more defined sets of signature taxa, sometimes computing microbial dysbiotic indices such as the SMDI, that may be clinically applied as biomarkers of dysbiosis.[138,139,149] This index was tested in one longitudinal study that profiled the subgingival microbiota by 16S sequencing and computed the SMDI in periodontal patients prior to and after scaling and root planing.[172] The authors demonstrated a significant decrease in the SMDI from baseline to day 1 after treatment, and a stabilization of the index for up to 3 months, indicating a steady suppression of the dysbiosis following treatment. Of note, associations between the SMDI and periodontal parameters or treatment outcomes were only limited as sampling did not extend beyond 3 months.[172] As early indicators of periodontal dysbiosis, these biomarkers could advantageously be employed for early diagnosis during recalls, or as predictors of disease relapse for patients in maintenance.[173] Evidence additionally indicates that such biomarkers should ideally cumulate microbial and host factors for maximum accuracy and predictability.[174–176] A particularly attractive application of this strategy is the detection of initial shifts from a healthy oral state toward gingivitis. Experimental gingivitis models have shown that changes in plaque community composition and metabolites, alongside alterations in host cytokine profiles, can be identified as early as 24 hours following the halt of oral hygiene.[177] The diagnostic power of these biomarkers warrants large-scale validation and the establishment of standardized protocols for sample collection, experimental methodologies, and analysis pipelines to ensure their reproducibility and clinical relevance.[178] When effectively implemented, they hold the potential to delineate an "at-risk" population for periodontitis that may benefit from early interventions to prevent tissue destruction. Figure 5 provides an example of microbial and host biomarkers that may be used to outline such an "at-risk" population prior to observing the first clinical signs of inflammation. There is value in contemplating analogous situations in medicine, where recognition of similar "at-risk" populations has prompted the development of prevention policies that drastically decreased the adverse impact of conditions such as osteoporosis or breast cancer.[179,180]

While this review focused on microbial analytical methods that have tangible clinical applications, there is value in this outlook to provide a glimpse into possible future approaches directed to ecological modulation. The concept of ecological modulation has evolved from our growing understanding of the metabolic networks that underlie the ecology of periodontal communities. Specifically, it has underscored the limitations of traditional infection control methods in reducing the pathogenicity of dysbiotic microbiota without causing adverse effects for the host. This realization prompted efforts to interfere within the ecological network to curve dysbiotic shifts back to homeostasis.[181,182] Promising strategies for ecological modulation encompass the use of prebiotics—substrates selectively metabolized by commensal

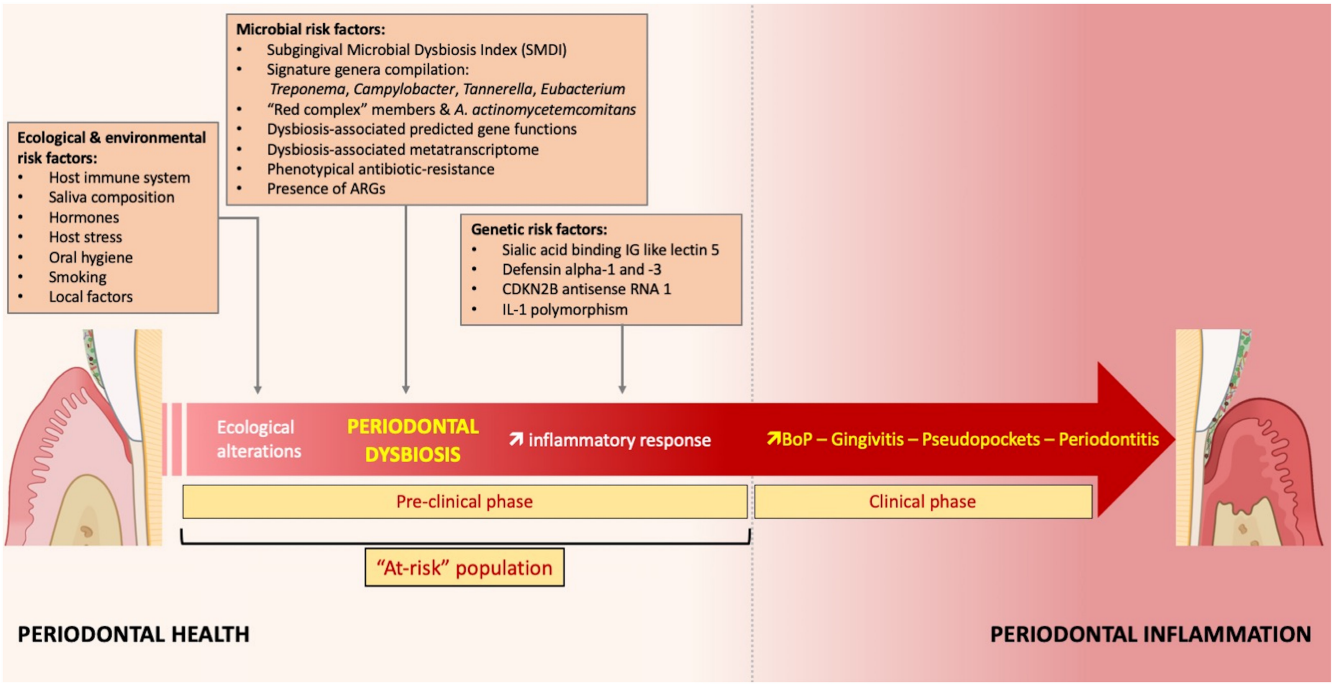

FIGURE 5 Schematic representation of the etiology and natural history of periodontitis. The scheme depicts the evolution from a healthy periodontium to periodontitis alongside the ecological, microbial, and host-derived factors known to play a role. The illustration highlights the possibility of delineating a preclinical phase, in which patients would be "at-risk" of developing further periodontal inflammation, as opposed to a clinical phase, in which inflammation becomes clinically evident. The data presented in this figure are synthesized from diverse scientific references.[54,76,138,139,149,165,201] The web interface BioRender was utilized in the design of this illustration.

taxa to promote their growth—or probiotics—live microorganisms whose functions contribute to maintaining homeostasis.[183,184] More recent approaches involve interfering with bacterial quorum-sensing communication or inoculating bacterial communities with lytic phages that target specific taxa.[185–188] Although these strategies are currently the subject of active research, their potential implementation would require the application of clinical microbial diagnostics for informed decision-making and monitoring their effectiveness.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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