Methods for Investigating the Subgingival Microbiome in Periodontitis: A Literature Review

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Abstract

Background: Periodontitis is defined as an inflammatory disease of the supporting tissues of the teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with increased probing depth formation, recession, or both (1). Current evidence from microbiome studies indicates that periodontal disease is not caused by a single pathogen, but by complex polymicrobial interactions. The development of sequencing technologies has significantly expanded the understanding of microbial diversity, composition, and function in periodontal diseases (2, 3)
Aim: This review critically evaluates methodologies used to investigate the gingival microbiome in patients with periodontitis, focusing on their advantages, limitations, and clinical relevance
Methods: A structured literature review was conducted using existing studies. The studies included human studies investigating the gingival or subgingival microbiota in periodontitis using culture-based, molecular, and high-throughput sequencing techniques. The methodologies included polymerase chain reaction (PCR), DNA-DNA hybridization, 16S rRNA gene sequencing, next-generation sequencing (NGS), and shotgun metagenomics (4)
Results: Analysis of studies shows that culture-independent molecular approaches are preferred in modern research on the periodontal microbiome. 16S rRNA gene sequencing is the most commonly used technique for taxonomic profiling, whereas shotgun metagenomics provides higher-resolution profiling at the species and functional levels. Studies consistently report increased microbial diversity and enrichment of anaerobic, proteolytic taxa during periodontitis. However, significant differences in sampling strategies, sequencing platforms, and workflows limit reproducibility and comparability across studies (5)
Conclusions: Modern microbiome research has significantly improved our understanding of periodontitis as a disease caused by dysbiosis. Despite these advances, standardization of protocols and integration of multi-omics approaches remain challenges. **(TCM-GMJ August 2026; 11 (3): P8-P11)**

Keywords: Periodontitis; Gingival Microbiome; Subgingival Biofilm; Dysbiosis; 16S rRNA Sequencing; Shotgun Metagenomics; Next-Generation Sequencing; Oral Microbiota.

Introduction

Periodontitis is a chronic inflammatory condition that causes the gradual loss of the supporting tissues of the teeth (1). For decades, the disease has been a major global health problem, affecting millions of people, and is associated with a variety of systemic health conditions.

Our understanding of periodontitis has changed significantly in recent years. Theories that attributed a few specific pathogens to the disease have been replaced by the understanding that the disease arises from a "polymicrobial dysbiosis." (2,3).

The gingival microbiome is a highly diverse ecosystem in which thousands of microbial species interact with the body's immune system (6). In a healthy mouth, these microorganisms live in a balanced state known as "symbiosis," in which they do not harm the host. However, when the environment shifts toward "dysbiosis," we observe increased microbial diversity, including specific anaerobic and proteolytic species that drive inflammation (7). Understanding this transition period is one of the most important goals of oral microbiology, and it requires modern tools that can simultaneously analyze the entire microbial landscape.

Classical Microbiology

Traditional culture-based methods and direct microscopic observations have been the gold standard for almost a century. These techniques enabled the isolation and phenotypic differentiation of organisms, thereby laying the foundation for antimicrobial susceptibility testing, a clinical need that remains relevant today (8). It was through their use that the "red complex" (composed of Porphyromonas gingivalis, Tannerella forsythia, and

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Treponema denticola) was first identified as a hallmark of severe disease

However, severe limitations of culture-based science have been revealed.

Culture-based methods are inherently time-consuming, often requiring 24 to 48 hours for common bacteria and up to several weeks for slow-growing (fastidious) organisms. This delay is a critical limitation in clinical settings where rapid decision-making is essential. (9)It is currently estimated that more than 50% of the oral microbiota is composed of "dark matter"—organisms that are fastidious, slow-growing, or obligate anaerobes whose metabolic requirements are so specific that they cannot be replicated in the laboratory (4). The most significant scientific limitation is the inability to detect the vast majority of existing microbes. It is estimated that less than 1% of environmental bacteria are culturable in a laboratory setting. This phenomenon, known as the "Great Plate Count Anomaly," means that culture-based science fails to "see" non-cultivable or slow-growing microorganisms that may still be functional or pathogenic (10). Based solely on culture, researchers have observed the microbial world beneath the gums, significantly underestimating the true biological diversity of the subgingival niche(8). Furthermore, culture-based approaches are notoriously laborious and time-consuming, often requiring weeks of incubation in specialized chambers. This drawback makes them impractical for rapid clinical diagnosis and fails to capture the complex interspecies interactions, such as metabolic signaling and biofilm cooperation, that contribute to periodontal pathogenesis (11).

One of the main limitations of direct microscopic observation in microbiome analysis is its low sensitivity, as it requires a minimum concentration of cells for reliable visualization (12). Furthermore, simple morphology is not enough to tell different species apart, resulting in poor taxonomic resolution. Additionally, traditional microscopy cannot effectively differentiate between live and dead cells without special staining, which can lead to inaccurate population estimates(12).

Molecular techniques: polymerase chain reaction (PCR) and DNA-DNA hybridization. These methods allow the detection of bacterial DNA, whether the cells are "live" or cultured (12).

Analysis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins is also performed using methods in molecular biology. The DNA profiling technique included restriction fragment length polymorphism (RFLP), which uses diversity in homologous DNA sequences; it is arduous, time-consuming, and costly, and requires a large sample size. A nucleic acid probe is a nucleic acid molecule that has undergone unnatural synthesis and labeling to identify a particular organism, while reducing cross-reactivity (1). Hybridization is defined as the interconnection of complementary DNA strands to create double-stranded nucleic acids, and the DNA-DNA hybridization technology with a chequered pattern is used in epidemiological investigations and ecological research, which require complex laboratory equipment and high skill.

Polymerase chain reaction (PCR), as a vast diagnostic method, does not have the restrictions stated above and has the capability of recognizing even one copy of the explored DNA targets from clinical microbiological samples (13,14).

The PCR method, owing to its greater precision, sensitivity, and rapidity, is used for the detection and quantification of periodontal bacterial counts (15). Q-PCR or real -time PCR containing species-specific primers can precisely quantify individual microbial species and total bacterial count in dental plaque (16). This technique, with its precision and sensitivity, is a valuable tool for research into the etiology of periodontal diseases.

To summarise, PCR offers unprecedented sensitivity, allowing the identification of pathogens even in samples with low pathogen content.

The 16S ribosomal RNA (rRNA) gene sequencing

The introduction of 16S ribosomal RNA (rRNA) gene sequencing has fundamentally changed the landscape of oral microbiology. By targeting the 16S gene—a component found in all bacteria that contains both highly conserved and hypervariable regions—scientists have finally obtained the universal "barcode" of life. This method allows the identification of complex microbial communities without prior knowledge of which species may be present (17).

The advantages of 16S rRNA sequencing are manifold. First, it can detect the "unculturable" majority that was previously overlooked (4). This has led to the discovery of many low-abundance taxa that may act as "core pathogens," modulating the environment to promote inflammation (5,18,19). The method is also highly scalable and cost-effective(17,20). With standardized workflows, researchers can now process thousands of samples in a single study, enabling large-scale cross-population and cross-disease-state comparisons.

However, 16S sequencing has its limitations. Because it only sequences a single gene fragment, its taxonomic resolution is often limited to the genus level(5,21). Distinguishing closely related species or identifying specific virulent strains within a species remains a challenge.

Shotgun sequencing is a laboratory technique for determining the DNA sequence of an organism's genome. The method involves randomly shearing the genome into small DNA fragments, which are sequenced individually. A computer program searches for overlaps among DNA sequences, using them to reassemble the fragments in their correct order to reconstitute the genome. Shotgun sequencing can be used to profile the taxonomic composition and functional potential of microbial communities and to recover whole-genome sequences (4).

Results and discussion

The shift from culture-based to high-throughput sequencing has fundamentally transformed our understanding. We now realize that the periodontal pocket is not merely a collection of "bad" bacteria, but a complex biofilm where metabolic cooperation plays a crucial role. Traditional methods have never been able to fully capture

these interactions because they isolate organisms from their social context.

PCR, as a powerful analytical technique, can play a significant role in validating the clinical diagnosis of periodontal disease, elucidating its cause, pathogenesis, clinical stages, progression, and prognosis. As a main example, PCR methods that amplify bacterial genes show great potential for clinical applications (22).

The polymerase chain reaction (PCR) methods have limitations. PCR primers can only detect the sequence to which they were designed. In experimental studies aimed at discovering new disease-causing factors, these targeted approaches act as filters, excluding organisms that do not meet predefined criteria (23). This limitation laid the foundation for the emergence of "unbiased" sequencing technologies.

Our findings indicate that 16S rRNA sequencing remains the most practical and reliable tool for broad profiling, despite the increasing popularity of Shotgun Metagenomics. Although shotgun sequencing provides higher resolution (down to the strain level) and offers insight into functional potential (bacterial behavior, not just identification), its cost and the enormous computational power required for data analysis limit its widespread use in clinical settings (24). For large-scale epidemiological studies, the cost-benefit ratio remains strongly in favor of 16S rRNA.

In addition, the move to next-generation sequencing (NGS) has allowed us to understand the "systems level" of disease. We can now see how the entire microbial network responds to treatment. Instead of looking for the absence of the "red complex," we can look for the restoration of the "green" and "yellow" complexes that are associated with health. This holistic view is essential for the development of personalized medicine approaches in dentistry.

Conclusion

The approach to studying the gingival microbiome has undergone a major shift, moving from basic isolate identification to a more advanced systems-level science. The shift from traditional culture methods to high-throughput sequencing has transformed our understanding of periodontology, focusing less on individual "bad" bacteria and more on intricate microbial biofilms where metabolic cooperation drives disease. While traditional culture remains a crucial and irreplaceable tool for antibiotic susceptibility testing, the future of periodontal diagnosis, prognosis, and research clearly depends on high-throughput molecular analysis.

Within this molecular landscape, 16S rRNA sequencing remains the most practical and reliable foundation for broad microbial profiling. While newer technologies like Shotgun Metagenomics offer impressive high-resolution data at the strain level and provide insights into functional potential, their widespread clinical use is currently limited by high costs, the technical challenge of host DNA contamination, and the extensive computational power needed for data analysis. As a result, for large-scale epidemiological studies and routine clinical monitoring, the cost-benefit ratio still favors 16S rRNA sequencing.

This holistic, NGS-based approach allows us to observe how entire microbial networks respond to treatment in real time. Instead of just screening for the absence of the pathogenic "red complex," clinicians can now monitor the restoration of the "green" and "yellow" complexes associated with oral health. By combining the taxonomic breadth of 16S rRNA sequencing with the functional depth provided by emerging technologies, we are beginning to understand the complex microbial language that governs the delicate balance between oral health and systemic disease. Ultimately, this comprehensive view is key to developing truly personalized approaches to medicine in modern dentistry.

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