Chapter-2

ORAL MICROBIOTA AND IMPACT OF SMOKING

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LEARNING OBJECTIVES

1. Define the oral microbiota and describe its composition, ecological diversity, and role in maintaining oral and systemic health.
2. Explain the concept of microbial homeostasis and dysbiosis, and discuss their significance in the development of oral and systemic diseases.
3. Describe the major ecological niches of the oral cavity and explain how variations in environmental conditions influence microbial colonization and community structure.
4. Discuss the physiological functions of the oral microbiota, including colonization resistance, immune modulation, metabolic activity, maintenance of epithelial integrity, and contribution to wound healing.
5. Identify the intrinsic and extrinsic factors that influence the composition and stability of the oral microbiome, with particular emphasis on lifestyle-related factors.
6. Explain the chemical composition of tobacco smoke and describe how its toxic constituents alter the oral environment, saliva, immune responses, and microbial ecology.

2.1 INTRODUCTION

The oral cavity harbors one of the most diverse and dynamic microbial ecosystems in the human body, collectively known as the oral microbiota. This complex community consists of bacteria, fungi, viruses, and archaea that colonize various anatomical sites, including the tongue, teeth, gingival sulcus, buccal mucosa, palate, and saliva. More than 700 microbial species have been identified within the oral cavity, where they exist primarily as highly organized biofilms. These biofilms facilitate microbial adhesion, communication, nutrient exchange, and cooperative interactions, enabling microorganisms to adapt to the constantly changing oral environment. Under healthy conditions, the oral microbiota exists in a balanced relationship with the host and contributes significantly to both oral and systemic health by preventing colonization by pathogenic microorganisms, regulating immune responses, maintaining mucosal integrity, and supporting normal physiological functions of the oral tissues. Maintenance of microbial homeostasis is essential for preserving oral health. [1-2] In a healthy oral environment, beneficial commensal microorganisms compete with potentially harmful pathogens for nutrients and attachment sites, thereby limiting their growth through ecological competition and metabolic interactions. In addition, these resident microorganisms actively communicate with the host immune system, helping to maintain immune tolerance while preventing excessive inflammatory responses. However, disruption of this finely regulated microbial equilibrium leads to a condition known as oral dysbiosis, in swhich beneficial microorganisms decline while pathogenic species become dominant. Such alterations in microbial composition and function contribute to tissue inflammation and destruction, ultimately increasing susceptibility to oral diseases. Dysbiosis has been strongly implicated in the pathogenesis of common oral conditions such as dental caries and periodontal disease, where shifts

toward acid-producing or proteolytic bacterial communities promote enamel demineralization, connective tissue breakdown, and alveolar bone loss. Increasing evidence also suggests that disturbances in the oral microbiota may influence systemic health and have been associated with cardiovascular diseases, diabetes mellitus, adverse pregnancy outcomes, and other chronic inflammatory disorders. [3,10] The composition and stability of the oral microbiota are influenced by a wide range of host-related and environmental factors. Host factors such as age, genetic background, immune competence, hormonal status, and salivary flow regulate microbial colonization and community structure. Similarly, environmental and lifestyle factors including diet, oral hygiene practices, medication use, alcohol consumption, and tobacco exposure play a major role in shaping the oral microbial ecosystem. Among these, tobacco smoking is recognized as one of the most significant and modifiable risk factors affecting oral microbial health. Smoking remains a major global public health concern and is responsible for substantial morbidity and mortality worldwide. Beyond its well-established systemic health consequences, tobacco use profoundly alters the oral environment, making it an important determinant of microbial imbalance and oral disease development. [2,4]

The oral cavity represents the first point of contact for tobacco smoke, exposing oral tissues and resident microorganisms directly to thousands of toxic chemicals. Tobacco smoke contains a complex mixture of carcinogens, reactive oxygen species, nicotine, aldehydes, and numerous other harmful compounds capable of altering the physicochemical characteristics of the oral environment. These substances modify oxygen availability, salivary composition, pH, nutrient availability, and redox potential, thereby creating conditions that favor the survival and proliferation of pathogenic microorganisms. At the same time, smoking suppresses several components of the host defense system by impairing salivary antimicrobial activity, reducing immune cell function, and compromising mucosal immunity. The combined effects of environmental alteration and immune suppression promote oral microbial dysbiosis, allowing disease-associated microorganisms to colonize and persist more effectively within oral biofilms. [5,6] Over the past decade, advances in molecular microbiology and next-generation sequencing technologies have provided compelling evidence that smoking significantly alters both the composition and functional characteristics of the oral microbiome. Numerous studies have demonstrated that smokers exhibit reduced microbial diversity together with increased abundance of anaerobic pathogenic bacteria, particularly species associated with periodontal disease. Conversely, several beneficial commensal microorganisms that contribute to microbial stability and host protection are consistently reduced in smokers. This shift from a symbiotic microbial community toward a dysbiotic state compromises the protective functions of the oral microbiota and creates an environment that promotes chronic inflammation, tissue destruction, and disease progression.

Consequently, smokers experience a substantially higher prevalence of severe periodontal disease, delayed wound healing, recurrent oral infections, and an increased risk of potentially malignant disorders and oral cancer compared with non-smokers. [7–9] Despite considerable progress in understanding the relationship between smoking and the oral microbiota, several important knowledge gaps remain. Differences in study design, sample collection methods, sequencing technologies, population characteristics, and bioinformatics approaches have resulted in variability among published findings, making direct comparisons between studies challenging. Furthermore, although strong associations between smoking-induced microbial alterations and oral diseases have been consistently reported, the precise biological mechanisms through which tobacco exposure reshapes the oral microbiome and contributes to disease progression are not yet fully understood. Future well-designed longitudinal studies integrating microbiome analysis with host immune responses, metabolomics, and functional microbial profiling will be essential to clarify these complex interactions and to identify novel microbiome-based strategies for the prevention, diagnosis, and treatment of smoking-related oral diseases. [10]

2.2 OVERVIEW OF THE ORAL MICROBIOTA

The oral microbiota represents one of the most diverse and complex microbial ecosystems in the human body. It consists of a highly organized and dynamic community of microorganisms that

includes bacteria, fungi, viruses, and archaea, all of which coexist within the unique environment of the oral cavity. [11] These microorganisms establish intricate ecological relationships with one another and with the host, contributing significantly to the maintenance of oral and systemic health. Among these microbial groups, bacteria constitute the dominant component of the oral microbiome and are responsible for most of its metabolic and physiological activities. At the phylum level, the oral bacterial community is primarily composed of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria, which collectively account for the majority of microorganisms inhabiting healthy oral tissues. [11,12] Within these phyla, several bacterial genera are consistently detected in healthy individuals, including Streptococcus, Actinomyces, Veillonella, Fusobacterium, and Prevotella. [13] These microorganisms contribute to the stability of the oral ecosystem by participating in biofilm formation, nutrient metabolism, and interactions with host immune mechanisms.

In addition to bacteria, the oral microbiota also contains numerous fungal species, viruses, and archaea that contribute to microbial diversity and ecological balance. Candida species are the most frequently encountered oral fungi and normally exist as harmless commensals, although they may become opportunistic pathogens under conditions of immune suppression or microbial imbalance. [10] Oral viruses include both human viruses and bacteriophages, the latter playing an important role in regulating bacterial populations through viral predation and horizontal gene transfer. Archaea, although present in relatively low abundance, are increasingly recognized for their potential involvement in oral microbial metabolism and periodontal disease.

Most oral microorganisms exist within structured biofilms, which are highly organized microbial communities embedded in a self-produced extracellular polymeric matrix. Biofilm formation enhances microbial adhesion to oral surfaces, facilitates communication through quorum sensing, promotes nutrient exchange, and increases resistance to environmental stress, antimicrobial agents, and host immune defences. [12] Consequently, biofilm formation is fundamental to both the maintenance of oral microbial homeostasis and the development of oral diseases.

2.2.1 Oral Ecological Niches and Microbial Distribution

The oral cavity is composed of multiple ecological niches, each possessing distinct anatomical, physical, and biochemical characteristics that influence microbial colonization and community composition. [14] These niches include the tongue dorsum, buccal mucosa, hard palate, soft palate, supragingival tooth surfaces, subgingival periodontal pockets, gingival sulcus, and saliva. Each site provides a unique habitat with specific oxygen concentrations, nutrient availability, moisture levels, pH, temperature, and epithelial characteristics, thereby supporting the growth of specialized microbial communities. The tongue dorsum possesses numerous papillae that create protected anaerobic microenvironments favorable for colonization by anaerobic microorganisms. In contrast, the supragingival tooth surfaces are continuously exposed to saliva and oxygen, making them more suitable for facultative anaerobic bacteria such as *Streptococcus* species. [14] The subgingival region, located beneath the gingival margin, provides an oxygen-poor environment rich in gingival crevicular fluid that supports the proliferation of obligate anaerobic pathogens, including *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Fusobacterium nucleatum*, organisms strongly associated with periodontal disease. [12] Saliva serves as an important ecological component of the oral cavity. Beyond acting as a transport medium for microorganisms, saliva provides mechanical cleansing, buffering capacity, antimicrobial proteins, immunoglobulins, enzymes, and nutrients that regulate microbial growth and maintain ecological stability. [15] The continuous interaction among these diverse oral habitats enables dynamic microbial exchange while preserving the overall balance of the oral microbiome.

2.2.2 Oral Microbial Homeostasis and Dysbiosis

Under physiological conditions, the oral microbiota exists in a state of microbial homeostasis, characterized by a balanced and mutually beneficial relationship between the host and resident microorganisms. [3] This equilibrium is maintained through complex ecological interactions among microbial species as well as continuous communication between the

microbiota and the host immune system. Beneficial commensal microorganisms compete with potentially pathogenic species for nutrients and adhesion sites, produce antimicrobial substances such as bacteriocins, regulate environmental pH, and stimulate protective immune responses. Together, these mechanisms prevent excessive colonization by pathogenic microorganisms and contribute to the maintenance of oral health. Disruption of this delicate microbial equilibrium results in oral dysbiosis, a condition characterized by reduced microbial diversity, depletion of beneficial commensal microorganisms, and overgrowth of pathogenic species. [16]

Dysbiosis alters the functional characteristics of the microbial community and promotes chronic inflammation, tissue destruction, and disease development. One of the most common consequences of dysbiosis is dental caries, which develops when acid-producing (acidogenic) and acid-tolerant (aciduric) bacteria metabolize dietary carbohydrates to produce organic acids. These acids lower the local pH, resulting in progressive demineralization of tooth enamel and dentin. Similarly, dysbiosis contributes to the development of periodontal disease, in which proteolytic anaerobic bacteria stimulate chronic inflammatory responses that destroy gingival tissues, periodontal ligament fibers, and alveolar bone, ultimately leading to tooth loss if left untreated.

2.2.3 Physiological Functions of the Oral Microbiota

The oral microbiota performs numerous physiological functions that are essential for maintaining both oral and systemic health. One of its primary protective roles is colonization resistance, whereby commensal microorganisms inhibit the establishment and growth of pathogenic microbes by competing for nutrients, adhesion sites, and ecological space. Many commensal bacteria also produce antimicrobial compounds that suppress opportunistic pathogens, thereby maintaining microbial equilibrium. Beyond microbial competition, oral microorganisms participate in several important metabolic activities. They ferment dietary carbohydrates, metabolize salivary glycoproteins, produce short-chain organic acids, and contribute to pH regulation within the oral cavity. [11] These metabolic processes help maintain environmental stability and support the ecological balance of the oral microbiome.

A. Role of the Oral Microbiota in Immune Regulation: The oral microbiota plays a fundamental role in regulating both innate and adaptive immune responses. Continuous interactions between resident microorganisms and oral epithelial cells promote immune tolerance toward beneficial microbes while preserving the host's capacity to recognize and eliminate pathogens. [3] Microbial-derived signaling molecules stimulate immune maturation, regulate cytokine production, and maintain mucosal barrier function, thereby preventing excessive inflammation and tissue injury.

B. Maintenance of Oral Tissue Integrity: In addition to immune regulation, the oral microbiota contributes to the maintenance of oral tissues by supporting epithelial integrity, facilitating tissue repair, promoting wound healing, and preserving mucosal homeostasis. These beneficial functions emphasize the importance of maintaining a balanced microbial ecosystem, as disruption of microbial homeostasis can compromise tissue health and increase susceptibility to oral disease.

2.2.4 Factors Influencing the Oral Microbiota

The composition, diversity, and stability of the oral microbiota are influenced by numerous intrinsic and extrinsic factors that collectively shape the oral microbial ecosystem. [2]

A. Intrinsic Factors: Host-related factors include genetic background, age, immune competence, hormonal status, salivary flow rate, salivary composition, and systemic health conditions. These factors determine the host environment available for microbial colonization and influence the ability of microorganisms to establish stable communities.

B. Extrinsic Factors: Environmental and lifestyle factors also exert profound effects on oral microbial composition. These include dietary habits, oral hygiene practices, medication use, antibiotic exposure, alcohol consumption, environmental pollutants, and tobacco smoking.

Diet plays a particularly important role in determining microbial composition. Frequent consumption of fermentable carbohydrates promotes the proliferation of acidogenic bacteria, thereby increasing the risk of dental caries. [16]

Similarly, inadequate oral hygiene allows accumulation of dental plaque biofilms, creating favorable conditions for pathogenic microorganisms associated with periodontal disease [12] Among all modifiable lifestyle factors, tobacco smoking has one of the most profound effects on the oral microbiome. Smoking alters oxygen availability, salivary composition, immune function, and the physicochemical properties of the oral environment, resulting in significant microbial dysbiosis. These changes favor colonization by pathogenic microorganisms while reducing beneficial commensal species, thereby substantially increasing the risk of periodontal disease, oral infections, delayed wound healing, and oral malignancies. Consequently, smoking is recognized as one of the most important environmental determinants of oral microbial imbalance and oral disease development.

2.3 SMOKING: BIOLOGICAL AND CHEMICAL BACKGROUND

A. Tobacco Smoke: Tobacco smoke is a complex chemical cocktail of over 7,000 compounds, some of them toxic, mutagenic and carcinogenic. [17] This includes nicotine, carbon monoxide, tar, polycyclic aromatic hydrocarbons (PAHs), tobacco-specific nitrosamines, aldehydes and reactive oxygen species (ROS). [5] Nicotine is the main addictive agent and plays a major role in cell signalling and vascular responses, whereas other compounds contribute to the generation of oxidative stress and tissue injury. [5,17] These compounds modify the oral environment, changing the oxygen, pH and nutrient status of the oral cavity. [5] These modifications can affect the microenvironment and potentially influence microbial survival, growth, and interactions in the oral ecosystem.

B. Effects of Smoking on Oral Tissues: Smoking affects the oral cavity, which is the first site of exposure to tobacco smoke. Cigarette smoking causes vasoconstriction of the gingival tissues, leading to decreased blood flow, oxygen tension and nutrient supply. [18] This promotes the growth of anaerobic bacteria found in periodontal disease. [8] Moreover, smoking results in changes in epithelial tissues through the process of keratinization as well as delayed wound healing. In addition, smoking reduces the activity of fibroblasts and production of collagen that are required for wound healing. [19]

C. Immunological Modulation: Smoking cigarettes impacts both the innate and adaptive immune systems. [6] Smoking compromises neutrophil function, phagocytosis, and cytokine secretion, thus limiting the host's ability to control the microorganisms. [6] In addition, smoking hinders adaptive immunity by impairing T-cell activity and antibody immunity. [6] Smoking also results in decreased salivary immunoglobulin A (IgA) levels, impairing mucosal immunity and promoting microbial survival. [20] These immune suppressive effects enable pathogenic microorganisms to colonise and create a dysbiotic microbial environment. [6,20]

D. Saliva and Oral Environment: Saliva is an essential agent for the maintenance of good oral health because of its buffer action, cleansing activity, and antimicrobial properties. [15] It has been found that cigarette smoke affects the production and quality of saliva by changing its pH and antimicrobial protein concentrations. [21] The alteration in properties makes saliva incapable of acting as a defense mechanism. [12,21] In addition to that, cigarette smoking itself creates oxidative stress and alters the biochemical composition of the mouth microenvironment, thereby promoting microbial dysbiosis. [5,17] This creates the appropriate milieu for the growth and multiplication of harmful microbes.

E. Tobacco Use and New Trends: Although traditional cigarette smoking is the most well-researched form of tobacco use, other forms, including cigars, smokeless tobacco and electronic cigarettes (e-cigarettes), are on the rise. [22] E-cigarettes are emerging as perceived "safe" alternatives; however, recent evidence supports the possibility that their aerosols can dysregulate the microbiome and cause inflammation. [22] Although more work is needed to determine the long-term consequences, there is evidence to suggest that they are active and can lead to oral dysbiosis [22] This shows the importance of recognizing different ways of using tobacco when evaluating their effects on oral health.

2.3.1 Effect of Smoking on the Oral Microbiome

A. Microbiota Composition: There is robust evidence that smoking is associated with changes in the oral microbiota, including a loss of diversity. [9,23] Molecular analyses (16S rRNA sequencing) have shown differences in microbial composition between smokers and non-smokers,

with reduced diversity and increased abundance of pathogenic bacteria. [9,23] Loss of diversity is a characteristic of dysbiosis and has been linked to reduced stability in the microbial community. [23] A notable aspect of the microbial changes seen in smokers is the presence of anaerobic and pathogenic bacteria. These include periodontal pathogens such as Porphyromonas gingivalis, Tannerella forsythia, and Fusobacterium nucleatum, which are strongly associated with periodontal disease. [7,8] These anaerobes favor hypoxic environments and play a role in disease via virulence factors and inflammatory pathways. [8] On the other hand, protective commensal bacteria such as Streptococcus and Actinomyces bacteria are frequently depleted in smokers, leading to loss of microbial balance and reduced host defence. [24] This transition from symbiosis to dysbiosis plays a key role in the development of smoking-associated oral disease.

B. Microbial Modulation: Effects of smoking on the oral microbiome involve an intricate relationship between the environment, the microbe, and the host. The main mechanism that drives these effects involves reduction in oxygen levels in the mouth due to smoking-associated vasoconstriction, promoting the growth of anaerobes. [8] Another mechanism is changes in saliva production and composition, which leads to decreased saliva antimicrobial activity and clearance of microorganisms. [21] This promotes bacterial adherence and biofilm formation, which protects bacteria and promotes resistance to host immune mechanisms. [12] Furthermore, tobacco smoke is able to control the expression of bacterial genes and increase their virulence. [25] Another adverse effect of smoking on microorganisms is the immunosuppressive impact it has on the host organism, which promotes the development of dysbiosis through cytokines and neutrophils. [6]

C. Functional and Metabolic Alterations: Aside from compositional shifts, smoking also affects the functional and metabolic properties of the oral microbiome. Researchers have demonstrated that the microbiota of smokers have higher expression of genes related to virulence, inflammation, and biofilm formation. These modifications increase the virulence of the microbiota, and disease development. Smoking also induces metabolic changes, leading to increased levels of proteolytic enzymes and inflammatory mediators that may damage the host and promote chronic inflammation. In addition, increased biofilm formation leads to a more mature, resistant microbiota with reduced responsiveness to environmental stimuli and therapeutic strategies.

2.4 MICROBIAL DYSBIOSIS RELATED TO SMOKING-INDUCED ORAL DISEASES

The smoking-induced changes in the oral microbiota are key factors in the progression of many oral diseases. These are achieved through a combination of microbial dysbiosis, altered host immune responses, and changes in the oral environment. [7,19]

A. Periodontal disease: The strongest link between smoking-induced changes and disease is with periodontal disease. Smokers have greater periodontal pocket depth, clinical attachment loss and alveolar bone destruction than non-smokers. [19] These effects are associated with increased levels of anaerobic periodontal pathogens, including Porphyromonas gingivalis, Tannerella forsythia and Fusobacterium nucleatum. [7,8]. Furthermore, smoking impairs the host's immune response, facilitating the persistence of these microorganisms and chronic inflammation, and consequent destruction of the tissues. [7,19]

B. Dental caries: Cigarette smoking can also lead to dental caries through both direct and indirect mechanisms. Alterations in the oral flora, with an increase in acidogenic and aciduric bacteria, lead to an increased production of organic acids that demineralise the enamel. [16] In addition, smokers have a lower salivary flow rate and buffering capacity, and this affects the remineralisation process, rendering them more vulnerable to caries. [16,21] Lifestyle factors, such as poor oral hygiene, often associated with smoking may also contribute.

C. Oral candidiasis: Candida albicans colonization is more common in smokers, which is associated with a higher incidence of oral candidiasis. [27] The effects of smoking on the oral environment and local immunity promote fungal colonization. [27] This is a serious condition in people with other predisposing factors such as immunosuppression, denture wear or systemic disease.

D. Potentially malignant disorders (PMDs): Potentially malignant disorders like leukoplakia and erythroplakia are strongly associated with smoking. [28] Dysbiosis could contribute to the development of these disorders through chronic inflammation and epithelial changes. [26,28] Chronic inflammation and microbial interactions can result in cellular alterations that predispose to

cancer.

E. Oral cancer: Recent research has shown that the oral microbiome contributes to the development of tumours in smokers. Some bacteria can generate carcinogenic products, trigger inflammation and interact with the human immune response. [26] The actions of these microorganisms may synergise with carcinogens derived from tobacco smoke to increase the risk of oral squamous cell carcinoma (OSCC). While the causal link is still being studied, the link between microbial dysbiosis and oral cancer is becoming apparent.

F. Halitosis (oral malodor): Halitosis is common among smokers and is primarily due to increased production of volatile sulfur compounds by anaerobic bacteria. [29] These compounds are produced by the breakdown of sulfur-containing amino acids and are indicative of oral dysbiosis. Although not a serious disease, halitosis is a sign of microbial dysbiosis and of oral ecosystem instability.

2.5 COMPARATIVE ANALYSIS: SMOKERS, NON-SMOKERS, AND SMOKING CESSATION

A. Microbiota in Smokers vs. Non-Smokers: There is clear evidence of distinct oral microbiota in smokers compared to non-smokers. [30,31] Smokers have lower microbial diversity and higher levels of pathogenic anaerobic bacteria than non-smokers, who have a more stable microbiome. [30] Lower diversity among smokers is associated with reduced stability of the microbial community, increasing the risk of dysbiosis. [31] Increased levels of periodontal pathogens, including Porphyromonas gingivalis, Fusobacterium nucleatum, and Tannerella forsythia, have been found in smokers compared to non-smokers, while commensals such as Streptococcus species are more abundant in non-smokers. [31,32] This suggests that smoking has a role in shifting the microbial ecology from symbiosis to pathogenicity.

B. Dose-Response Relationship: Smoking has a dose-response relationship with changes in oral microbiota, such that increased smoking causes more dramatic shifts in the composition of microorganisms. Existing heavy smokers have greater amounts of pathogenic microorganisms than existing light and occasional smokers. [33] This dose-dependent effect indicates the cumulative effects of smoking on microbial ecology and implies that both the duration and intensity of smoking are key factors in the development of oral dysbiosis.

C. Impact of Smoking Cessation on Oral Microbiome: Quitting smoking has been shown to result in partial restoration of the oral microbiota to a healthier profile. [30] Ex-smokers have higher microbial diversity and lower abundance of pathogenic microorganisms than current smokers. [30,34] But the degree of recovery may be variable and influenced by smoking history and time since quitting. [34] Other studies show that some microbial changes may remain after smoking cessation, suggesting a long-term or potentially irreversible impact on the microbiome. [34] This suggests that quitting early is recommended to avoid long-term changes.

D. Assessment of the Evidence: Although there is an increasing body of research in this area, there are some limitations. Cross-sectional designs prevent claims that smoking has a causal effect on the microbiome. [31] Variations in sampling, sequencing, and population characteristics also contribute to inconsistencies in results. [31] Furthermore, diet, alcohol, oral hygiene and general health status can also affect the results, but are not always accounted for. [2] Further, there are no longitudinal studies examining the long-term recovery of the microbiome after quitting smoking. [34] Future studies should also be conducted using standardised methods, longitudinal and new technologies (such as metagenomics and metabolomics) to understand the intricate relationship between smoking and oral microbiota. [31]

2.6 APPROACHES TO INVESTIGATE ORAL MICROBIOTA

Understanding the composition and function of the oral microbiota requires accurate and reliable laboratory techniques capable of identifying the diverse microorganisms that inhabit the oral cavity. Over the past two decades, remarkable advances in microbiological, molecular, and computational technologies have transformed oral microbiome research. Traditional culture-based methods have been complemented by high-throughput sequencing, multi-omics technologies, and bioinformatics, enabling comprehensive characterization of microbial communities that were previously impossible to study. The selection of appropriate sampling methods, laboratory procedures, sequencing platforms, and

analytical tools is essential for generating reproducible and clinically meaningful data. Together, these approaches provide valuable insights into microbial diversity, ecological interactions, functional activities, and the relationship between the oral microbiome and diseases such as periodontal disease, dental caries, oral candidiasis, and smoking-associated dysbiosis.

2.6.1 Sampling Techniques

Accurate sample collection is the foundation of oral microbiome research because the quality and representativeness of the specimen directly influence downstream microbial analysis. Different anatomical sites within the oral cavity harbor distinct microbial communities; therefore, the choice of sampling technique depends on the specific research question or clinical objective. Commonly collected specimens include saliva, oral swabs, supragingival plaque, subgingival plaque, tongue scrapings, and mucosal swabs, each representing a unique ecological niche within the oral cavity. [35,36] Saliva is one of the most frequently used specimens because it provides a comprehensive overview of the oral microbiome by containing microorganisms shed from multiple oral surfaces. Supragingival and subgingival plaque samples are preferred for investigating site-specific diseases such as dental caries and periodontal disease because they directly reflect the microbial populations associated with tooth surfaces and periodontal pockets. [36,37] Oral swabs are particularly useful for studying microorganisms colonizing the buccal mucosa, palate, tongue, or lesions. To ensure reliable and reproducible results, standardized sampling protocols are essential. Factors such as the time of collection, fasting status, oral hygiene practices, recent food intake, storage temperature, transport conditions, and DNA preservation methods can all influence microbial composition and sequencing outcomes. Consequently, strict standardization of sample collection, handling, and storage procedures is recommended to minimize technical variation and improve comparability across studies [38,39]

2.6.2 Culture-Dependent Methods

Culture-dependent microbiological techniques have historically been the cornerstone of oral microbiology and continue to play an important role in clinical diagnostics. These methods involve growing microorganisms on selective or non-selective culture media under controlled aerobic or anaerobic conditions, followed by identification based on colony morphology, biochemical characteristics, and antimicrobial susceptibility testing [40] Culture-based methods are particularly valuable for isolating pathogenic organisms, determining antibiotic sensitivity, and investigating microbial physiology. They remain indispensable in routine clinical microbiology laboratories for diagnosing bacterial and fungal infections. However, a major limitation is that a large proportion of oral microorganisms are fastidious or unculturable under conventional laboratory conditions. Many species require highly specialized nutritional requirements or exist in complex biofilm communities that cannot be adequately replicated in vitro. As a result, culture-based techniques capture only a fraction of the total microbial diversity present in the oral cavity [40,41] Consequently, these methods are now commonly used alongside molecular approaches to validate sequencing results and investigate specific microorganisms in greater detail.

2.6.3 Molecular and Sequencing Techniques

Molecular diagnostic techniques have revolutionized oral microbiome research by enabling culture-independent identification of microorganisms. These approaches detect microbial nucleic acids directly from clinical samples, allowing researchers to identify both culturable and unculturable organisms with high sensitivity and specificity [42] Polymerase chain reaction (PCR) is widely used for the rapid detection of specific microbial species, virulence genes, and antimicrobial resistance markers. Quantitative PCR (qPCR) further enables the estimation of microbial abundance by measuring gene copy numbers in real time. The introduction of 16S ribosomal RNA (16S rRNA) gene sequencing has dramatically expanded our understanding of bacterial diversity within the oral cavity. Because the 16S rRNA gene contains both conserved and variable regions, it serves as an ideal molecular marker for bacterial identification and taxonomic classification. [43] This technique allows simultaneous identification of hundreds of bacterial species within a single sample. Next-generation sequencing (NGS) technologies have further transformed microbiome research by generating millions of

sequencing reads in a single experiment. These high-throughput platforms provide comprehensive information regarding microbial composition, diversity, and relative abundance across complex microbial communities. [43] Whole-genome shotgun metagenomic sequencing represents an even more advanced approach by sequencing all microbial DNA present within a sample rather than targeting only the 16S rRNA gene. This enables identification of bacteria, fungi, viruses, and archaea while simultaneously providing information regarding microbial genes, metabolic pathways, antimicrobial resistance, and functional potential. [44]

2.6.4 Multi-Omics Approaches

Recent advances in systems biology have introduced multi-omics technologies that provide functional insights beyond microbial identification alone. Rather than determining which microorganisms are present, these techniques investigate what microorganisms are actively doing within the oral ecosystem [45] Metatranscriptomics analyzes messenger RNA (mRNA) to determine which microbial genes are actively expressed under specific physiological or disease conditions. This provides information regarding microbial activity and adaptation within the oral environment. Metaproteomics examines proteins produced by microbial communities, allowing investigators to study enzyme production, virulence factors, host–microbe interactions, and microbial metabolic functions. Metabolomics evaluates small-molecule metabolites produced during microbial metabolism. These metabolites provide direct evidence of biochemical activity within the oral cavity and may serve as valuable biomarkers for disease diagnosis. Metabolomic studies have become increasingly important for investigating metabolic alterations associated with smoking, periodontal disease, oral cancer, and other oral disorders. [45,46] When integrated, these complementary technologies provide a comprehensive understanding of microbial composition, gene expression, protein synthesis, and metabolic activity. Such integrated multi-omics approaches offer deeper insights into host–microbe interactions and disease mechanisms than any single analytical technique alone.

Bioinformatics and Data Analysis: The enormous volume of data generated by modern sequencing technologies requires sophisticated computational methods for accurate analysis and interpretation. Bioinformatics combines computer science, statistics, mathematics, and molecular biology to process raw sequencing data into clinically meaningful information [47] Following sequencing, bioinformatics pipelines perform multiple analytical steps, including quality assessment, removal of sequencing errors, taxonomic classification, microbial abundance estimation, diversity analysis, phylogenetic reconstruction, functional prediction, and visualization of microbial communities. Widely used software platforms such as QIIME, Mothur, Kraken, and MetaPhlAn facilitate comprehensive microbiome analysis by identifying microbial species, comparing microbial communities, evaluating ecological diversity, and predicting functional characteristics. Statistical analyses further enable researchers to identify disease-associated microbial signatures, compare microbial populations between healthy and diseased individuals, and discover potential microbial biomarkers. Machine learning and artificial intelligence are increasingly being incorporated into microbiome research to improve disease prediction, patient stratification, and personalized therapeutic decision-making. Despite these advances, considerable challenges remain regarding data normalization, computational pipelines, reference databases, and analytical reproducibility. Standardized bioinformatics workflows are therefore essential for generating reliable and comparable microbiome studies [47,48]

2.7 LIMITATIONS AND CHALLENGES

Despite remarkable technological progress, several challenges continue to limit the clinical application and interpretation of oral microbiome research. Variability in sample collection methods, DNA extraction protocols, sequencing platforms, and bioinformatics pipelines can introduce substantial differences between studies, reducing reproducibility and limiting cross-study comparisons [38,47] Most currently available studies are observational and cross-sectional, making it difficult to establish causal relationships between microbial alterations and disease development. Numerous confounding variables including age, diet, oral hygiene practices, smoking status, medication use, systemic diseases, genetics, geographical location, and

environmental exposures can significantly influence microbial composition and complicate data interpretation [2] Furthermore, the oral microbiome represents a highly dynamic ecosystem that changes continuously in response to physiological and environmental factors. Distinguishing normal biological variation from disease-associated dysbiosis therefore remains challenging. Additional limitations include incomplete microbial reference databases, high computational requirements, complex data interpretation, and the lack of universally accepted analytical standards [48] Future research should focus on developing standardized sampling protocols, harmonized sequencing methodologies, improved bioinformatics pipelines, and large-scale longitudinal studies. Such advances will improve reproducibility, facilitate clinical translation, and enhance our understanding of the role of the oral microbiome in health and disease

2.8 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The emerging knowledge of the association between smoking and oral microbiota has implications for clinical practice and public health. Microbiological changes associated with smoking play a prominent role in the development of oral diseases, such as periodontal disease, dental caries and oral cancer, and therefore need to be addressed by specific prevention strategies and treatments [7,19] Clinically, smoking status should be an important consideration in oral health assessments. Health-care providers should identify smoking as a prime risk factor for both tissue and microbial damage, which may affect disease progression and therapeutic success. [19] Understanding the microbiological aspects of diagnosis and treatment planning may enhance the treatment of smoking-induced oral diseases. Manual smoking cessation is a key strategy to improve oral health.

Quitting smoking has been shown to result in partial restoration of the oral microbiota and a decrease in the pathogenic microbial load, but not necessarily a full reversal. [9,30] Thus, dentists have a significant role in smoking cessation advice and promoting behavioural changes in a holistic approach to patient care. Moreover, new strategies such as microbiome-based diagnosis and therapies hold potential for clinical use. Next-generation sequencing may enable early detection of dysbiosis and risk assessment of oral diseases. [47] Interventions such as probiotics, prebiotics and antibiotics may alleviate dysbiosis and enhance therapeutic efficacy. Research-wise, future research should be longitudinal to gain better insight into the temporal dynamics of smoking and microbiome changes, and the reversibility of these changes when smoking ceases. [30] The use of multi-omics approaches will help to better understand the functional and metabolic features of oral microbiome and its interaction with host. [45] Further, research should also focus on understanding the impacts of novel tobacco use such as e-cigarettes to understand their impact on oral microbiota [22] Additionally, it is essential that the methodology used in such studies be standardized. [47,48]

2.9 CONCLUSION

The oral microbiota is a complex and dynamic community that plays a critical role in oral and overall health. In a state of balance (homeostasis), this microbiota supports host immunity, immune regulation, and oral health. But when this balance is disturbed, as in the case of smoking, this results in microbial dysbiosis and disease. This chapter has outlined the effects of smoking on the composition, diversity and function of the oral microbiota. These include an increase in pathogenic and anaerobic microbes, a decrease in commensal microbes, and upregulation of virulence factors and biofilm formation. The mechanisms of these changes are multifaceted and involve interactions between the chemical constituents of tobacco smoke, immune suppression, and environmental changes. The effects of smoking-induced dysbiosis are reflected in heightened risk and severity of oral diseases such as periodontal disease, dental decay, oral thrush and cancer. This research highlights the importance of the oral microbiome as a link between environmental exposures and disease. Moreover, recent developments in molecular and multi-omics technologies have contributed to a better understanding of the composition and function of the oral microbiome, albeit with limitations regarding method consistency and a lack of longitudinal studies. Comparisons among smokers, non-smokers, and ex-smokers indicate that although the microbiota may recover to some extent after smoking cessation, persistent changes are observed, suggesting lasting effects of

smoking. Clinically, understanding the impact of smoking on oral microbiota is crucial for enhanced prevention, diagnosis and treatment. The application of microbiome-based diagnostics and therapeutic strategies, combined with smoking cessation programs, can greatly improve oral health. In conclusion, the connection between smoking and the oral microbiome constitutes an important field of study with clinical implications. This information will help in developing treatment methods that will benefit this particular patient group.

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