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Oral Microbiology in Periodontitis

Edited by Sonia Bhonchal Bhardwaj

ORAL MICROBIOLOGY IN PERIODONTITIS

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Meet the editor

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She received grants from the Department of Science and Technology (India) and worked on biofilm formation by *E. faecalis* in periodontitis, *S. mutans,* and phage therapy.

Contents

Preface

The oral diseases are predominantly microbial diseases. Most lesions which arise in the oral cavity arise because of the microbial infections. A comprehensive attempt has been made to cover the aspects of common oral bacterial diseases like their identification and the immune mechanism related to periodontitis.

This book will serve as a brief yet exhaustive guide to the role of oral microbes in health and disease. It will be useful to dental and medical students and to microbiologists.

My wholehearted thanks go to Intech Publisher pioneer in the field of medical publication for taking up this work for publication.

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Introduction

Chapter 1

Introductory Chapter: Oral Flora and Oral Diseases

Sonia Bhonchal Bhardwaj

Additional information is available at the end of the chapter

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1. Introduction

The mouth is colonized by 200–300 bacterial species. The indigenous microbiota plays an important role in health and disease of humans. Indigenous bacteria are often associated with the etiology of two major oral diseases: the dental decay (caries) and periodontal disease. This book attempts to describe the oral ecosystems, factors controlling the oral microbiota, identification of the oral microbes, and basic aspects of immune system with a particular emphasis on periodontal disease.

2. Periodontal disease

Periodontitis occurs when the plaque-induced inflammatory response in the tissue results in actual loss of collagen attachment of the tooth to the bone, to loss of bone, and to deep periodontal pockets.

2.1. Etiology

Periodontal infections are mixed often involving anaerobes such as *Treponema denticola, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Tannerella forsythia, Prevotella intermedia, Fusobacterium nucleatum, Peptostreptococcus micros, Campylobacter rectus,* and *Spirochetes* and other aerobic species such as *Pseudomonas aeruginosa, Enterobacteriaceae, Candida albicans, Staphylococci,* and *Enterococci* have also been found in association.

2.2. Pathogenesis and clinical manifestation

These bacteria in plaque secrete compounds such as H_2S, NH_3, amines, toxins, and enzymes, which initiate an inflammatory response responsible for loss of periodontal tissue, pocket

formation, and loosening and loss of teeth. Although there is no manifestation of pain, bleeding gums and bad breath may occur.

Periodontitis in systemic disease of systemic disease-severe periodontitis has been observed in patients with defective neutrophils such as in Chediak–Higashi syndrome, Down syndrome, Neutropenia, Leukocyte adhesion deficiency, etc.

2.3. Microbiologic diagnosis

The traditional culturing techniques along with DNA-based methodology for identification and detection of specific bacteria and viruses, which have advantages in time and cost. Also the number of samples and the number of microorganisms identified and detected that cannot be cultivated have become possible by molecular biology techniques. The other methods commonly used are darkfield examination for spirochetes and enzyme assays.

3. Dental caries

Dental decay is due to the degradation of tooth mineral by acids derived from bacteria which form a dental plaque that accumulates on the tooth surface.

3.1. Etiology

The specific plaque hypothesis proposes that species such as *Streptococcus mutans* and *Streptococcus sobrinus* are actively involved in the disease. Other spp. such as *Veillonella, Actinomyces, Bifidobacterium*, and *Lactobacillus fermentum* are associated with caries. However, in advanced caries in adults, *S. mutans* is not commonly detected but species in the genera *lactobacillus, Prevotella, Selenomonas, Dialister, Fusobacterium, Eubacterium, Olsenella, Bifidobacterium, Propionibacterium*, and *Pseudoramibacter* are abundant. Finally to understand the mechanism involved in caries and periodontal diseases, it is important to understand the microbial ecology of oral cavity and the factors responsible for transition of the commensal flora to the pathogenic microflora in the host. The concept of bacterial succession is important in oral microbiology.

The normal flora benefits the host as it occupies the normal oral flora occupies colonization sites in the mouth and it becomes difficult for the noncommensal flora to establish, oral flora also contributes to host nutrition, low levels of circulating immunity cross reacting with the pathogens. Thirdly, microbial antagonism of normal oral flora by secreting fatty acids, bacteriocins, and peroxides is also a beneficial effect in the host.

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Section 2

Normal Oral Flora and Oral Pathogens

Chapter 2

Influence of the Oral Microbiome on General Health

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Abstract

The prevalence of edentulism is common worldwide. While improvements in access to healthcare and dental care are reducing the prevalence rate of edentulism, the rapidly growing number of elderly as a percent of the global population will sustain a need for denture therapy for the foreseeable future. While denture use has positive impacts on the quality of life, their use is associated with some problems and risks. Denture stomatitis, a chronic infection-related inflammatory disorder of the oral mucosa, is extremely common and has been reported to occur in up to two-thirds of denture wearers. Importantly, epidemiology studies have shown edentulism and denture wearing, while not proven as causative factors, to be associated with significant increases in risk for serious systemic diseases, such as chronic obstructive pulmonary disease (COPD), cardiovascular diseases, diabetes, and arthritic disorders. A common linkage across these diseases is an association between increased risk for the disease and chronic inflammation. The nature of surface properties and porosity of denture materials contributes to the attachment of microorganisms and the establishment and growth of the adherent biofilm. Hence, proper denture cleansing is critical in maintaining oral hygiene and general health and perhaps to reduce the risk factors for systemic disease.

Keywords: *Candida*, biofilm, stomatitis, chronic obstructive pulmonary disease, edentulism

1. Introduction

Loss of natural dentition and use of removable dental prostheses is extremely common worldwide. While improved global access to oral care is decreasing the incidence of partial and complete edentulism, the prevalence of edentulism remains high and, among the elderly, can

exceed 50% in many countries. Furthermore, over the next few years, the global population of elderly individuals will dramatically increase, and this will require the ongoing management of edentulism, at least for the foreseeable future. Edentulism adversely impacts nutrition and quality of life. For example, edentulism is associated with decreased masticatory performance, and this limits the types of food which individuals can chew and eat; furthermore, edentulous individuals report limiting their social function due to negative perceptions related to self-appearance and/or embarrassment and discomfort when eating in social settings.

Restoring dentition by use of an appropriate prosthesis is the treatment approach to edentulism. Denture prostheses can significantly improve masticatory performance, but their impact on changing and improving dietary habits is much less clear. Similarly, dentures can positively impact quality of life regarding appearance and social function, but limitations related to functional improvement often remain. Finally, denture wearing can uniquely impact oral health. Denture surfaces rapidly develop a complex biofilm of bacteria, yeasts and other microorganisms, which can contribute to oral mucosal pathologies. For example, denture stomatitis, a chronic inflammatory disorder, is one of the most common adverse conditions associated with denture use and is associated with contamination of denture surfaces and the underlying oral mucosa by *Candida albicans*, an opportunistic yeast pathogen. Hence, appropriate denture hygiene is beginning to be recognized as critical for maintenance of oral health and perhaps has a role in reducing risk of systemic disease as well. More recently, the potential that denture contamination may also impact systemic disease has been hypothesized and is an ongoing area of research.

This review provides an update of recent developments related to edentulism and denture use. The summary initially focuses on the demographics of edentulism and denture use and potential relationships between edentulism and increased risk of comorbid disease. Current understanding of the role of the denture biofilm as a contributory factor to disease risk is discussed, as are the relationships between biofilm formation and denture materials. Finally, the critical importance of denture cleansing to control the formation of the denture biofilm is summarized with a focus on approaches which can help maintain oral health and potentially reduce risk for systemic disease.

2. Results and discussion

2.1. Demographics and risk of comorbidities

Edentulism and use of dentures is very common among the elderly. This is of critical importance, as the elderly represent a dramatically increasing segment of the world population. In 2000, only 6% of the global population was estimated to be 65 years of age or older. In contrast, by 2030, the percentage of the world population who are at least 65 years of age is estimated to double to 12%, with the largest increases occurring in North America, Europe, Asia and South America [1]. Similarly, in 1998, the World Health Organization reported 390 million people worldwide to be >65 years of age and estimated that this would double by 2025 [2]. Hence, the rapid growth of the elderly as a percentage of the world population will

outpace changes in oral health management designed to reduce edentulism and will sustain a significant incidence of edentulism, the need for denture prostheses, and the requirement to manage the oral and systemic health of denture wearers.

The global prevalence of edentulism varies widely across countries. Current estimates range from 12 to 15% in Hong Kong, India, and several European countries to >60% reported in a survey of residents from Botucatu, Brazil [3, 4]. In the USA, the prevalence of edentulism is estimated to be 36% based on a national population-based survey (NHANEs III) [5]. This survey also reported that the prevalence of edentulism increased with age. In a separate population-based study, Felton reported that 26% of the US population between the ages of 65 and 74 are completely edentulous [6]. A report summarizing data from a 2003 population-based survey conducted in Canada illustrated the dramatic association between increased denture usage with increasing age among both men and women [7]. Similarly, 32 and 59% of residents of Botucatu, Brazil, aged 60–64 are reported to use complete lower and upper dentures, respectively. This increases to 52 and 82%, among those ≥75 years of age [3]. In general, the prevalence of edentulism is generally shown to be positively associated with having lower income or socio-economic status, lower education, and in some countries living in rural areas [8–14].

There are also well-demonstrated relationships between edentulism, denture wearing, poor oral health and increased risk of systemic disease. While associations between denture use and some oral diseases, such as denture stomatitis, are well known and have been widely reported and reviewed in the literature, associations between edentulism, denture use, and their potential to increase the risk for non-oral systemic diseases are less well understood. In a review, Felton reported increased risk for several systemic diseases, including asthma (odds ratio [OR] was 10.52), coronary arterial plaque (OR was 2.32), rheumatoid arthritis (OR was 2.27), diabetes (OR was 1.82), and various cancers (OR was 1.54–2.85) to be associated with edentulism [6]. A study conducted in Thailand among patients wearing either removable complete or removable partial dentures demonstrated a correlation between the presence of oral mucosal lesions or denture-related lesions with several different systemic conditions. In this study, denture patients were found to have significant comorbidities, including bone and joint disorders (26.5% of complete denture wearers), hypertension (23.2%), diabetes (19.4%), cardiovascular disease (8.4%), as well as other illnesses [15]. The study did not, however, include a reference or control group of dentate individuals. Thus, odds ratios for any increase in risk among denture users cannot be determined. Overall, there appears to be an association for significant increases of risk of comorbid disease among denture wearers; however, whether these relationships are causal or casual remains unknown.

2.2. Structure/function relationships between denture material and microbial adhesion

Two factors associated with denture structure and material, surface roughness and the presence of surface pores within the material matrices appear to be the major material-related factors which are associated with microbial adhesion. Both surface roughness and porosity provide mechanisms for the attachment of various microorganisms, and this can promote their colonization within the denture biofilm, which develops on the denture surface. The biofilm

is a complex matrix of various microorganisms [16]. In addition, some of the biofilm microorganisms can colonize within pores which open on the denture surface and hence penetrate into the material matrix. Colonization of these microscopic pores is of critical importance, as common denture cleaning approaches, such as brushing or the use of various antimicrobial rinse products, may be less able to access these sites and remove or kill these organisms. Hence, the microbes which reside within the pore structures may serve as a reservoir of residual organisms which can lead to rapid regeneration of the biofilm following surface cleaning.

2.3. Early colonizer: *Streptococcus oralis*

Different dental materials, such as acrylic, porcelain, and hydroxyapatite, have differing surface roughness; however, denture acrylic, which is the most commonly used denture material, has the highest level of surface roughness. Even smooth acrylic has a surface roughness approximately fourfold greater than that of smooth porcelain [17]. Charman et al. demonstrated more extensive in vitro colonization by *Streptococcus oralis*, an early colonizer which initiates the formation of denture biofilm, on rough (surface Ra 1.14 μm) versus smooth surface (Ra 0.07 μm) denture acrylic [18]. This supports the concept that an increase in roughness of the acrylic surface or other denture materials would promote more rapid establishment of the biofilm.

2.4. Denture biofilm composition

Denture biofilms are complex matrices containing many microorganisms. Using molecular biology approaches, Sachdeo et al. and Campos et al. characterized the microbiota in the oral cavities of healthy denture wearers as well as in denture stomatitis populations [19, 20]. As reported by Campos et al., a total of 82 bacterial species were identified in both the healthy subjects and the patients with denture stomatitis. Twenty-nine bacterial species were present exclusively in patients with denture stomatitis, and 26 species were detected only in the healthy subjects.

Using scanning electron microscopy, Glass et al. recently published images which exemplify the microbial complexity of these biofilms [21]. These images show a range of different microorganisms inhabiting the biofilm matrix and even penetrating into the pores of the denture acrylic [21]. In what may be the first study of its kind, these authors further characterized the biofilm population, identifying potential pathogens and disease-causing microorganisms. Biofilm samples isolated from the dentures of 51 individuals living in different regions of the USA were obtained. Techniques allowing the differential growth of specific microorganisms identified 916 unique microbial isolates from these dentures, of which 711, 67, 125, and 13 were aerobic bacteria, anaerobic bacteria, yeasts, and amoebae, respectively. Interestingly, no two dentures harbored the same microbiota; in addition, no association between biofilm composition and denture cleanliness could be demonstrated [21]. Hence, the microbiology of denture biofilms is complex. Biofilms occur on both complete dentures and partial dentures. Since the potential involvement of the biofilm in the disease is determined by the composition of the organisms contained within the biofilm, controlling and limiting the growth of this matrix by stringent and appropriate cleaning of dentures are critical.

2.5. Oral microbiome and systemic disease

Oral bacteria have been implicated in bacterial endocarditis, aspiration pneumonia, gastrointestinal infection, and chronic obstructive pulmonary disease. Dentures provide a reservoir for microorganisms associated with these infections, in particular respiratory and systemic opportunistic pathogens. As such, they may present an environment for antibiotic-resistant bacteria [22]. Because dentures on occasion may spend time in non-hygienic environmental conditions, non-resident oral microorganisms including *Streptococcus pneumoniae, Haemophilus influenzae, Klebsiella* spp., *Pseudomonas* spp., and *Staphylococci* including MRSA strains have been isolated [23, 24]. The continuous aspiration of microorganisms from denture plaque exposes patients to the risks of infection and the role of dentures may be significant [25].

It is not our purpose in this chapter to provide an extensive review of our knowledge and understanding of denture stomatitis, which has been broadly and extensively reviewed by others [26–36]. Denture stomatitis is a common disorder, occurring in up to 65% of denture wearers. It is characterized by a chronic inflammation of the oral mucosa, most often on mucosal areas which lie beneath the denture base. While denture stomatitis was originally considered to be, at least in part, a traumatic disorder due to poorly fitting dentures, it is now recognized as an inflammatory disorder. If there is any causative role of traumatic injury from poor-fitting dentures in denture stomatitis, it is minor. Emerging evidence suggests that ill-fitting may be a risk factor for the development of oral cancer [37]. The degree of inflammation varies and for diagnostic purposes is graded by using the well-established three-point Newton score [38]. Importantly, regardless of the severity of the inflammatory score, patients with denture stomatitis may be symptomatic or asymptomatic.

2.6. *Candida albicans* association with denture stomatitis

Denture biofilm has a role in denture stomatitis as there is a clear association between the occurrence of denture stomatitis and the presence of *Candida albicans* colonizing both denture materials and the oral mucosa. Indeed, *C. albicans* has been reported to have a selective affinity for colonizing biofilms formed on denture acrylic, with about fourfold greater biomass within biofilms on this substrate as compared to hydroxyapatite [17]. In the pathogenesis of denture stomatitis, *C. albicans* is considered an opportunistic pathogen. While *C. albicans* manifests a significantly greater presence on denture surfaces of patients with denture stomatitis, a clear causal relationship for *C. albicans* as the primary infectious agent responsible for development of this disorder has not been demonstrated. Hence, our current understanding is that *C. albicans* infection is not the single cause of denture stomatitis but has an association with the disorder and may have a role in increasing the likelihood of, or sustaining the associated, oral mucosal inflammation. Denture-related factors associated with denture stomatitis include poor denture cleanliness and hygiene, age of dentures, and continual denture wearing [28, 34, 39–41]. All of these have been reported to significantly increase the risk of denture stomatitis. All of these factors also promote formation of the adherent biofilm on the denture surfaces and hence provide conditions which increase the likelihood of the presence of *C. albicans*. Typical treatment strategies include efforts to improve denture cleanliness and oral hygiene among patients, which can also include replacing old dentures with new prosthetic

devices as well as treatment with topical or oral antifungal agents. In general, treatments can eradicate fungal infection and reduce inflammation, but stomatitis rapidly recurs once treatment is halted unless there has been a successful concomitant effort to clean and subsequently maintain the cleanliness of patients' dentures.

2.7. The importance of denture cleansing in reducing microbial biofilms and disease risk

The development of denture adherent biofilm provides the opportunity for colonization of a wide range of pathogenic and opportunistic pathogenic microbial organisms. Since the microbiota may contribute to both oral and systemic infectious disease, maximizing their eradication from the denture surfaces during routine denture cleansing could be of critical importance in improving the health of denture wearers. A number of studies evaluating different denture cleanser methods on bacterial survival have been reported [42]. These studies suggest that differences between denture cleaning methods exist and that there are simple approaches which can potentially maximize eradication of contaminating pathogens from denture surfaces.

Brushing dentures with standard toothpastes remains the most common approach to denture cleaning; however, this is inadequate. Combining brushing and use of a soaking cleanser is superior for killing bacteria and removing the adherent biofilm and plaque [30, 31, 33]. Furthermore, toothpastes generally contain abrasive components, and cleaning dentures by brushing with dentifrices has been shown to increase surface roughness [43]. Increased roughness of denture surfaces has been shown to increase adherence of microorganisms and development of the adherent biofilm. In addition, others have reported a positive correlation between denture surface roughness and colonization with *C. albicans* [44, 45]. Hence, the method used to clean dentures may be important in controlling future microbial adherence. Use of denture cleansers which can effectively eradicate or remove microbial contaminants and disrupt the denture biofilm without the use of abrasive cleansers may offer significant benefits for denture wearers.

A study by Li et al. reported differences in eradication of *C. albicans* biofilms when evaluated by different denture cleansing methods [46]. The study compared several popular denture cleansing products used in China including (a) soaking with Kyoshin denture cleanser tablet (Kyoshin Company Ltd., Japan); (b) brushing with Colgate Cavity Protection toothpaste (Colgate, NY, USA); (c) brushing with Bamboo Salt & UDCA toothpaste (LG, Beijing, China); (d) brushing with Yunnan Baiyao toothpaste (Yunnan Baiyao Group Co., Kumming, Yunnan, China); (e) brushing with Zhonghua Aloe toothpaste (Unilever, Heifei, Anhui, China); (f) soaking with Polident denture cleanser (GSK, Brentford, UK); and (g) soaking with sodium bicarbonate (0.5 g, Neptunus, Fuzhou, Fujian, China). Compared to the control (PBS) and all other treatments, only Polident, which combined soaking with a commercial denture cleanser and brushing using the same solution, resulted in almost complete removal, or eradication, of *C. albicans* from the denture acrylic disks. Furthermore, no significant regrowth of *C. albicans* was noted over a subsequent 24-h incubation following treatment with Polident. In comparison, the other procedures resulted in some reduction in *C. albicans*; however, rapid regrowth and reestablishment of *C. albicans* and the denture biofilm were observed within 6–24 h.

Lee et al. evaluated six different cleaning methods for dentures including (a) mechanical—brushing with Colgate Extra Clean toothpaste (Colgate-Palmolive, Guangzhou, China); (b) chemical—soaking with a Polident denture cleanser (GSK, Dublin, Ireland); (c) combined chemical and mechanical; (d) chemical—soaking in a commercial chlorhexidine gluconate mouthwash (Parmason Shining, Taipei, Taiwan); (e) UV irradiation (ADH Health Products, Seoul, Korea); and (f) soaking in water [47]. Compared to the control (water), brushing, soaking with a denture cleanser, and the combination mechanical-chemical method were found to be superior to soaking in a commercial mouthwash or irradiation with UV light [47].

In 2009, the American College of Prosthodontists convened a task force to establish evidence-based guidelines for the care and maintenance of dentures. Based upon a review of several hundred abstracts and articles, the recommendation put forth by the task force for effective denture cleaning was daily soaking and brushing with an effective, non-abrasive denture cleanser [48].

3. Conclusions

The relationships between oral and systemic health are complex. As illustrated in **Figure 1**, various societal factors, such as attitudes, beliefs, education and income, and behavioral factors such as oral hygiene, diet, general health maintenance, and engaging in high-risk activities, contribute to oral health. Specifically, these factors will impact dental caries and the development of periodontal disease. While not addressed specifically in this review, periodontal disease is associated with a chronic inflammatory condition and has been shown to have a relationship for increasing risk and contributing to the development of chronic systemic disorders, including cardiovascular disease, stroke, diabetes, renal disease, and respiratory diseases. This review has focused on the health impacts of edentulism and denture wearing and how we can control and improve adverse risks associated with denture wearing. Eventual tooth loss and the requirement for denture prostheses are generally considered an outcome of dental caries. The use of removable dentures, whether complete or partial dentures, is associated with changes in eating and social habits and alterations in the microbiota (or oral ecology). It is well established that the sustained presence of novel pathogenic and opportunistic pathogens in the denture biofilm, especially *C. albicans*, clearly contributes to an increased risk for denture wearers to develop denture stomatitis. In addition, the range of pathogens which colonizes denture surfaces also appears to contribute to increasing the risk for several systemic diseases. The risk potential appears to be related to the potential for these pathogens to support chronic systemic inflammation.

Hence, there is a critical need for the education of both professionals and denture patients on the importance of maintaining denture hygiene and the most appropriate and effective means for doing so. Recent studies have demonstrated differences between denture cleansing methods on removal of surface-contaminating microorganisms. In general, the use of a commercial denture cleanser appears to provide better removal and eradication of microorganisms from the denture surface and also slows the rate for regrowth of specific organisms on the dentures. The effects of denture cleansers combined with brushing using the cleaning solution appear to exceed that of brushing with an abrasive dentifrice alone.

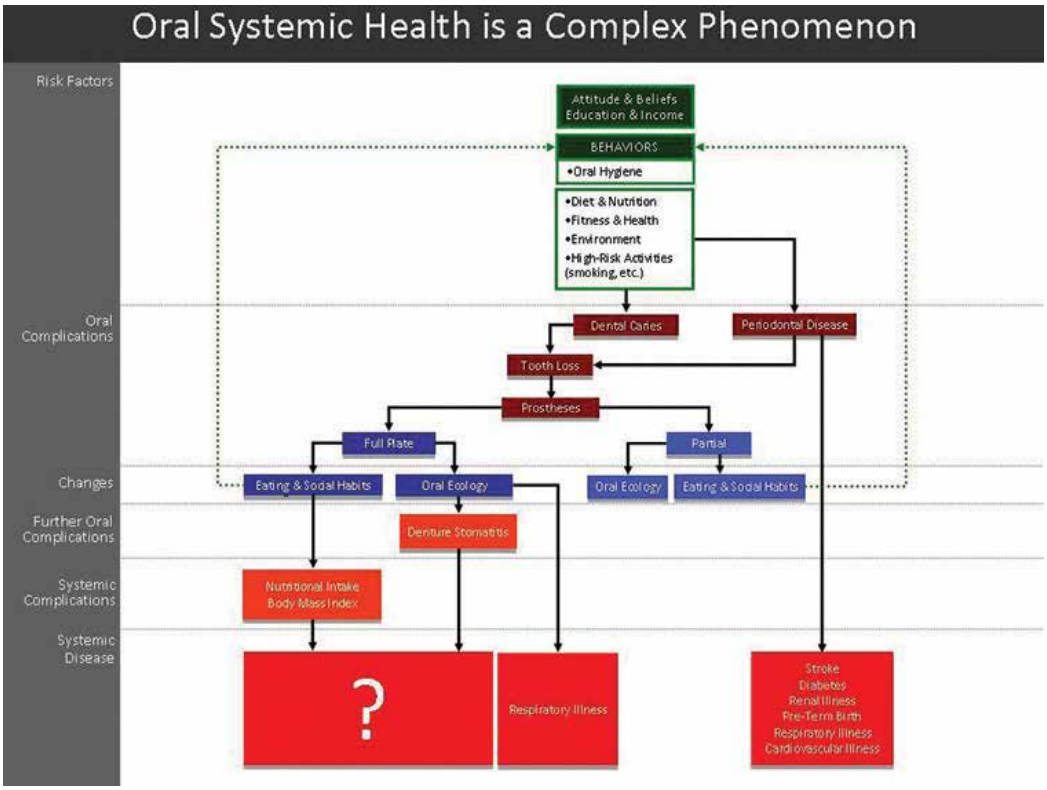

Figure 1. The oral and systemic health linkage has been pioneered by evaluating the relationship between periodontal disease and systemic diseases including diabetes, cardiovascular and stroke as summarized on the right side of this chart. Oral and systemic health as it relates specifically to the denture wearer is summarized on the left side of the chart. Initial systemic targets have included stomatitis and respiratory disease.

In summary, the age distribution of the world population is changing. Over the next 1–2 decades, there will be a significant increase in the number of elderly individuals worldwide. In many countries, this will be associated with a significant increase in the percent of their respective populations who are edentulous and who will rely on denture prostheses. There is an association between denture wearing and adverse impact on systemic health. This may become more profound with the ongoing demographic population shift we are experiencing. Improving hygienic maintenance of dentures, especially among the growing population of elderly, may reduce their risk of developing systemic disease. Furthermore, relatively simple approaches, such as the regular use of denture cleansers to clean dentures, may greatly improve denture hygiene, reduce accumulation of denture biofilm and plaque, and reduce chronic inflammatory conditions which can contribute to oral disorders, such as denture stomatitis and various systemic diseases.

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Conflict of interest

The authors of this manuscript have no conflict of interest with the subject matter of this chapter.

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Chapter 3

Anaerobic Bacteria Associated with Periodontitis

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Additional information is available at the end of the chapter

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Abstract

Oral bacteria are highly associated with oral diseases, and periodontitis is a strongly prevalent disease, presenting a substantial economical burden. Furthermore, there is a strong association between periodontal bacteria and other diseases, such as cardiovascular disease, rheumatoid arthritis, or diabetes, so it becomes clear that efficient periodontal cure would be of good medical benefit to general health. Periodontally, Healthy loci show a low number of bacteria which are cultivable by individual sulcus, 10^2–10^3 microorganisms with almost Gram-positive microbiota, including *Streptococcus* and *Actinomyces* species. In gingivitis, it is characterized by an increased bacterial number, 104–105 microorganisms by periodontal sulcus, besides an increased diffusion of Gram negative bacteria (15–50%).The increased number of oral bacteria could be associated with the decreased role of the innate and adaptive immunity; so, this chapter will focus on the most prevalent bacteria associated with the oral disease on the one hand and the role of innate immunity and adaptive immunity (Interleukin 1 Beta Il-1β and Tumor necrosis factor-alpha TNF-α) in oral diseases on the other hand.

Keywords: anaerobic bacteria, oral bacteria, oral diseases, periodontitis, oral immunity

1. Introduction

Oral bacteria are highly associated with oral diseases; periodontitis is a strongly prevalent disease, presenting substantial economic problem [1]; and oral disease are associated with other diseases, such as cardiovascular, rheumatoid arthritis, or diabetes, so it becomes clear that good periodontal cure would be of excellent medical interest to general health [2]. Periodontally, healthy sites show a low number of bacteria which are cultivable by individual sulcus, 10^2–10^3 microorganisms with almost Gram-positive microbiota, including

Streptococcus and *Actinomyces* species. In gingivitis, it is characterized by an increased bacterial number, 10^4–10^5 microorganisms by periodontal sulcus besides an increased diffusion of Gram-negative bacteria (15–50%) [3]. The increased number of oral bacteria could be associated with the decreased role of the innate and adaptive immunity; so, this chapter will focus on the most prevalent bacteria associated with the oral disease on the one hand and the role of innate immunity and adaptive immunity (interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α)) in oral diseases on the other hand.

2. Historical review on the classification and identification of oral bacteria

The initial date for the identification of oral bacteria belongs to 1680, when Antonie van Leeuwenhoek noticed, described, and isolated the microorganisms from his teeth plaque by using a primitive microscope. He drawn the noticed microbes and, when he established with the current knowledge, these drawings represented the most plentiful bacteria found within the oral cavity, including fusiform, spirochetes, and cocci bacteria [4].

Record research, a wide range of clinical studies on animals, engaged these oral bacteria with two common diseases, periodontitis, and dental caries. Even long before the visual observations of microorganisms, about 5000 BC, the Sumerians accused certain form of living (called as tooth worm) as a causative agent of caries on teeth [5]. Limited microbiological cultivation procedures and isolation techniques beginning of the nineteenth century forbid scientists to identify the exact causative agent of the disease. But this finding was partially done in 1925, by Clarke [6]. Unlike dental caries, another human oral disease is called periodontitis, and it is considered as the second most common disease worldwide. The early studies including oral bacteria in the pathogenesis of periodontitis were done on a hamster. Administration of penicillin inhibited-periodontitis in hamster gives a clear evidence of a bacterial agent [7]. Some studies isolated bacteria from dental caries, called *Streptococcus mutans* and described its ability to ferment many sugars and produce acids in glucose broth (pH of 4.3). However, he was not able to prove that *S. mutans* actually produces dental caries, but this finding was experimentally proven later in 1960 [8]. Whereas the infectious case of periodontitis appeared by demonstration of its transmissibility during infection from a person to another [9]. For a long time, periodontal disease researchers aimed to determine specific bacteria from a complex microbial plaque that may be considered a sole causative agent of periodontitis. The big problem was the cultivation of oral bacteria in laboratory. Most of the oral bacteria are anaerobic that died by air and considered fastidious microbes. This was recognized by researchers at that time. Major progress in the anaerobic culture was done in 1960 by designation of anaerobic glove boxes (a primitive form of now widely used anaerobic chambers), and it was used for the first time by Socransky [10]. This invention improved anaerobic cultivation techniques and was combined with optimized complex culture media; it allowed the invention of a pure and a good culture of more than 300 oral bacteria types in the period of 40 years ago, including clinical samples from supragingival and subgingival dental plaque taken from diseased and healthy subjects [11].

The studies on healthy subjects who agreed to take toothbrushing for a prolonged period appeared direct association between assembly of dental plaque and the initiation of gingiva diseases, mild form of oral diseases [12, 13]. After 28 days without basic oral hygiene in periodontally healthy subjects, there was a rapid assembly of bacterial plaque on the surface of teeth, and gingivitis was developed in all subjects within 10–21 days. These damages were reversible when toothbrushing was reintroduced. The researchers analyzed the smear of dental plaque specimen taken during the 28th day, and they found, at first, colonizing bacteria on the surface of the teeth, bacteria which belonged to the Gram-positive cocci and rods, Gram-negative cocci and rods, filaments, and fusobacteria, respectively, while finally spirochetes and spirilla were taken place in some times during colonizing. The outside of clinical gingivitis linked with the manifestation of the Gram-negative bacteria, and other studies on the microbial rotation in oral plaque formation confirmed these outcomes [14]. Through the years' progress, many other culture-based and molecular methods were given a huge information about the type of species included in periodontitis. A passionate dentist, W. D. Miller, studied hard for a long time in the of Robert Koch's laboratory trying to discover the microorganisms which were responsible for teeth decay; he published his research in 1980, with a book called *Microorganisms of the Human Mouth*; and in the same book, he suggested a chemoparasitic theory. According to that theory, in a sensitive host, carbohydrates fermentable oral microorganisms convert carbohydrates into acid, then the acid demineralizes tooth structure specially enamel [15, 16].

The classification of periodontal pathogens was tried to figure out by many researchers. The most understanding classification divided the periodontal pathogens into color-coded clusters published by Socransky and his team in 1998. This division resolves and identifies many problems and complexes of bacteria and clears their series of infection in the oral plaque and their role in periodontitis. Biofilm structure, which extends away from the tooth surface, was essential in this classification, and the bacteria responsible for dental plaque were classified into six clusters (red, orange, yellow, green, blue, and purple). *Actinomyces odontolyticus* and *Veillonella parvula* represented the "purple" form, while species of *Streptococci* including *S. sanguinis* and *S. oralis* refer to the "yellow" form [17].

The first colonizers of the surface of the teeth with *Actinomyces* species are purple and yellow form of this classification. The next complex, designated with green, included *Capnocytophaga* spp., *Campylobacter concisus*, *Eikenella corrodens*, and *Actinobacillus actinomycetemcomitans*, the bacteria contributing to the primary changes in the host. The "bridging species" formed the orange cluster are as follows: *Prevotella* spp., *Micromonas micros*, *Fusobacterium* spp., *Eubacterium* spp., and *Streptococcus constellatus*. That cluster included the species capable of using and secreting nutrients in the biofilm, in addition to expressing cell surface molecules facilitating binding to early colonizers, and the individual of the red complex. Finally, *P. gingivalis* and *T. denticola* in addition to *Tannerella forsythia* refer to the red cluster, and these are considered the prevalent pathogens in periodontitis progression; however, there is a clear association between the prevalence, number of these bacteria, and periodontitis clinical parameters [17, 18]. These three bacteria (in particular *P. gingivalis*), besides individuals of the orange cluster also linked with periodontal lesions, have been heavily studied in vitro, aiming to the identification of their key virulence mechanisms [18].

3. Most prevalent diseases caused by oral bacteria

Many major periopathogens can be seen in healthy individuals of all ages, indicating the coexistence of these bacteria as a normal flora in the host. These bacteria increase their numbers over time, and this change depends on the conditions of the internal or external environment, and it induces chronic periodontal inflammation that can cause the teeth loss as an outcome destroying the alveolar bone [19]. The inflammation of the tissues around the tooth due to accumulation of dental plaque is considered the main characteristic of acute and chronic periodontitis. The current classification of oral disease included the following [20]:

- Gingivitis: Plaque triggers inflammation in the gingivae that are characterized by red, swollen tissues and bleeding while brushing or probing.
- Chronic periodontitis: The connective tissue attachment of the teeth and destruction of junctional epithelium are damaged. Periodontal pockets and alveolar bone destruction occurred, and this state leads to chronic periodontitis.
- Aggressive periodontitis: It is a severe condition that represented the high proportion of younger cohort patients, the progression of disease is rapid, and the degree of destruction of the tissue (connective tissue) is high. The higher the level of the plaque, the higher the level of the disease.
- Necrotizing ulcerative gingivitis (NUG): Painful ulceration of the tips of the interdental papillae. Grey necrotic tissue is visible and there is an associated halitosis. The condition is termed necrotizing ulcerative periodontitis (NUP).
- Periodontal abscess: Inside the periodontal pocket is a different species of bacteria when the immune system responded to infection, and the periodontal abscess is form. Acute or chronic condition may occur, and in some time, the condition is asymptomatic.
- Perio-endo lesions: Lesions may be coalescing or independent, and the periodontal pathogen source originates either in the root canal system or in the periodontium.
- Gingival enlargement: The thickness occurs in response to irritation caused by plaque or calculus, and the other responses are repeated friction or trauma changes in hormone levels or in some time the effect of a drug.

The most common periodontopathogen correlated with aggressive forms of periodontitis is *Aggregatibacter* (previously *Actinobacillus*) *actinomycetemcomitans*. This small Gram-negative coccobacillus, capnophilic and non-motile have been determined as the most causative factor of aggressive periodontitis in young individuals and adults [21]. *A. actinomycetemcomitans* has been divided into six serotypes, and it has been postulated that some serotypes are correlated with periodontitis more frequently than periodontal health. Exemplifying this relationship, serotype C has appeared more repeatedly from healthy subjects and serotypes A and B more frequently in periodontitis [22]. But differences are pointed in *A. actinomycetemcomitans* serotype distribution when ethnicity and geographic location are taken into account; still, 3–8% of strains have remained nonserotypeable [23].

Gram-negative obligate anaerobe asaccharolytic bacteria (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*) have been extensively correlated with periodontitis [17]. *P. gingivalis* has been detected in correlation with periodontal damages and has an arsenal of virulent factors that can affectively stimulate the host responses [18]. *T. forsythia* was first described at the Forsyth Institute, and it became a recognized periodontopathogen because of its repeated detection from sites with periodontitis and its huge correlation with the formation of pocket with deep size [24]. *T. denticola* is also frequently presented in periodontitis subgingivally sites, and their number is decreased after appropriate treatment [25]. Other bacteria that have been related with periodontitis include *Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum*, *Selenomonas*, *Eubacteria*, *Eikenella corrodens*, *Campylobacter rectus*, and *Parvimonas micra* [26].

Molecular microbiological studies have shown that many of the bacteria species are recognized in correlation with periodontitis and expanded to include uncultivated and less-often-identified phylotypes [27].

4. Mechanisms of destruction in periodontal tissues

Bacteria can cause damage directly and indirectly. Various mechanisms are described in the steps below. Cytotoxic cellular immune responses to self- and pro-inflammatory responses involving release of interleukin-1 beta (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) could lead to tissue destruction [28].

- Crevicular epithelium is destroyed by Porphyromonas gingivalis, Treponema denticola, and Aggregatibacter actinomycetemcomitans.
- Leukotoxin is secreted by A. actinomycetemcomitans, and it is impaired with polymorphonuclear (PMN) function (chemotaxis, phagocytosis, and intracellular killing) and other leukocytes.
- P. gingivalis is dysregulated of cytokine networks by their R1 proteinase activity.
- Capnocytophaga spp. are degraded of immunoglobulins.
- P. gingivalis, *P. intermedia*, T. forsythia, and T. denticola increase the mucosal permeability and degradation of collagen by fibroblastic collagenase by volatile sulfur compounds from Gram-negative anaerobes in addition to disaggregation of proteoglycans by disrupting SH (sulphydryl) bonds or impaired host cell function.
- Destruction of periodontal tissues proteins by proteolytic enzymes (collagenases and trypsin-like proteinases) to peptides and amino acids provides nutrients for Gram-negative bacteria. While the extracellular matrix is destroyed by other type of enzymes that called hydrolytic enzymes.
- The complement is activated when infection occurs by bacteria in response to LPS.
- Lipoteichoic acid from Gram-positive bacterial cell walls stimulates bone resorption.

5. Immunopathological factor associated with periodontal pathogens

The pathogenesis of periodontal disease is categorized into four stages, based on histopathological examination of the development of periodontal inflammation due to plaque accumulation. These stages are called **(a)** the initial, **(b)** the early, **(c)** the established, and **(d)** the advanced lesions [28, 29]. The description of stages in periodontal damage progression is listed below:

(a) Initial lesion

Without normal oral hygiene measures, within 2–4 days of plaque accumulation, the first inflammatory response is observed histologically. It is characterized by vasodilatation, loss of perivascular collagen, and active migration of monocytes and neutrophils into the periodontal tissues and junctional epithelium mediated by endothelial leucocyte adhesion molecules (ELAM) and intercellular adhesion molecules (ICAM) that are observed. The exudation of serum proteins from the dilated capillaries leads to an increase in gingival crevicular fluid (GCF) flow.

(b) Early lesion

The early lesion presents after 4–7 days of plaque accumulation. This is clinically detectable as gingivitis, with more pronounced vascular changes and an increase in extravascular neutrophils. Histologically, the inflammatory infiltrate consists of numerous lymphocytes (predominantly T lymphocytes), immediately below the proliferating basal cells of the junctional epithelium. Destruction of the gingival connective tissue occurs through apoptosis of fibroblasts, and a reduction in the collagen fiber network of the marginal gingivae occurs via host- and pathogen-derived MMP.

(c) Established lesion

This is similar to the early lesion with a shift in the cell population in the inflammatory (2–3 weeks of plaque accumulation). Here, plasma cells are the main histological features in older patients, whereas in younger patients, the infiltrate continues to be dominated by lymphocytes. Clinically, inflammation will become more pronounced with an increase in swelling, and the false pocket will form. T and B lymphocytes, antibodies, and complement are found in the inflamed marginal gingival and gingival sulcus.

(d) Advanced lesion

At this stage the inflammatory lesion expands into the periodontal ligament and alveolar bone. There is a destruction of a tissue linked to the teeth. The junctional epithelium migrates down the root surface to form a true periodontal pocket. MMP has the ability to destroy periodontal ligament and the surrounding alveolar bone through enhanced osteolytic activity. The direct cytotoxicity of bacterial products leads to direct tissue damage. Proteinases, collagenases, epitheliotoxin, cytolethal distending toxin, hemolysin, hydrogen sulfide, and ammonia are examples of bacterial products. Moreover, dysregulation of the factor derived from the host such as proteinases and proteinase inhibitors; MMPs and tissue inhibitors to metalloproteinases (TIMPs); pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α, and

others; prostaglandins; and the products of polymorphonuclear leukocytes leads to the damage of the connective tissue attachment.

5.1. Innate immunity response to periodontal pathogens

The innate host response primarily involves the recognition of microbial components such as LPS by the immune cells of the host, and the result of activation produced inflammatory mediators. The Toll-like receptors (TLRs), which are synthesized by leukocytes and resident cells in the periodontal tissues, can activate the innate immunity response by binding to numerous bacterial components [30–31]. The developing biofilm consists of initially Gram-positive cocci in health, changing to the increased numbers of motile Gram-negative anaerobes in gingivitis and periodontitis [17].

Endotoxin (LPS) of Gram-negative bacteria is considered a huge stimulator of TLR4. LPS from Gram-negative bacteria cell wall can be released through cell lysis. It becomes linked to the extracellular acute-phase protein LPS-binding protein before binding to the cluster of differentiation 14 (CD14). The outcome is transferred from LPS to the extracellular domain of the TLR4 receptor and subsequent TLR4 signaling [32]. Gram-negative bacteria also activate TLR2 through their cell membrane proteins, TLR5 through flagella, TLR9 through the determination of bacterial cytosine-phosphate-guanine (CpG) DNA, and nucleotide-binding oligomerization domain-containing proteins 1 and 2 (NOD 1, NOD 2) through peptidoglycan derivatives [32, 33].

Periodontal pathogens have been reported to stimulate TLRs in vitro, such as LPS of *P. gingivalis*, and fimbriae is a potent TLR2 agonists [34–36]. *A. actinomycetemcomitans* and whole *P. gingivalis* will stimulate TLRs [37–40]. Moreover, many bacteria can initiate an immune response via TLR9, which also detects viable bacterial DNA [41]. It is therefore clear that the myriad of bacteria that are found in both health and increasing hardness of periodontitis will present a challenge to the response innate immunity. Following TLR activation, an intracellular signaling cascade occurs which can result in stimulation of transcription factors, subsequent inflammatory cytokine expression, leukocyte migration to the infection locus, and tissue damaging [42, 43]. The nucleotide-binding oligomerization domain (NOD) and the inflammation system have been submitted as possible accessory molecules in the induction of response of innate immunity against periodontopathogens [44–46]. The junctional epithelium is the front line between the oral normal flora and the host. It is well equipped to recognize invading pathogens, some studies showed that the present of mRNA encoding TLR2, TLR3, TLR4, TLR5, TLR6, and TLR9 in gingival epithelial cells is a clear indication of the existence of the infectious agent [47]. Within the gingival epithelium and between the connective tissue, Langerhans cells and tissue dendritic cells are also found. TLRs are produced by antigen-presenting cells and appear on their surface including TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR8, and TLR10. The response of adaptive immunity against bacterial products is monitored by these receptors [30, 33].

The alveolar bone is the supporting structure into which the periodontal ligament inserts that is ultimately destroyed by the inflammatory lesion of periodontitis. Osteoblasts and osteoclasts included in bone turnover also express TLR1, TLR4, TLR5, TLR6, and TLR9 [35] and TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9, respectively [48]. It is therefore possible that TLR signaling within the bone can generate an inflammatory response to invading pathogens,

leading to pathological resorption of the bone through excessive or prolonged production of osteolytic host molecules, including IL-1, tumor necrosis factor-α (TNF-α), and prostaglandin E2 (PGE2), which stimulate osteoblast inhibition and osteoclast activation and maturation through the receptor activator of nuclear factor kappa-B ligand/osteoprotegerin (RANKL/OPG). Many biological events in periodontal disease are obligatory regulated by cell–cell interactions, which may be grouped into two forms: cognate (adhesive) interaction, achieved by mutual recognition between membrane-bound cell surface molecules, and cytokine-mediated interactions [49].

Intercellular adhesion molecule-1 (ICAM- 1, CD54) and ITGB2 (integrin beta 2, CD18), which stabilize cell–cell interactions and facilitation of leukocyte migration across the endothelial barrier, are achieved by ICAM-1 (intercellular adhesion molecule-1, CD54) and ITGB2 (integrin beta 2, CD18); therefore, they are called adhesion molecules [22].

5.1.1. Adaptive immunity cytokine (pro-inflammatory cytokines) response to periodontal pathogens

Cytokines are a large and diverse family of soluble mediators including interleukins. Cytokines play a major role in various biological activities such as differentiation, proliferation, regeneration, development, repair inflammation, and homeostasis. Cytokine networks are an important side of periodontal inflammation and subject to several excellent reviews [50].

The IL-1 family of cytokines (IL-1α and IL-1β) has different roles in immunity, tissue homeostasis, tissue breakdown, and inflammation. Monocytes and macrophages are released TNF-α in huge amount in responses for infection. It induces the production of collagenase and is secreted by fibroblasts to make damages on the cartilage and bone, and it has been involved in the damage of the periodontal tissue in periodontitis [51].

5.1.2. Interleukin-1α and interleukin-1β (IL-1α/IL-1β) role in periodontal pathogens

IL-1 is a polypeptide, which has diverse activities and roles in immunity, inflammation, tissue breakdown, and tissue homeostasis [52]. IL-1 is synthesized by various cell types, such as fibroblasts, lymphocytes, skin cells, macrophages, monocytes, vascular cells, and osteocytes, following its activation. IL-1α and IL-1β belong to the IL-1 family of cytokines which have similar biological functions and bind to the same receptors found on many cell types. Fibroblast cells in periodontal ligament are triggered by IL-1 to stimulate them to release cellular mediators, prostaglandin E2 (PGE2), and matrix-degrading enzymes which destroyed the connective tissue and lead to attachment loss [53]. Some studies refer that IL-1 is involved in the pathogenesis of periodontitis and also associated with bone destruction. Together, IL-1α and IL-1β have appeared to stimulate bone resorption and bone inhibition in cooperation with TNF-α. IL-1β has appeared to be significantly more potent in mediating bone resorption compared with IL-1α and TNF-α. IL-1 can also stimulate elevated production of matrix metalloproteinases (MMPs), procollagenase, and plasminogen activator [54].

5.1.3. Tumor necrosis factor-alpha (TNF-α) role against periodontal pathogens

TNF-α is a pro-inflammatory cytokine released by activated monocytes and macrophages [55]. TNF-α functions include the upregulation of attachment molecules and chemokines which

are involved in the cell migration to inflamed and infected sites [56]. Collagenase secreted by fibroblasts, resorption of the cartilage and bone, and damaging of the periodontal tissue all are stimulated by cytokine production [57]. Both GCF and periodontitis tissues have shown high levels of TNF-α, and it has shown positive correlation to MMP and RANKL expression [58, 59]. Animal studies also demonstrated that TNF-α plays a key role in inflammation and periodontal tissue damaging including bone resorption and loss of connective tissue attachment [58, 60]. Pro-inflammatory cytokines produced during infection (IL-1β and IL-6) are upregulated by TNF-α, this production linked with cell migration into the site of infection, and finally bone resorption occurred [55, 61]. New studies was done by Alwaeli and Abd [62, 63] who tried to interpret the relation between concentration of TNF-α and IL-1β and polymorphism of their genes, and they found some of SNPs (single-nucleotide polymorphisms) that trigger the production of TNF-α and IL-1β, by increasing the activity of their genes so the high concentration level of TNF-α and IL-1β leads to additional damage in periodontal tissue, while the other SNPs decrease the production of TNF-α and IL-1β, for this reason the termed "SNP-genotype combination principal" for this phenomena by Alwaeli and Abd (62–63).

List of abbreviation

BC	Before Christ
CD	cluster of differentiation
CpG	cytosine-phosphate-guanine
ELAM	endothelial leukocyte adhesion molecules
GCF	gingival crevicular fluid
ICAM	intercellular adhesion molecules
IL-1β	interleukin-1 beta
ITGB2	integrin beta 2
LPS	lipopolysaccharide
MMP	matrix metalloproteinases
NOD	nucleotide-binding oligomerization domain
NUG	necrotizing ulcerative gingivitis
NUP	necrotizing ulcerative periodontitis
OPG	osteoprotegerin
PGE2	prostaglandin E2
PMN	polymorphonuclear
RANKL	receptor activator nuclear factor kappa-B ligand

SNP	single-nucleotide polymorphism
TIMP	tissue inhibitors to metalloproteinases
TLRs	toll-like receptors
TNF-α	tumor necrosis factor-alpha

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Chapter 4

Oral Microbiology in Periodontal Health and Disease

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Additional information is available at the end of the chapter

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Abstract

Oral microbial community is one of the most complex bacterial florae associated with human body. Up to now, more than 700 different bacterial species have been identified from human oral cavity. Oral bacteria form communities on distinctly different surfaces, such as hard enamel and cementum, as well as on soft epithelial cells. These communities are biofilms, which are characterized by their species composition, their surface or substratum composition, and the conditioning films coating the surfaces on which they form. The composition of the resident oral microflora shows local variations in composition on distinct surfaces (e.g., tongue, cheek, teeth) due to differences in key environmental conditions. Many studies have found that certain microbial flora may be compatible with a state of periodontal health and variations in oral flora is associated with varying degrees of periodontal disease. Information about the composition and the assembly processes of oral microbiota could be used to develop effective strategy and monitoring protocols for periodontal therapy.

Keywords: oral microbiology, periodontal health, periodontal diseases

1. Introduction

Mammals are complex gatherings of mammalian and bacterial cells structured into functional organs, tissues, and cellular communities [1]. Cell-rich bacterial communities are more numerous than human cells in each person with a ratio of 10 bacterial cells to each human cell. In other words, approximately 90% of the cells in and on the human body are microbial cells [2].

The birth of the oral microbiology had been signaled by the fascinating observation of Antony Van Leeuwenhoek (1632–1723), a Dutch dry goods merchant, who observed and described first microorganisms in tartar from his teeth with his primitive microscope. These microorganisms

are now known as some of the abundant bacteria reside in the oral cavity including cocci, spirochetes, and fusiform bacteria [3].

Oral microbial community is one of the most complex bacterial floras associated with human body. Up to now, more than 700 different bacterial species have been identified from human oral cavity. For a long time, the study of oral microbiology has gone through phases of "reductionism" and "holism." In reductionism, the strategy was to understand the whole by examining smaller components. Whereas in holism, microbiologists took the approach of system thinking that helps in understanding of microbial physiology which in turn will have a great impact on oral microbiology by providing invaluable insight into the etiology of dental and periodontal diseases [3].

The human mouth is profoundly colonized by microorganisms, comprising viruses, protozoa, fungi, archaea and bacteria. The normal microbiota of the mouth can act as opportunistic pathogens, and as a consequence of this, many oral diseases such as dental caries and periodontal diseases start to develop [4].

The use of culture-independent methods in determining the composition of the oral microbiome, together with next generation DNA sequencing methods is offering a far deeper analysis than hitherto possible. A combination of phylogenetic, metagenomic, transcriptomic, proteomic and metabolomic methodologies may be required to fully understand oral host-microbiome interactions relevant to health and disease [4].

The purpose of this chapter is to review the properties of the mouth that influence its function as a microbial habitat together with giving a description of the oral microflora associated with periodontal health and disease.

2. The mouth as a microbial habitat

The characteristics of mouth are ecologically different from all other surfaces of the body and control the types of microbes that are able to persist, so that not all of the microorganisms that enter the mouth are able to inhabit in it. The simple presence of the oral microbiota in the mouth inhibits colonization by pathogens, the phenomenon of colonization resistance [5].

The mouth has heterogeneous environments for microbial colonization, diverse habitats exist including, the mucosal surfaces (such as the lips, cheek, palate, and tongue). The properties of these habitats change during the life of an individual.

The growth of distinctive microbial communities is enhanced by the presence of different biological features of these surfaces [6]. Microbial ecology is concerned with the interrelationships between microorganisms and their environments. The most important concept in microbial ecology is the ecosystem which is considered as a complex of organisms in a specified environment associated with nonmicrobial surroundings. Different ecosystems with different assemblage of species and organic and inorganic constituents have been recognized at different sites in the oral cavity.

The site at which a population or a community of microorganisms grows, reproduces or survives is called a habitat, and the function of the microorganism in a habitat is its niche.

The properties of some of the major habitats in the mouth will alter throughout the life of an individual. These changes can be manifested during the first few months of life as the mouth at this time consists only of mucosal surfaces for microbial colonization. Another change will happen when hard nonshedding surfaces appear with the development of the primary dentition, providing a unique surface in the body for microbial colonization. The eruption of teeth also generates another habitat via the development of gingival crevice where the tooth emerges from the gums, and an additional major nutrient source for that site will be obtained from the gingival crevicular fluid (GCF) [6].

In addition, ecological conditions within the mouth will also be affected by the eruption and loss of teeth, the insertion of prostheses such as dentures as well as any dental treatment including scaling, polishing and restorations.

Further fluctuations in the stability of the ecosystem can be induced by external factors including the types of food ingested, periods of antibiotic therapy, and variations in the composition and rate of flow of saliva [6].

The health of the mouth is reliant upon the integrity of the mucosa which acts as a physical barrier by preventing penetration of microorganisms and antigens. In addition to the host defense, factors such as saliva and GCF play an important role in maintaining the integrity of these oral surfaces. For example, saliva contains several anti-bacterial factors, including salivary immunoglobulin A (SIgA) which can reduce or prevent microbial colonization of oral surfaces. Moreover saliva encompasses different types of antimicrobial peptides, including histidine-rich polypeptides (histatins), and cystatins, which may control the levels of yeasts, and a range of active proteins and glycoproteins (lysozyme, lactoferrin, sialoperoxidase) [7].

On the other hand, GCF contains large numbers of viable neutrophils as well as a minor number of lymphocytes and monocytes. Also, GCF can control the ecology of the site in many ways for example removing weakly adherent microbial cells, introducing additional components of the host defenses, and acting as a novel source of nutrients for the resident microorganisms [6].

3. Development of the resident microflora

The human fetus inhabits a sterile environment and from a microbiological point of view, acquisition of resident microflora of any surface influences by successive transmission of microorganisms to the site of potential colonization. It is noteworthy that the human birth is a turning point to its environment from the one that is free of microbe to the one that is microbes dominated.

Within a very short time of delivery, microbes are detectable on those surfaces of the baby that are exposed to the external environment, that is, the eyes, skin, respiratory tract, genito-urinary system, and oral cavity [8].

What is surprising is that despite the neonate's exposure to such a variety of microbes, only a limited number of species are able to permanently colonize the various body sites available, and each site harbors a microbial community comprised of certain characteristic species, that is, the microbes display "tissue tropism."

The mouth is highly selective for microorganisms even during the first few days of life. Only a few of the species common to the oral cavity of adults, and even less of the large number of bacteria found in the environment, are able to colonize the mouth of the newborn [9].

Pioneer organism is a term that defines the organisms to colonize first in a developing ecosystem. The pioneer organisms are capable to alter their environment and make it suitable for colonization by other species [10].

In the mouth, the predominant pioneer organisms are Streptococci and in particular *Streptococcus salivarius*, *Streptococcus mitis*, and *Streptococcus oralis* [11, 12].

The pioneer species are often replaced by other species after they have altered the habitat, making it suitable for colonization by other species by a process called a microbial succession.

There are two kinds of microbial succession. The first one is the autogenic succession in which, the sequence of species is brought about because the resident populations alter their surroundings in such a manner that they are replaced by species better suited to the modified habitat. The second type of succession is the allogenic succession where one type of community is replaced by another because the habitat is altered by nonmicrobial factors for instance changes in the physical or chemical properties of the region or changes in the host [10].

Gradually, the metabolic activity of the pioneer community changes the environment, in that way providing conditions suitable for colonization by a succession of other populations. Factors contributing to succession include changing the local Eh or pH, modifying or exposing new receptors on surfaces for attachment as well as generating nutrients as end products of metabolism (lactate, succinate, etc.) or as break down products which can be used as primary nutrients by other organisms [13].

The early colonizers organisms consist of mainly aerobic and facultative anaerobic species are able to tolerate the high oxygen concentrations and to battle the various removal mechanisms of the oral cavity such as swallowing, chewing, nose blowing and salivary, nasal and crevicular fluid outflow [14].

In a study of 40 full-term babies, a range of streptococcal species were recovered during the first 3 days of life, and *Streptococcus oralis*, *S. mitis biovar 1*, and *S. salivarius* were the numerically dominant species [15].

The replication of early colonized organisms allows the subsequent adhesion of other bacterial species, which though unable to stick to tooth hard surfaces, are quite capable of attaching themselves to already present microorganisms. This is so-called "secondary colonization." As the number of plaque layers' increases, nutritional and atmospheric gradients are created, the oxygen level decreases and the anaerobes can survive [16, 17].

As the multiplicity of the pioneer oral community increases, several species of Gram-negative Anaerobes start to appear.

In a study of edentulous infants with a mean age of 3 months, *Prevotella melaninogenica* was the most frequently isolated anaerobe, as it was recovered from 76% of infants. Additional commonly isolated bacteria were *Fusobacterium nucleatum*, *Veillonella* spp., and non-pigmented *Prevotella* spp. [18].

When the same infants were followed up longitudinally during the eruption of the primary dentition Gram-negative anaerobic bacteria were isolated more commonly, and a greater diversity of species were recovered from around the gingival margin of the newly erupted teeth. These findings confirm that a change in the environment, such as the eruption of teeth, has a major ecological impact on the resident microflora [19].

4. Dental plaque

Communication is a crucial part in successful organizations. Communication between oral microorganisms is essential for initial colonization and subsequent biofilm formation on the enamel surfaces of teeth and necessitates physical contact between colonizing bacteria and between the bacteria and their host [20].

Retention of bacteria to tooth surface prevents it from being swallowing by saliva. Through retention, these bacteria can form organized, intimate, multispecies communities referred to as dental plaque [21].

Dental plaque is structurally and functionally organized biofilm adheres resolutely to tooth surfaces as well as restorations and prosthetic appliances. It is a multi-species biofilm comprising of hundreds of bacterial species, salivary polymers, and bacterial extracellular products. The microbial species colonize the teeth, hard palate, tongue, carious lesions, oral mucosa, and periodontal pockets [22].

The distribution of the microbial species in these plaque biofilms varies depending on the anatomical locations and environmental factors [23].

Dental plaque is classified into supra-gingival and sub-gingival plaques, and both of them have significant contributions to dental and periodontal diseases [22].

The predominant microorganisms of supragingival plaque are Gram-positive facultative anaerobic bacteria particularly *Actinomyces* species, *Streptococci* and *Capnocytophaga* species. The Gram-negative species including *Veillonella* species, *Prevotella* species as well as *Porphyromonas gingivalis* and *Tannerella forsythia*. Whereas the subgingival plaque comprises the following species, *Streptococci, Prevotella denticola*, *Porphyromonas endodontalis*, and *Porphyromonas gingivalis* [24].

The difference between sub- and supragingival plaque as well as between periodontal disease and health is characterized by less proportions of *Actinomyces* spp. and higher proportions of *Prevotella intermedia*, *Prevotella nigrescens*, *Peptostreptococcus micros* and *Fusobacterium* spp. [25].

4.1. Formation of dental plaque

Dental plaque forms through a well-organized sequence of events, ensuing in a structurally- and functionally organized, species-rich microbial community [26].

The stages of plaque biofilm formation include acquired pellicle formation; reversible adhesion involving weak long-range physicochemical interactions between the cell surface and the pellicle, which can lead to stronger adhesin-receptor mediated attachment; co-adhesion resulting in attachment of secondary colonizers to already attached cells; and formation of mature, sub-gingival plaque biofilms [23].

Once dental plaque is formed, the overall composition of its climax community is varied with many species being identified at individual sites. The composition of microbial species in dental plaque is characterized by a degree of stability or balance among the component species. This stability is termed microbial homeostasis, and it is due to a balance carried out by numerous microbial interactions, including examples of both synergism and antagonism [27].

Essential inter-bacterial relationships have been detected in mature biofilms. Such relationships may affect the entire biofilm in general and to some extent the virulence of certain species. These relations are classified as positive or negative.

The positive relationships are known as symbiosis and are classified into three subclasses: mutualism, synergism, and commensalism. Mutualism is a symbiosis in which the bacterial species have equal benefit from their coexistence (*Porphyromonas gingivalis* and *Treponema denticola*; *Tannerella forsythia* and *Fusobacterium nucleatum*). Synergism is the interbacterial relation when the pathogenic potential of both species is superior to the sum of their individual potentials (*Porphyromonas gingivalis* and *Fusobacterium nucleatum*). Commensalism is a bacterial interaction that favors one of the two species (*Porphyromonas gingivalis* and *Campylobacter rectus*).

On the other hand, negative relationships between bacterial species exist in the form of antagonism (*Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans*; *Streptococcus sanguis* and *Aggregatibacter actinomycetemcomitans*) and competitive relations (*Porphyromonas gingivalis* and Gram-positive *Actinomyces viscosus*, *Actinomyces naeslundii*, *Actinomyces israelii*, *Streptococcus mutans*, *Streptococcus mitis*, *Corynebacterium* spp.) [28].

4.2. Quorum sensing in plaque biofilms

As many as 700 diverse species of bacteria have been isolated from the oral cavity [29]. These bacteria exhibit coordinated group behaviors and are responsible for causing periodontal infections as well as dental caries. Bacteria in biofilms come across much higher local cell densities than free-floating, planktonic cell populations (**Figure 1**) [30]. An apparent consequence of this is the elevated levels of metabolic by-products, secondary metabolites and other secreted or excreted microbial factors that biofilm cells encounter. Of particular interest are intercellular signaling molecules called the "quorum-sensing molecules" [31].

Quorum sensing is a process that allows the bacteria to sense one another and to regulate variety of physiological activities and biofilm formation. It was first described for the luminous

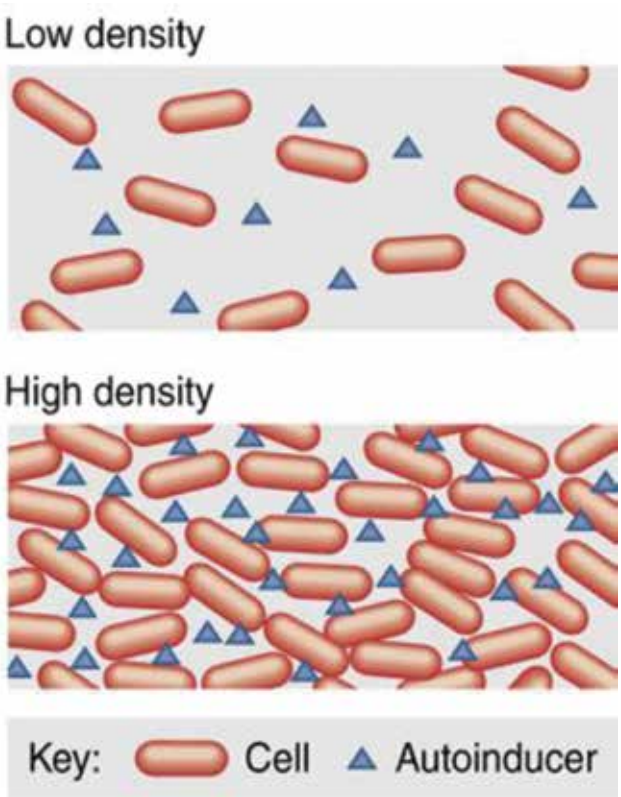

Figure 1. The ability of a cell to produce a signaling molecule (an autoinducer) and sense its extracellular concentration can enable the cell to sense changes in population density [30].

marine bacterium Photo-bacterium fischeri (*Vibrio fischeri*) in 1970 by Kenneth et al. who observed that these bacteria do not luminesce until they reach a high population density. Based on this observation, they postulated that bioluminescence in this organism was possibly controlled by molecular messengers that moved between cells. These messengers were called "autoinducers" [32, 33].

Quorum sensing relies upon the interaction of a small diffusible signal molecule (autoinducers) with a sensor or transcriptional activator to initiate gene expression for coordinated activities. It is extensively used by a variety of Gram-positive and Gram-negative bacterial species to coordinate communal behavior [31].

Quorum sensing systems in bacteria have been generally divided into three classes namely: LuxI/LuxR-type quorum sensing in Gram-negative bacteria, oligopeptide-two component-type quorum sensing in Gram-positive bacteria and luxS-encoded autoinducer 2 (AI-2) quorum sensing in both Gram-negative and Gram-positive bacteria (**Figure 2**) [34].

Quorum sensing permits the bacteria to sense one another and to regulate variety of physiological activities like symbiosis, virulence, motility, antibiotic production, and biofilm formation. Additionally, quorum sensing plays a role in expressing genes for antibiotic resistance

and in promoting the growth of beneficial species to the biofilm and discouraging the growth of competitors [35].

The physiological and clinical aspects of quorum sensing have received considerable attention. It was found that quorum sensing improves the ability of bacteria to increase bacterial defenses against eukaryotic hosts. Furthermore, the gene expression of some bacteria differs in biofilms formed on different dental surfaces and stressful circumstances of adjustment to the surface may persist enhancing intercellular signaling between bacteria [36].

Obviously, many genes and pathways are involved in biofilm formation in different bacteria; moreover, various quorum sensing systems are present in different bacteria. The use of proteomic and genomic techniques should help to elucidate the phenotypes associated with quorum sensing and the mechanisms by which these pathways work in causing periodontal diseases [31].

4.3. The bacterial composition of biofilm in relation to periodontal health

As in other environments, a substantial proportion of the total oral microbiota remains unculturable; therefore, nonculture methods are required to designate the overall species richness of the oral microbiome. Sequence analysis of 16S ribosomal RNA has been the method of choice because of its universal presence in all organisms.

The application of this methodology has led to the description of 11 phyla in the domain Bacteria in the oral microbiome in addition to methanogenic species of the Methanobrevibacter genus from the domain Archaea [37].

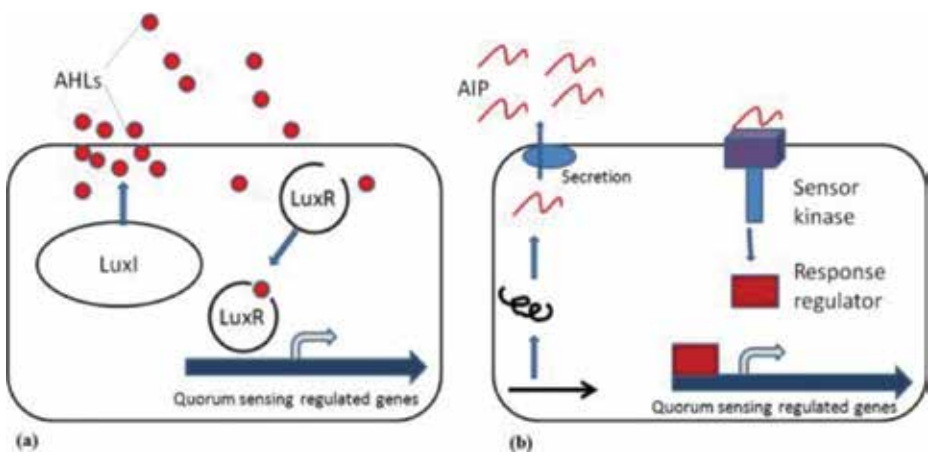

Figure 2. Schematic presentations of bacterial quorum sensing systems. (a) In Gram-negative bacteria, AHLs (filled circles) are produced by the LuxI synthase and will bind to the cognate LuxR receptor. The AHL-LuxR protein complex will bind to promoter DNA elements and regulate transcription of QS-regulated genes. (b) Gram-positive bacteria synthesize AIP (curvy lines) that are post-translationally modified and secreted. AIP detection occurs via a two-component signal transduction circuit, leading to the ATP-driven phosphorylation of a response regulator protein, which then binds to promoter DNA and regulates transcription of QS-regulated genes [34].

The periodontal microbiota is mostly heterogeneous and over 400 species have been defined in this habitat alone using a 16S rRNA amplification, cloning and Sanger sequencing approach [38].

Normally, the periodontal tissues remain healthy owing mainly to the numerous host protection mechanisms that work in the oral cavity [39].

Conceivably, the utmost unique and major host protection mechanism in the periodontium is the continuous passage of neutrophils from the underlying highly vascular periodontal tissue, through the connective and epithelial cell layers and into the gingival crevice. It has been estimated that approximately 30,000 polymorphonuclear neutrophils (PMNs) travel through periodontal tissue every minute and by this mean a constant contact between host neutrophils and the dental plaque biofilm will be facilitated [40].

The junctional epithelium surrounds the tooth surface and forms the "junction" between the tooth and host tissue. It is highly porous with large intracellular spaces and it contains no tight junctions and a lower number of desmosomes than the adjacent oral or sulcular epithelium [41].

Likewise, clinically healthy junctional epithelial tissue expresses high levels of IL-8, a potent neutrophil chemoattractant, that draws neutrophils to the adjacent dental plaque biofilm inhibiting biofilm growth (**Figure 3**) [42]. Additional host defense mediators associated with neutrophil exit from the vasculature and transit through the connective tissue, such as ICAM-1 and E-selectin, are also expressed in the appropriate tissues in clinically healthy periodontal tissue [43].

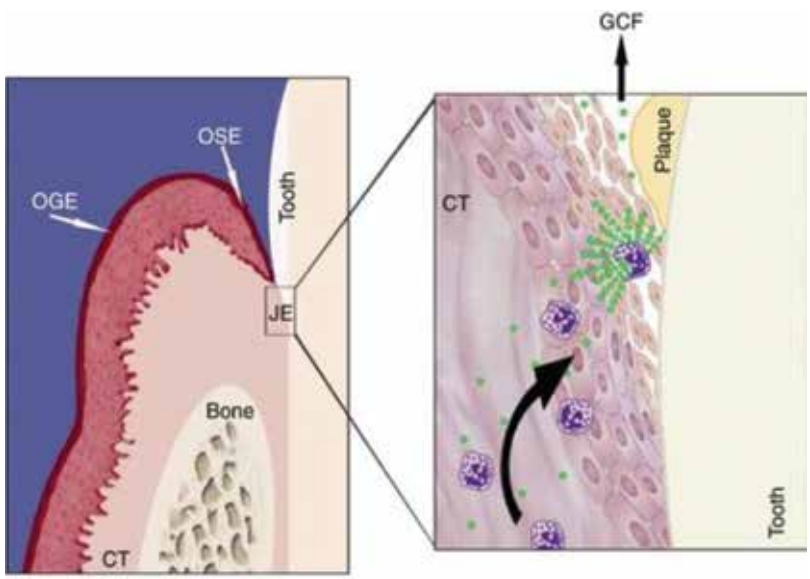

Figure 3. The junctional epithelium exemplifies a polymorphonuclear neutrophil degranulating upon bacterial stimulation [42].

More additional immunohistochemical and in situ studies have discovered that clinically healthy periodontal tissue also expresses human β defensin molecules 1, 2, and 3 along with soluble and membrane bound CD14 and lipopolysaccharide binding protein [44–46].

These innate defense proteins function in either bacterial killing or bacterial elimination, in line with the concept that healthy periodontal tissue is armed by the innate host defense system to protect against bacterial infection.

A study conducted by Beklen et al. defined the expression of TLR's 1–10 in both clinically healthy and diseased tissues [47].

Also the expression of antimicrobial peptides in response to microbial challenge as a result of the synergistic action of NOD1 and NOD2 with select TLRs has been described by Uehara and Takada [48].

Healthy periodontal tissue has been accompanying with a very simple supragingival plaque composition: few [1–20] layers of predominantly Gram-positive cocci (Streptococcus spp.: *S. mutans, S. mitis, S. sanguis, S. oralis*; *Rothia dentocariosa*; *Staphylococcus epidermidis*), followed by some Gram-positive rods and filaments (*Actinomyces* spp.: *Actinomyces viscosus*, *Actinomyces gerencseriae*, *Corynebacterium* spp.) and very few Gram-negative cocci (*Veillonella parvula*; *Neisseria* spp.). These latter are aerobic or facultative aerobic bacteria, capable to adhere to the non-exfoliating hard surfaces; initial adhesion is endorsed by surface free energy, roughness and hydrophilia, and is mediated by long- and short-range forces [49, 50].

4.4. Dental plaque mediated periodontal disease

Recent data from a number of laboratories propose that different types of periodontal disease may possibly have specific microbial etiologies.

Striking differences in microbial composition have been revealed upon examination of the microbiota in healthy and diseased periodontal tissues [51].

There have been two main hypotheses that explain the role of plaque bacteria in the etiology of periodontal diseases. The "Specific Plaque Hypothesis" proposed that, out of the diverse collection of organisms comprising the resident plaque microflora, only a few species are actively involved in disease [52].

This suggestion focused on controlling disease by targeting preventive measures and treatment against a limited number of organisms. In contrast, the "Non-Specific Plaque Hypothesis" considered that disease is the outcome of the overall activity of the total plaque microflora [53].

More recently, an alternative hypothesis has been proposed the "Ecological Plaque Hypothesis "that reconciles the key elements of the earlier two hypotheses. Significant features of this hypothesis are that, the selection of" pathogenic" bacteria is directly coupled to changes in the environment in addition diseases need not have a specific etiology; any species with relevant traits can contribute to the disease process [54].

A vital element of the ecological plaque hypothesis is that the disease can be prevented by direct targeting of the putative periodontal pathogens together with modifying the environment that is responsible for their enrichment [23].

5. The bacterial composition of biofilm in relation to periodontal disease

Microbiological analyses revealed that the composition of commensal oral bacteria and the bacterial load isolated from healthy sites are considerably different from that found in diseased sites.

Characterization of the periopathogenic microbial flora has shown that the microbial load is higher in periodontal pocket than in normal sulcus, also there is an increase in the number of Gram-negative organisms (15–50%) when compared to clinically healthy sites [55].

In the mid-1960s, Le et al. demonstrated the positive association between dental plaque and gingivitis [56].

Socransky modified Koch's postulates and, through associative and eliminative studies, identified a group of Gram-negative anaerobic bacteria able to induce periodontal deterioration [57].

He also classified several complexes of bacteria dividing them into groups, labeled by colors. The categories were based upon the pathogenicity of the bacteria and their role in inflammation and periodontal destruction (**Figure 4**) [58].

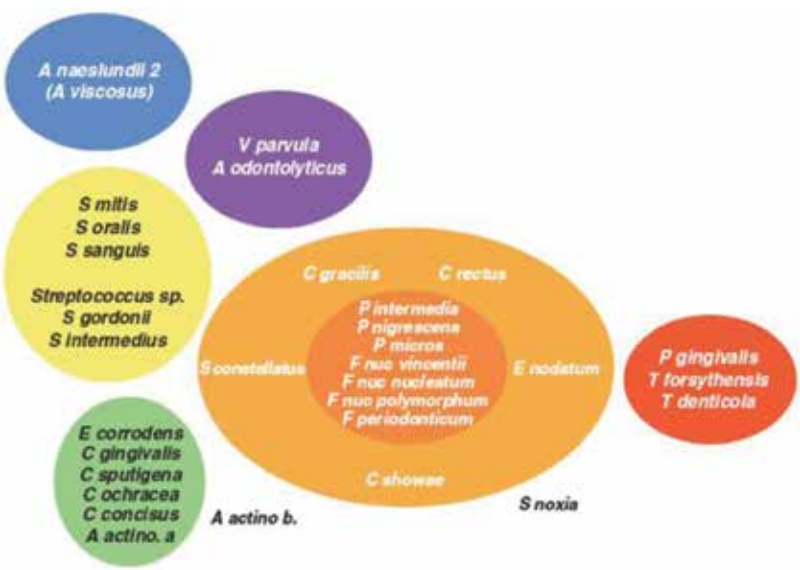

Figure 4. Microbial complexes in subgingival biofilm.

Early cultural analyses and current culture-independent molecular analyses of the periodontal microbiota have revealed profound ecological shifts in community structure associated with the transition from health to disease [59].

Recent advances based on independent metagenomic and mechanistic approaches propose that the pathogenesis of periodontal disease involves polymicrobial synergy and dysbiosis [60].

The dysbiosis of the periodontal microbiota indicates a change in the relative abundance of individual components of the bacterial community compared to their abundance in health, leading to alterations in the host-microbe crosstalk sufficient to mediate destructive inflammation and bone loss [61].

There is epidemiological evidence that plaque-induced gingivitis is the most prevalent periodontal disease and is more severe in individuals with poor oral hygiene [62].

Clinical gingivitis is associated with the development of a more organized dental plaque. Such biofilms are characterized by several cell layers (100–300), with bacteria stratification arranged by metabolism; besides the Gram-positive cocci, rods and filaments associated with healthy gingivae, the number of Gram-negative cocci, rods and filaments increases and anaerobic bacteria appear (*Fusobacterium nucleatum*, *Centruroides gracilis*, *Tannerella forsythia, Capnocytophaga* spp.) [63, 64].

The species involved vary depending on local environmental characteristics, but the colonization pattern is always the same [65].

5.1. Bacterial biofilm and the development of periodontitis

Periodontitis is a chronic inflammatory disease affecting tooth-supporting structures including the alveolar bone, connective tissue attachment, and gingiva [66].

The transition from gingivitis to periodontitis does not come about automatically, either in every patient or every site, but determined by three main factors: host susceptibility, pathogenic bacteria and "protective bacteria" [14].

Pathogenic bacteria possess virulence features that decrease the effectiveness of the host response by causing tissue breakdown and hindering tissue healing. Pili, fimbriae and blebs allow adhesion and colonization, and host defenses are impaired through a number of mechanisms: proteases that inhibit polymorphonuclear leukocyte (PMN) chemotaxis; capsules that mask LPS or increase resistance to phagocytosis; inhibition of PMN superoxide production.

The biofilm associated to periodontitis is complex and formed by many cell layers. The composition of the bacterial population in the active, destructive phase differs slightly from that during the remission period, adding support to the theory of the high specificity of pathogenic plaque; a preponderance of *Tannerella forsythia, P.gingivalis, T. denticola, C.rectus, P.intermedia* is associated with increasing probing depth and bleeding on probing (BOP) [58, 67, 68].

Based on classification system of periodontal disease and condition, two major forms of periodontitis are found, chronic periodontitis (CP) and aggressive periodontitis (AgP), which differ in clinical presentation, rate of progression, and, perhaps, age of onset [69].

5.2. The bacterial composition of biofilm in chronic periodontitis

Chronic periodontitis is an oral infection that results in destruction involving the gums, cementum, periodontium and alveolar process bone. The primary etiological factor of chronic periodontitis is bacterial plaque [70].

Chronic periodontitis is associated with heterogenic subgingival flora; however, the bacteria most cultivated in higher levels *are P. gingivalis, T. forsythia, P. intermedia, C. rectus, Eikenella corrodens, F. nucleatum, A. actinomycetemcomitans, P. micros, T. denticola, and Eubacterium* spp. Gram-negative anaerobes and capnophiles are dominant; spirochetes may also be present. In the sequence of initiation and progression of the inflammatory process, the subgingival bacteria increase in numbers and invade the pocket epithelial cells and, consequently, the underlying tissues. It has been proven that *A. actinomycetemcomitans* and *P. gingivalis* can invade the gingival tissues and this fact is distinctive for the more severe chronic periodontitis and aggressive periodontitis. Some recent data reveal that some herpes viruses present in the periodontal pockets, for example, Epstein-Barr virus-1 (EBV-1) and human cytomegalovirus (HCMV) [28].

5.3. The bacterial composition of biofilm in aggressive periodontitis

Aggressive periodontitis (AgP) is a form of periodontitis described by rapid and severe periodontal destruction in otherwise young healthy individuals. The etiology of periodontitis is very complex including the dental biofilm, which triggers the immuno-inflammatory response in a susceptible host [71].

The predominant microbiota in aggressive periodontitis is Gram-negative capnophiles and anaerobic rods. In localized aggressive periodontitis, *A. actinomycetemcomitans* is frequently present; this microorganism may comprise up to 90% of the cultivable microflora but essential levels of other microorganisms (*Capnocytophaga, E. corrodens, P. gingivalis*) have been found in periodontal pockets. In generalized form of aggressive periodontitis, *A. actinomycetemcomitans*, *P. gingivalis, P. intermedia, C. rectus* are prevailing. Herpesviruses, including Epstein-Barr virus-1 (EBV-1) and human cytomegalovirus (HCMV), can also be come across [28].

6. Systemic consequences of oral dysbiosis

Dysbiosis in periodontal disease as a trigger of bacteremia likely facilitates systemic dissemination of oral bacteria, and therefore good oral hygiene is crucial for controlling the total bacterial load. The link between oral pathogens and systemic effects has been evidenced by a recent study in animals, which found a direct effect of oral administration of *P. gingivalis* on the composition of the gut microbiome as well as inflammatory changes in various tissues and organs. Oral bacteria have been proposed to play a role in a number of systemic diseases, including cardiovascular disease, rheumatoid arthritis, adverse pregnancy outcomes, stroke, inflammatory bowel disease and colorectal cancer, respiratory tract infection, meningitis or brain abscesses, lung, liver or splenic abscesses, appendicitis, pneumonia and diabetes [72, 73].

7. Controlling oral communities

Oral biofilms play a major role in the etiology of oral diseases and have wide effects on quality of life and systemic health.

Many hypotheses were developed describing the ways by which dental plaque can exert its pathogenic potential. These hypotheses have been changed over time.

New understandings of the structure and composition of oral microbial communities have implicated shifts in the composition of the resident microbiota in the development of periodontal diseases and in that way the entire microbial communities could be considered as pathogenic [74].

Self-performed and professionally administered plaque controls are the mainstay in prevention of periodontal diseases.

Scaling and root planning together with self-performed plaque control have been shown to reverse the microbial shifts associated with periodontal diseases and reform subgingival microbiota similar to those found in periodontal health.

In addition to conventional approaches used to control oral biofilms, adjunctive treatments for periodontal diseases include systemically administered antibiotics, antiseptics and host-modulating agents have been developed with improvement in the clinical outcome of periodontal therapy [74].

As progress in the field of oral communities has increased, a new inhibitor or antagonist for dental plaque biofilm has been developed [75].

These are aimed to manipulate the structure or function of communities, endorsing health as opposed to disease. Some of these new methodologies target bacterial adhesion to host tissues, some target co-adhesion or co-aggregation and others struggle to harvest the natural armaments of commensal bacteria to affect the retention of others.

These successes in controlling the growth of specific periodontal pathogens in dental plaque pave the way for the development of strategies for manipulating more complex communities that are not so accessible (e.g., periodontal microflora) and that are more closely integrated with host tissues and host-cell functions [76].

Recently, transcriptional profiling of gingival epithelial cells stimulated with oral pathogens, for example P. gingivalis or A. actinomycetemcomitans, has revealed that specific responses for species predominate and that the core transcriptional response to oral organisms is limited [77].

The signal transduction within oral epithelial cells is designed to combat the challenging organism. Therefore, it might be possible to modulate host-cell signaling path ways to maintain a situation compatible with a healthy periodontal community [76].

8. Chapter summary

Oral microbial habitat is composed of wide variety of species. These species play a significant role in maintaining the health of the oral cavity by contributing in various ways. Resident microorganisms have coevolved and coexisted in a mostly harmonious symbiotic relationship.

The oral microflora can act as opportunistic pathogens when the habitat is altered or when microorganisms are found at sites not normally reachable to them.

In dysbiosis, the balance of the oral ecosystem is disrupted, allowing disease-promoting bacteria to manifest and cause conditions such as gingivitis and periodontitis.

Analysis of the microbiota reside in the oral cavity may be a useful approach to diagnose systemic diseases that have periodontal manifestations. The control of the total oral microbial load is important to prevent dissemination to other body sites.

Methods for the control of oral biofilms that are less dependent upon compliance and regular access to professional dental care are needed.

Approaches that intended to inhibit the attachment of oral microorganisms on oral surfaces or create long-lasting shifts in the oral microbiota hold much promise.

Future research exploring these and other possibilities will provide guidance on how to better prevent and manage periodontal diseases.

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Section 3

Identification of Oral Microbes

Chapter 5

Discriminating Life Forms in Oral Biofilms

Vishakha Grover and Anoop Kapoor

Additional information is available at the end of the chapter

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Abstract

The bacteria colonizing the hard and soft tissues of the oral cavity are known to significantly influence oral health and disease. Recent studies of subgingival dental plaque, based on different identification methods, provide direct evidence of substantial diversity of plaque microbiota. Till date only about 280 bacterial species have been isolated by cultivable methods, characterized and formally named out of this enormous microbial diversity of oral biofilms. As a consequence, there is a complete lack of information about the properties of a substantial proportion of the plaque microbiota, apart from their position in the taxonomic hierarchy of bacteria. This limited knowledge about the behavior and properties, combined with recognition of the considerable diversity that exists within individual species, raises serious questions to the foundations on which previous conclusions, concerning the etiology of periodontal diseases, rest. The emerging realization is it is impossible to fully understand oral health and disease without identifying and understanding the pathogenic potential of all of the bacteria that colonize the oral cavity. The current chapter shall provide an update on current status of oral microbiota, ecological significance of their biofilm life style and various methods to study microbes residing in oral biofilms.

Keywords: biofilm, dental plaque, microbes, methods, identification

1. Introduction

Upon formation of earth about 3.5 billion years ago life began under anaerobic conditions, which resulted in current form as a result of evolution that is continued with the time. Initially, earth was colonized by unicellular prokaryotic bacteria that could survive under anaerobic conditions and eventually facilitated aerobic conditions that turned into evolution. However, till date we can find these microbes in various anaerobic environments. The estimated microbial diversity on earth constitutes 1.2×10^{29} in oceans, 2.6×10^{29} in terrestrial environment [1].

Remarkably, much more diversity was observed in subsurface environment with an estimate of 2.5×10^{30}, suggesting the adaptation of these microbes to such conditions at the formation of earth. Thus, microbial diversity constitutes a significant mass on earth among living organisms. However, most of the microbial diversity remained undisclosed due to limited knowledge on their adaption strategies and functions under diverse environments. In fact, their association has been observed with higher forms of living organisms including plants, animals and humans. There were various projects dealt with understanding the role of these microbes in host. Among those human microbiome project is considered to be important that helped in understanding the ecology of microbes in human including their disease causing abilities. Various habitats on human body are composed of vast microbial flora which include both autochthonous and allochthonous populations. Among those the oral microbiome is known to contain more than 700 different prokaryotic species with distinct subsets prevailing at different habitats of oral niche including periodontic and endodontic environments. Attempts were made for extensive characterization of this microbiome using both cultivation and culture-independent molecular methods. Unfortunately, most of the culture-independent methods revealed vast majority of oral taxa as uncultured clone and referenced by their 16S rRNA GenBank accession numbers [2, 3]. Application of recent advances in technology provided new insights in understanding the oral microbiome complexity and their role in both health and disease. In this chapter we have made an attempt to compile all updated information and current status of oral microbiota their biofilms, ecological significance and various methods to study microbes residing in oral biofilms. In 1978, Costerton invented the word "biofilm", referring to the matrix-enclosed bacterial community [4]. However, the first biofilm described by Antonie van Leeuwenhoek.

2. Oral microflora – general aspects

The "oral microbiome" represents a group of microorganisms that includes mutualistic, symbiotic, commensal and pathogenic microorganisms which determine oral health and disease1. Though babies are protected inside the amniotic sac during pregnancy and born with germ free oral cavity, various microbes of the vaginal environment of the mother comes into contact at the time of birth and subsequently establish their niche in oral cavity. Thus, the initial microbial flora of oral cavity resembles the mother's vaginal flora. Despite the possibility of contamination from the environment and surrounding personnel, the mouth of a newborn baby is usually sterile and microbes start invading with residential flora during feeding process. The natural history of oral bacteria acquisition and potential determinants of oral microbial composition are beyond the scope of this chapter. With direct exposure to the environment, oral cavity possesses a complex microbial ecosystem where wide variety of microbes including bacteria and fungi are continually involved in their establishment upon attachment to the surfaces like teeth, tongue, restorations and soft tissues. These varying colonizers primarily cause polymicrobial infection in the form of biofilm i.e., dental plaque with ecologic succession and inter-bacterial interactions between commensals, opportunistic pathogens and pathogenic microbes leading toward homeostasis in oral microflora [5]. Microbial studies of

human dental plaque carried out by Socransky clearly showed that the oral health depends on the type of microorganisms present [6], however, interspecies interactions among these microbes determines healthy or diseased condition [7]. In fact, dysbiosis of microbial communities leads to dental caries or periodontitis [8, 9]. Commensal bacteria persist in oral habitat for long duration upon colonization and thus, they co-evolve with host and prevent access to pathogenic microorganisms by stimulating the immune response [10]. Dental caries are actually result of disequilibrium between acid and alkali producing microorganisms or acid producers and utilizers [11].Thus, paradigm of microbial dysbiosis revealed significance of autochthonous or resident microflora in maintaining healthy oral environment [11, 12].

3. Dental plaque

Dental plaque is a sticky film comprising multiple bacteria assembled as biofilm on surface or periphery of teeth. It consists of highly structured complex that allows sequential bacterial/microbial succession. Dental plaque development studies under in vitro and in vivo investigations revealed occurrence of early and late colonizers. While early colonizers with ability to produce biochemical components that adhere to target tissue initiates biofilm formation on tooth surface including periodontal tissue. Subsequently, they allow the adhesion of late colonizers that are capable to adhere with early colonizers to impart metabolic and competitive advantages to biofilm. Usually, early colonizers include species of *Streptococcus, Lactobacillus, Lactococcus, Eikenella, Veillonella, Provetella*, *Propionobacterium* and *Hemophilus*. Late colonizers represented by members of the genera like *Actinomycetes, Eubacterium, Treponema* and *Porphyromonas*. A mature dental plaque biofilm contain bacterial species that are well bound to bacterial strains located adjacent to form a unique structure that improves their adherence ability and provides protection from adverse conditions. Previous comprehensive reviews by Kolenbrander et al. should be consulted for assessment of these important properties [13–15].

3.1. Microbial composition of dental plaque

Dental plaque represents a microbial community with high genetic diversity. Moreover, it maintains a stable structural complexity, despite the continuous exposure to external environment and various stress factors. The microbial composition largely remains constant as a result of balanced antagonistic and synergistic associations [16, 17]. This indicates specific contribution of physiological functions by individual participating microorganisms in biofilms. In addition, their physiological functions contribute to facilitate growth of other organisms such as anaerobic microbes. The biofilms formed on tooth are divided into supra- and subgingival biofilms. While supragingival biofilm is formed above the gum, subgingival biofilm formed under the gum. Most of the bacterial strains described from oral environment were isolated from these biofilms. With more than 700 Gram-positive and Gram-negative bacterial species oral ecosystem represents a complex ecosystem after gut environment [18, 19]. It is often observed that supragingival plaque contained Gram-positive bacteria, including members belonging to genera *Streptococcus Lactococcus*, *Lactobacillus*, *Veillonella*

and the subgingival plaque revealed primarily gram negative anaerobic bacteria such as *Actinobacteria, Tannerella, Campylobacter, Treponema, Fusobacterium, Porphyromonas, Prevotella,*. Majority of these microbes belongs to the phyla like *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, *Fusobacteria* as well as uncharacterized phyla like SR1and TM7 [18, 20]. Despite such huge diversity, only very limited number of bacterial species have been isolated and characterized by cultivable methods till date and this may be due to lack of understanding of microenvironment associated with these microbes [3, 19].

3.2. Dental plaque – a highly specialized host associated biofilm

As mentioned earlier, dental plaque is a biofilm attached not only to tooth surface but also under gums. Diverse community of microbes exists in the form of biofilm where all microbial strains bound tightly between them as well as to the tooth surface. Dental plaque is a form of biofilms, which engulf diverse bacterial populations adherent to each other and primarily results in formation of dental caries. Their structure is influenced with high and low bacterial biomass interlaced with aqueous channels formed to provide nutrients to the bacterial strains [21, 22]. Biofilms permit association of diverse species with increased metabolic efficiency, enhanced virulence and higher resistance to stress and antimicrobials as a result of entirely different expression of genes in comparison to planktonic form. Ability to adhere on surface, strong binding between cells gene regulation and genetic transfer are some of the important properties that define biofilm formation. In fact, extensive metabolites exchanges, signaling trafficking and different levels of interactions among different species were usually observed in biofilms [16, 23, 24]. However, introduction of biofilm theory into oral microbiology provided insights to understand the roles of different bacterial species at different time intervals. Most important is these biofilms proven to provide protection by increasing the antibiotic and acid tolerance, a property indicating it as a marker for caries production. Though biofilms consist of millions of cells of multiple species in thousands of layers, they behave like a single organism. These microbial cells are also encompassed in polysaccharide complex to stay together and acquire resistant properties to survive under stress environment.

4. Clinical relevance of biofilms in disease etiology

Planktonic microbes existing in dental ecosystem often involved in acute infections that can be diagnosed and treated appropriately before the establishment of disease. In contrast, bacteria existing in biofilms demonstrate an infectious course in disease establishment as observed in dental caries, where large quantities of acid formed as a result of increased acid tolerance. Dental plaque biofilm also increase the expression of virulence factors as differential expression of participants lead to formation of noxious products that initiates inflammation and development of periodontal disease. Biofilm exhibit all genetic network required for these activities as evident in global analysis of gene expression during biofilm formation. This state also imparts global adaptation to stress condition i.e. crowded environment. Thus, this lifestyle appears most important adaptation to any form of environmental stress and gain increased tolerance. This cooperative behavior among the participating species in a biofilm

covered by extracellular matrix with coordinated management between cells using quorum sensing signal molecules for communication mimics an integrated multicellular organism. Additionally, the virulence was increased in multispecies participating biofilms in comparison to their mono-species counterparts [24]. Most of the bacterial cells exhibit attachment sites on their surface for an effective attachment to abiotic and/or neighbor microbial cells and thereby multiplies inside the extracellular matrix. This amplification in biofilms results in formation of aggregates that play important role in virulence in establishment of diseases like endocarditis, dental caries, middle ear infections, osteomyelitis, chronic lung infections in cystic fibrosis patients [25–27]. Remarkably about 80% of all microbial infections are found to develop biofilms on host tissues associated with different organs. Cells residing in biofilms termed as persister cells that are mostly exist in dormant stage with minimal active metabolism to cause chronic infections. These infections include production of exo- and endotoxins, metabolites like acids and other products involved in inflammation of dental tissue. However, the intensity of infection is directly related to their antibiotic resistance and ability to modulate host immune system [28, 29].

5. Biofilm characterization methods

5.1. Methods to discriminate oral microbial flora

Several attempts made to discriminate oral microbial flora by cultivable and non-cultivable methods have provided limited information. Though several taxa have been reported to present, only few microbes could grow in pure culture. Cultivation of individual strains in pure culture through the perspective of Koch's postulates. Further, identification of these microbial isolates helps in understanding infection process and disease establishment. To achieve this numerical taxonomy was practiced earlier, however, it has been replaced with molecular taxonomy and polyphasic taxonomy (**Table 1**).

However, in the recent past microbiologists have refocused on microbial communities' identification instead of planktonic form as they developed disease in the form of biofilm. In fact, oral diseases like caries and periodontitis are reported to be outcome of a consortia of organisms in a biofilm. Therefore, detailed analysis of a microbial community is essential to understand their pathogenicity. It is pertinent to mention that our understanding of the microbial world is very limited due to the intrinsic limitation of the culture-dependent methods. Thus, only less than 1% organisms could be revived in pure culture form under in vitro conditions. Considering the fact that several microbial species involved in biofilm formation, comprehensive understanding on complexity and genetic diversity of these communities are severely hampered due to non-availability of cultivation techniques [30]. Furthermore, uncultured status of these microbes also intervening in completion of understanding human microbiome and thereby effects on human health and disease [31]. Various culture-independent techniques such as cloning and amplification of total DNA obtained from samples can be used to understand the total microbial taxa. For which various housekeeping genes like 16S rRNA gene have been employed in molecular cloning and sequence methods to reveal their exact

Phenotypic methods	Genotypic methods
Expressed, Characteristics	Amplification of housekeeping genes like 16S rRNA and rpoB genes
Colony morphology Size Shape Color	Phylogenetic analysis of gene sequences
Cell morphology Gram Staining Shape	Analysis of fragments obtained from random amplification of polymorphic DNA (RAPD)
Motility	DNA separation by pulsed-field gel electrophoresis (PFGE)
Biochemical, enzymology acid-gas Production, Oxidation – Fermentation	Multilocus sequence typing (MLST)
Whole cell protein analysis	Restriction fragment length polymorphism (RFLP) of DNA
Utilization of Carbon compounds	Nucleic acid base composition Mole % G + C
Antibiotic sensitivity	DNA–DNA hybridization
Susceptibility to phages	
Susceptibility to bacteriocins	
Chemotaxonomic (Lipids, Fatty acid methyl esters (FAMEs), Isoprenoid quinones, Mycolic acids, Pigments, Peptidoglycan, Cell wall sugars	

Table 1. Phenotypic and genetic methods used for identification of bacterial strains.

identity [3]. However, cultivation of individual strains in pure form is essential to fully understand their role in health and disease thereby to carry out meaningful clinical research.

The development of 16S rRNA gene as molecular chronometer by Woese and co-workers has transformed the microbial taxonomy as the alignment of these sequences and construction of their phylogenetic trees have allowed cataloging of microbial strains and establishment of novel species [32]. The 16S rRNA gene exhibits clocklike behavior, broad phylogenetic range and appropriate size and accuracy, and these properties made this gene to the best molecular chronometer. Moreover, rRNAs are essential for protein synthesis and readily isolated from all forms of life, they are structurally and functionally conserved. They display highly variable and conserved regions to distinguish into distinct. They appear to incorporate changes in sequence very slow and do not exhibit horizontal gene transfer. This finding in combination with various PCR methods opened the door for culture-independent analyses for exact identification of microbial strains present in different microbial communities, including the uncultured bacterial species. They allowed understanding of total number of species, their richness and distribution. During the past two decades, development of high throughput tools for microbial community analysis has further improved identification process. Most of these methods include nucleic acids isolated from samples being investigated. These techniques include both nucleic acids and their PCR products. While techniques like fluorescent in situ hybridization (FISH) with fluorescently-labeled taxon-specific oligonucleotide probes and checkerboard DNA–DNA hybridization method [33] used nucleic acid, others such as random amplified polymorphic DNA (RAPD), denaturing gradient gel electrophoresis (DGGE) [34] or temperature gradient gel electrophoresis [35], terminal restriction fragment length polymorphism (T-RFLP) [36] and automated ribosomal intergenic spacer analysis [20] were carried out using PCR amplified products to analyze environmental microbial communities. Application of these techniques has revealed large number of microbial species within dental plaque with

great genetic diversity. However, these techniques also showed limitations like cell lysis efficiency, nucleic acid extraction, differential amplification of target genes and differences in copy number of target genes, primer specificity and hybridization efficiency. Therefore, combining imaging tools such as the scanning electron microscope [37] and confocal laser scanning microscope [38], with molecular techniques can provide most effective identification [39].

5.2. Specific methods to discriminate oral microflora in biofilms – detection and quantification

Formation of biofilm containing multiple pathogens embedded in an extracellular polysaccharide matrix is a big threat to human health. Though biofilm formation is regulated by expression of various genes, there are multiple systems such as extracellular polysaccharides, lactones, pilin- or flagellin-like proteins, adhesins and other small molecules involved in quorum sensing and biofilm formation. Thus, considering the complexity of biofilm structure they are discriminated in qualitative and quantitative methods. The amount of EPS, types and total number of bacterial cells in biofilm must be considered as different "methods" requiring different experimental approaches. The biofilms are largely quantified using spectrophotometric and microscopic methods. The crystal violet (CV) staining method [40] is among the mostly used and also achieved by cangored method. CV staining can be performed as tube method or using microtitre plate.

5.2.1. Microtitre plate method

The microtitre plate method is most widely used method for detection of biofilm formation. It was initially developed as tissue culture plate method by Christensen et al. [40]. This method is used to test the influence of different media and addition of various sugars in media on biofilm production. Individual wells of sterile, polystyrene, 96 well-flat bottom microtitre plates were filled with 200 μl of diluted cultures in respective sterile media. They were incubated under optimal conditions required for the growth of microbes being tested. After incubation contents of wells were removed by gently tapping the plates and washed with sterile distilled water or buffer to remove free-floating bacteria. The biofilms formed by adherent mechanisms were stained with CV (0.1% w/v). Excess stain was removed by washing with deionized water and subsequently wells were air-dried. Adherent cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. The crystal violet was solubilized using absolute ethanol and the quantity of biofilm quantified by measuring the OD at 595 nm. Sterile uninoculated medium is usually used as a control.

5.2.2. Tube method (TM)

This method allows qualitative assessment of biofilm formation as described by Christensen et al. [41] The medium is inoculated with loopful of culture from plates that are overnight incubated at optimal conditions. Upon incubation these tubes are decanted, washed with distilled water or PBS (pH 7.3) and air-dried. They are stained with CV (0.1%). Excess stain removed as mentioned in microtitre plate method and observed for biofilm formation. Biofilm formation was detected by a visible film lined the wall and/or bottom of the tube.

5.2.3. Congo red agar method (CRA)

This is an alternative method of screening biofilm formation by microbes [42]. Microbes being screened are grown on solid medium supplemented with 5% sucrose and Congo red. Congo red usually added as concentrated aqueous solution. Plates were inoculated and incubated under optimal conditions. While positive result was indicated by black colonies slime producers showed pink colonies.

5.3. Other qualitative straining methods to detect biofilm

5.3.1. LIVE/DEAD BacLight assay

This method is performed using a bacterial viability kit for microscopy based on the use of two different nucleic acid binding stains. Two dyes employed are green fluorescent (SYTO 9) and propidium-iodide that should be used with appropriate care. While intact cells fluoresced green with Syto9, damaged or dead cells in biofilm stains red. These stained samples are usually observed under a fluorescent microscope. The main limitation to apply this method for quantification is low quantities of the representative sample used for the total population and it does not allow tracking of individual bacteria.

5.3.2. Immunofluorescence staining

Immunofluroscent staining is used to observe biofilms under optical fluorescence microscopes and is commonly used to stain biofilms under in vivo conditions. This method employs specificity displayed by antibodies toward antigens. Usually fluorescent dye-labeled antibodies are used to fluoresce specific target molecules within a cell. This method is often used in experiments that use cell lines or tissue culture studies. Immunofluorescence is also used with other non-antibody methods by using stains like DAPI and analyzed on epifluorescence or confocal microscope. Diverse florophore molecules are used to link with antibodies. Biofilms used for image analysis using electron microscope are treated with various staining and fixing protocols using fixative or stain like glutaraldehyde, osmium tetroxide, ruthenium red etc., and observed under electron microscope. A variety of fluorescent molecules like lipophilic styryl compounds (ThermoFisher) involving plasma membrane and vesiculation was also used for biofilm detection. These water soluble and exhibit fluorescence when interact with surface of microbial cell membrane.

5.4. Metabolic assays

Biofilms can be measured by different vital or non-vital dyes that interact with metabolic products.

5.4.1. Resazurin assay

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is a blue non-fluorescent biological dye that also known as Alamar Blue. It is used to quantify biofilms in microtiter plates as it gets converted to the pink-fluorescent resorufin upon reduction as a result of cellular metabolic

activity. The resorufin can be measured spectrophotometrically and intensity of fluorescence is directly proportional to number of cells or biofilm concentration [43, 44]. However, the test is highly susceptible to bacterial respiratory efficiency and calibration of curves established with planktonic cells is much lower than signal detected in biofilm [45]. Further, this assay also reveals the presence and efficiency of antimicrobial and antibiofilm compounds [46].

5.4.2. XTT and TTC assay

Tetrazolium dyes also can be used as resazurin assay to quantify metabolically active cells in biofilm by spectrophotometric method. Tetrazolium slats like 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) has been used to detect biofilm [47, 48] and another salt 2,3,5-triphenyl-tetrazolium chloride (TTC) also sued for the detection of biofilm [49] in microtitre plates by measuring absorbance. In fact, this method can be used to determine minimum biofilm inhibitory concentration (MBIC). Though these assays are highly sensitive and economical, the complexity and heterogeneity of biofilm structure of mature biofilm reduces the release of final products.

5.4.3. BioTimer assay

Bio Timer assay (BTA) is a biological method used to count adherent viable bacteria in biofilm life-style on any abiotic surface without manipulation of sample. BTA employs a specific reagent, phenol red that changes color from red-to-yellow based on microbial metabolism. This is specifically considering the microbes that produce diverse organic acids as their metabolic end products of fermenting bacteria. The time required for color change is determines the number of microbes as higher number of the organisms performs faster metabolism. Time required for initiation of color switch is correlated to the number of bacteria at time zero (N0) through a genus specific correlation described by equation $t^* = \log(1 + a/N0)/k$, where a represents metabolic product involved in color change and k is growth rate [50]. Though this technique is applied in microbiological quality analysis of foods and to evaluate antibiotic susceptibility of biofilm, is not applicable for the evaluation of multispecies biofilm.

5.5. Genetic assays to determine biofilms

Genetic assays have been used to assess the biofilm formation with focus on molecular mechanisms involved in biofilm formation. In particular, early stage of biofilm formation including attachment to surface, which is driven by expression of various genes in different microorganisms. Therefore, biofilms are associated with proteins and amplification or quantification of various genes including chaperone-usher fimbriae, outer membrane proteins, poly-N acetyl glucosamine, adherent proteins and pili proteins [51–53].

5.5.1. Polymerase chain reaction (PCR)

The most important diagnostic method used in genetic techniques is Polymerase chain reaction (PCR). PCR screening is often employed to detect the genes involved in biofilm formation. The amplified products are sequenced and analyzed using various bioinformatics tools such as BLASTp (NCBI) to align with homologous sequences. This method

allows to identify specific genetic sequences based on primer sequences used for individual bacterial species. The extracted DNA of the biofilm can be used for RAPD analysis by using specific oligonucleotide primers [54]. Amplification of genes like icaA, icaD, aap. The reaction mixture contains in general Taq polymerase enzyme, deoxynucleotides, primers, template DNA and MgCl2 in PCR buffer. The amplification is carried out in a gradient mastercycler with a program that includes initial denaturation of DNA at 95°C for 5 min. It is followed by 40 cycles of program at 94°C for 1 min, optimal temperature required for the binding of primers for 1 min, 72°C for 2 min (optimal enzyme activity and amplification) with a final extension at 72C for 5 min. Primers used in amplification of gens are as follows: icaA, 5′-AACAAGTTGAAGGCATCTCC and 5′-GATGCTTGTTTGATTCCCT [55]. Forr icaD, 5′-CCGGAGTATTTTGGATGTATTG (forward primer) and 5′-TTGAAACGCGAGACTAAATGTA (reverse primer). According to Vandecasteele et al. [56], for the detection of the aap gene, following primers were used: 5′-ATACAACTGGTGCAGATGGTTG (forward primer) and 5′-GTAGCCG TCCAAGTTTTACCAG (reverse primer). Nevertheless, PCR as such is not a suitable to quantify biofilm as it amplifies the DNA of both viable and dead cells, as well as any contamination leading to false positive results.

5.5.2. Fluorescence in situ hybridization and confocal laser scanning microscopy

Fluorescence in situ Hybridization (FISH) is a cytogenetic techniques that use fluorescent labeled oligonucleotide probes (like rRNA gene fragments) to detect microbes by hybridization of DNA with highly identical complementarity. This method allows direct visualization of species specific bacteria in a multispecies biofilm. These bacterial strains can be observed using confocal laser scanning microscopy. The technique can be modified with the samples to be observed, for example a modified version of the technique developed to identify based on peptides and termed as peptide nucleic acid-fluorescent in situ hybridization (PNA-FISH). Similarly, Flow-FISH employees flow cytometry to identify the microbes. Histo-FISH was developed to detect probiotic bacteria in gastrointestinal tract [57]. Interestingly, FISH can detect not cultivable bacteria and persister or dormant bacteria in biofilm. FISH technique is usually combined with confocal microscopy to visualize different species in a multispecies biofilm.

The aforementioned high throughput tools for microbial community analysis are largely based on PCR amplification of 16S rRNA gene sequences from microbial communities, which are relatively short, often conserved but varied enough to differentiate bacteria at species level. Although these approaches can provide us with the microbial composition within the community, unless we have genomic or other research data on those identified species, it reveals very limited information regarding what functions they might carry out within the flora.

5.6. Physical assays – biofilm imaging

5.6.1. Confocal laser scanning microscopy (CLSM)

It is a microscopy technique used in biology to study thick samples such as microbial biofilm, by processing images. Samples under investigation are stained with fluorescent dyes as mentioned in FISH so that the object can be illuminated and transformed by a photodiode

in electrical signal processed by a computer. Some systems use motorized computer assisted device control for adjustment or sectioning of the biofilm and automated image acquisition. This technique often used to understand the role of EPS components, live biofilms and their in situ gene expression studies [58, 59]. The main disadvantages are semi-quantitative investigation, limited fluorescent dye usage for few stains and expensive method.

5.6.2. Mass spectrometry (MS)

A powerful analytical technique used for detection of various molecules. MALDI-TOF showed to be a strong tool for proper identification bacterial strains in biofilms. This technique utilizes the protein profile of bacterial strains for identification with a reference database. In fact, it is used for accurate identification of clinical strains in biofilms with high resistance to antibiotics [60]. In this method the object under investigation is exposed to a beam of electrons to form ions that are separated based on mass that are detected by a spectrometer and identified by their mass/charge ratios. It fulfills both qualitative and quantitative analysis of the unknown compounds. However, many steps in MS are highly invasive for the sample: high vacuum environment, aggressive chemical solvent etc. To overcome this problem,

5.6.3. Desorption-electro-spray-ionization (DESI)

This method has been proposed to overcome the disadvantages of MS like chemical solvent exposure and vacuum environment. It is carried out at atmospheric pressure and the sample is maintained under ambient conditions and can be used to for the analysis of mixed biofilms [61, 62].

5.6.4. Electron microscopy (EM) techniques

Electron microscopic technique was used to understand microbial flora in dental environment [63]. This method provide high resolution and technique is used for both scanning electron microscopy (SEM) and transmission electron microscopy (TEM). While SEM used to visualize biofilm surface TEM is used to image inner of biofilm [64]. For SEM analysis objects prepared on coverslips are washed (2–3 times) with buffer (pH 7.2) and fixed in 1% osmium tetroxide in the absence of light. Later, washed with distilled water and dehydrated in crescent concentrations of acetone baths. Upon drying, samples were mounted and analyzed on a scanning electron microscope. For TEM, the sample to be prepared as ultra-thin slices to acquire accurate images of bacterial cells and biofilms. Atomic force microscopy (AFM) is another technique used for morphological characterization. This method is used to check microbial cells in both planktonic and biofilm forms. The objective is fixed using 1 ml of modified Karnovsky fixative (containing 2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose, and 0.1 M cacodylate buffer, pH 7.2) at room temperature. The analyses were performed at ambient temperature on an atomic force microscope equipped with a scanner. All images obtained were processed on specific softwares.

5.6.5. Micro-scale biogeography

Micro-scale biogeography is upcoming technique to understand the microenvironment of microbes by biofilm imaging [65]. This method also includes mimicking microenvironment including chemical ingredients and oxygen. It provides insights in understanding physiology and

ecology of community their attachment with other microbes and spatial structure. Neighboring strains in physical contact play significant role in physiology such as protecting from stress conditions and secretion of metabolic end products as substrate for subsequent colonizers [66–68].

6. Metagenomics to understand complex microbial communities

The introduction and application of "metagenomics" by Jo Handelsman [69] has greatly enhanced our ability to study microbial communities including dental plaque. It includes understanding the microbial communities directly in their natural habitat using genomics approach The method do not require isolation and cultivation of any microbial strains. The basic components involved in metagenomics are PCR amplification of DNA, sequencing, bioinformatics with enhanced computational power to analyze large datasets obtained in sequencing [70]. The approach is simple and involves isolation of total DNA from sample, which is subsequently used for amplification of various genes and their subsequent analysis to gain functional and metabolic understanding [71]. Further, comparative genetics with expression microarrays and proteomics provides insights on network life style of microbes within the community such as dental plaque. Such studies provide information on potential pathogens that remained unidentified due to cultivation limitations [72].

7. Adjunctive novel technologies for biofilm study methods to complement microbial identification

7.1. Microfluidics

Miniaturization approaches to biofilm cultivation by using techniques like microfluidics studies are used to understand the natural habitat in laboratory conditions. It is performed in micro-scale channels by allowing fluid flow of growth media or chemicals with remarkable degree of control over the physical and chemical environment of microorganisms. Thus, allows manipulation of microenvironments of bacteria as these devices are made with microscopic compatible materials. It is developed as a new approach to understand cultivation method and dynamics of biofilms. It is also used as high throughput system to determine bacterial antibiotic resistance [73], cell variability in bacterial persistence, quorum sensing and chemotaxis in bacteria [74].

8. Concluding remarks

Since the initial observations of bacteria within dental plaque by Antonie van Leeuwenhoek using his primitive microscopes in 1680, our ability to identify the resident organisms in dental plaque and decipher the interactions between key components has rapidly increased. It is further increased significantly with the advent of imaging and molecular techniques

during the past decade. These new techniques will have a great impact on oral and periodontal microbiology. We envision that in the future, new diagnostic tools developed with metagenomics methods would allow early detection and effective methods to combat the diseases. It also provides insights to prevent the cariogenic, endo and periodontic diseases.

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Conflict of interest

Authors declare there is no conflict of interest.

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Oral Immunity

Chapter 6

Trojans in Oral Environments: Evidence of Molecular Mimicry in Oral Immunity

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Additional information is available at the end of the chapter

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Abstract

Oral microbiome possesses more than 1000 microbial species that co-exist with human oral cavity. However, when there is an imbalance in microbial ecosystem, infection and inflammation occurs. Chronic inflammation produces constant antigen-cell presentation and reactivity T and B cell results in an adaptive immune response with high specificity cell-cell and antibody response producing an autoimmune disease by molecular mimicry. In this chapter, using just BLAST, shows self-epitopes (autoantigens) from different autoimmune diseases such as Systemic lupus erythematosus, Sjögren's syndrome, neuromyelitis optica, Stiff-Person syndrome, autoimmune diabetes, autoimmune thyroiditis, myasthenia gravis, autoimmune gastritis, autoimmune hepatitis, myositis and rheumatoid arthritis that possess similarities with microbial epitopes belonging to oral microbiome acting has a computer trojan occult in a software package.

Keywords: molecular mimicry, autoimmunity, autoantingens, inflammation

1. Introduction

Inflammation is a physiological response to any aseptic or septic injury to provoke the activation of immune response to enhance the healing [1]. This event began firstly by the recognition of pathogens-associated molecular patterns (PAMPs) [2], microbiota-associated molecular patterns (MAMPS) [3] or damage-associated molecular patterns (DAMPs) [4] by macrophages [1], mainly, leading the stimulation of innate immune response and the generation of acquired immune response producing a cellular and humoral immunity [5]. In this way, inflammation recognized pathogens via toll-like receptors (TLRs) to stimulate an immune response to remove these pathogens from the body [1] and acquired immunity memory by the antigen presentation mechanisms [6]. However, chronic inflammation can last for weeks, months, even

years, provoking cycles of injury and healing causing irreversible tissue damage, being a risk factor for the development of autoimmune disease [7, 8].

Oral microbiome contains innumerable epitopes similar to self-epitopes than cause cross-reactivity immune response provoking the kill of microbe and self-tissue injury generating an autoimmune disease [9–11]. This phenomenon is known as molecular mimicry [12] or epitope mimicry [13].

2. Molecular mimicry

Molecular mimicry, term proposed firstly by Damian, is the theoretical probability that exist similarities in the molecular structures (amino acid sequence or conformational structure) between pathogens and the host producing a cross-reactivity immune response turn a defensive immune response into autoimmunity [8, 12–16].

However, molecular mimicry has been demonstrated as a common mechanism by microbes to elude immune response and may modulate biosynthetic or metabolic pathway of the host involved in the regulation of apoptosis, cell proliferation, inflammation and immune response [14, 17]. Pathogens imitate host proteins and their interactions interfering with the cell functions at four different levels [18]:

- Full length protein or domain.
- Structure with apparently sequence similarity.
- Short motif.
- Interface mimicry.

The Toll/Interleukin-1 receptor (TIR) domain is an example of full length protein mimicry. When pathogens stimulate the TIR domain signalosome, a molecular pathway is activated to reach the NF-kB to produce inflammatory cytokines to modulate an immune response. In this manner, pathogens can interfere or inhibit this downstream pathway by the production of similar structures producing a negative regulation of TIR pathway, evading the host immune system neutralizing the TLR signaling for survival and proliferation [18].

In other way, structures with apparently sequence similarity can be interfered with the immune regulation, inflammation and wound healing [19]. In this manner, viral chemokine of Kaposi's sarcoma-associated herpesvirus is very similar to human chemokine CX3CL1 [20] causing the activation or inhibition of immune modulation in the host [21].

Pathogens have homologs of short amino acid sequences known as motif mimicry [22, 23] composed of 3–10 residues with the capability to altered immune molecular pathways of the host [18]. One example of this mimicry is the bacterial guanine nucleotide exchange factors (GEFs), as Map and EspM2 of E. coli than can activated GTPases in the host [24, 25], who regulates many cell function as proliferation, survival, differentiation, migration and apoptosis [18].

Interface mimicry is produced by short linear motif than may adopt altered conformations altering the global protein conformation, generating the pathogen evasion [18]. Human GTPases and Map of E. coli and SopE of Salmonella, can serve as an example of interface mimicry.

2.1. The molecular mimicry mechanism

During T cell development, naïve cells moved from the bone marrow to the thymus. In this organ occurs the positive selection, when T cell CD4 + CD8+ recognized the MHC on cortical thymic epithelial cells, they receive signals than let a CD4- and CD8- differentiation according to their affinity to MHC class I or II [26]. The process of thymic selection eliminates 99% of precursor cells by apoptosis, leaving 1% to reach the periphery [27].

In this case, if an external peptide (such as microbe) present similarity with the host peptides, activate T cells can be presented by dendritic or macrophages cells. And if the host peptide possesses similar structure, the T cell becoming autoreactive with self-antigen [27], could originate an autoimmune disease (**Figure 1**).

The importance of interaction of peptide-MHC-TCR cannot be underestimated, because, antigen presentation plays an important role for autoimmune disease. The MHC class I binding area is closed, limiting the length of the presented peptides to 8–10 amino acids [28], however, MHC class II binding site is open and led peptides with 14–18 aa in length [28], but under certain conditions shorter peptides can be presented [29].

3. Autoimmunity

Autoimmunity is defined as a condition of loss of immune tolerance to self-antigens causing an autoreactive immune T and B cells that attack own organs provoking an aseptic inflammation and comprised more than 80 chronic diseases characterized by inflammatory reactions that can either be systemic or organ specific [30] and no cure exist for the majority autoimmune diseases and the treatment is based by control disease symptoms [31].

The early event in autoimmunity is the presentation of self-antigen derived peptides in complex with MHC class II to self-reactive T cells in an inflammatory environment where antigen-presenting cell, dendritic cell mainly, is activated and drives co-stimulation and development of pathogenic autoreactive T cell and autoantibodies, playing a critical role in breaking tolerance to self during an autoimmune disease, leading tissue and organ damage [31, 32], produced by susceptible and aberrant genes, environment exposure, and failed immune regulation [30].

Dendritic cells are the responsible for the initiation of primary T cell responses imprinting the phenotype Th1, Th2, Th17, Treg population in response to environmental signals mediating the breach of T cell tolerance in many autoimmune conditions [31] involved in the activation of other autoreactive B cells [33]. Indeed, T cell help for antigens and can lead the activation of B

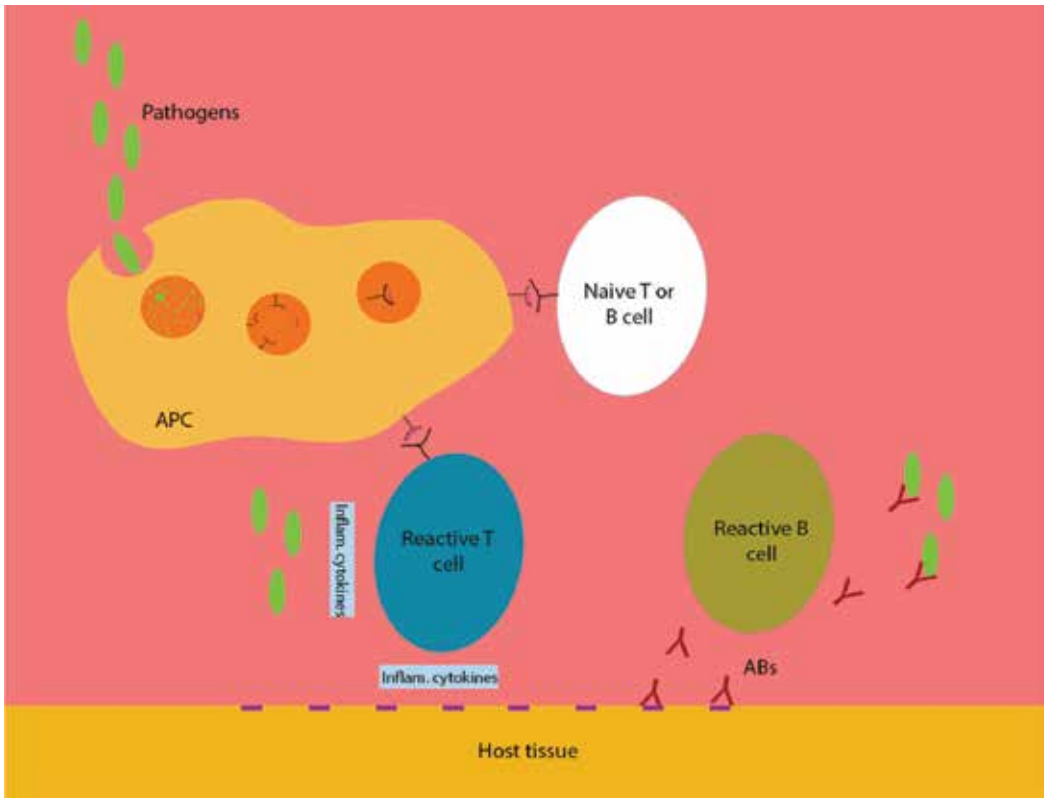

Figure 1. Molecular mimicry. Pathogens are recognized via TLRs by APC and they are phagocytosed in phagosome, digested and many microbial epitopes are exposed. MHC-II is mounted and microbial epitope is coupled in the MHC-II. In this case, epitope with similar characteristics with self-epitope (in violet) is mounted in MHC-II. The epitope is presented to naïve T or B cell, and a specific immune response is initiated. This immune response provokes the production of antibodies and cell-cell response by liberation of proinflammatory cytokines to the antigen presented. In this case, this immune response is addressed to pathogens and host tissue, with similar epitope, originating an autoimmune disease.

cells that recognized the foreign antigen but also cross-react with self-antigen [34] producing and autoimmune disease.

T cells, for example, are important for the pathogenesis of rheumatoid arthritis (RA), particularly in the initial phase of autoimmune response, inducing the joint inflammation of the joints [3]. The Th17 cells are very important because they promote the development of autoimmune diseases by producing IL-17 promoted osteoclastogenesis in RA by upregulating RANK-RANKL expression on osteoblast, macrophages and synovial fibroblast [3, 35] (**Figure 2**).

3.1. Autoantigens

Autoantigens can be defined as antigens that can be assumed to be targeted in an autoimmune disease [28] by the production autoantibodies by autoreactives B cells. Indeed, autoantibody-producing B cell originated from T cell responses to foreign antigens thought molecular mimicry between microbial antigens and self-antigens [33].

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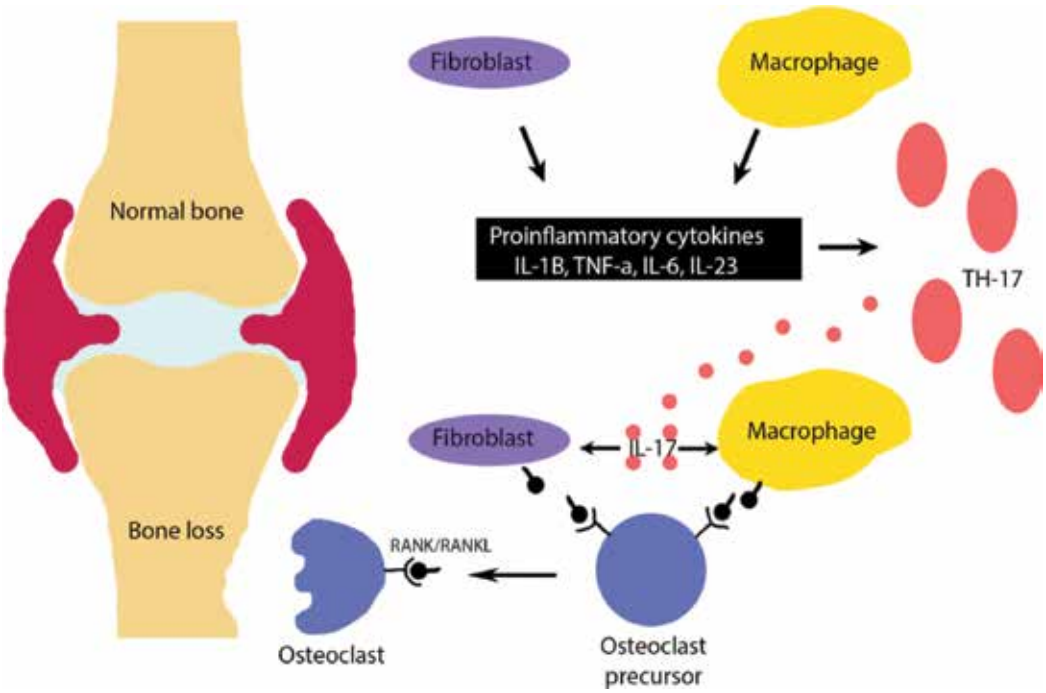

Figure 2. Synovial macrophages and fibroblast in a stress (aseptic or septic injury) released proinflammatory cytokines causing the production and release of IL-17 that provokes the overproduction of RANK by fibroblast and macrophages. RANK/RANKL stimulates osteoclast precursor to form an active osteoclast. The continued presence of RANK, produce the active form of osteoclast, reabsorbing bone.

The literature describes many autoantigens for each autoimmune disease. Type 1 diabetes mellitus (T1DM) is a metabolic disease that is explained as an autoimmune disease in which the B-cells in the Langerhans islands of pancreas are destroyed by autoreactive T and B cells resulting in a null production of insulin [28]. Zinc transporter 8 protein, pancreatic and duodenal homeobox 1, chromogranin A, islet amyloid polypeptide are new discovered autoantigens that explain the pathogenesis of T1DM [36].

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects connective tissue [37, 38], involved multiple systems, organs and autoantigens [38]. Autoantigens acidic ribosomal phosphoprotein (P0)-4, acidic ribosomal phosphoprotein (P0)-11, DNA topoisomerase 1 (full length)-1, and U1-SnRNP, were founded in clinical tests and are using as markers for clinical diagnoses [38].

Rheumatoid arthritis (RA) is a chronic inflammatory disease with a strong autoimmune component that affect bones and joints with the concomitant destruction, associated with adverse morbidity, mortality, and socioeconomic consequences [39]. Autoantibodies such as rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs) founded in serum samples obtained years before the onset of clinical disease [40, 41].

Autoantigens may cause a self-reactivity of T and B cells by dysregulation of homeostasis of immune response acting as a trojan horses harming own body producing an autoimmune disease.

3.2. Searching trojans in oral microbiome

Microbiome is defined by Lederberg as "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" [42]. *In silico* tools have provided a powerful means of understanding the contribution of the human microbiome to health and disease opening a great field for oral immunologist. In the era of computer trojan horse, microbial epitopes with high similarities (in sequence and structure) with the host, can act as little sequences for the evasion of host immune system, even more, this trojans may cause a T and B reactive cells provoking an immune response for the microbial elimination and the origin of an autoimmune disease [43].

3.2.1. Trojans against connective

Systemic lupus erythematosus (SLE) is an prototype of autoimmune disease, affecting the connective tissue [44], with a great spectrum of clinical symptoms such as joints, kidneys, skin, to other manifestation, in fact, SLE is a nonpreventable disease and may be life-threatening [45, 46]. Many autoantigens have been described to induce cross-reactivity immune response to SLE such as Ro52, Ro60, La, RNP-A, Sm-D3, and RNP-70 K. RNA-A and RNP-70 K, however, oral microbiome contain epitopes with similarities against SLE autoantingens (**Table 1**).

Ro ribonucleoprotein 60 KDa (Ro60) is an autoantigen most prevalent in systemic autoimmune diseases as SLE and Sjögren's syndrome, and exist in unabundant ribonucleoprotein complexes stabilizing small RNA to prevent degradation [47, 48]. This protein has a 6 aminoacids (aa) similarity against VWA domain-containing protein of *Prevotella denticola* (**Table 1**). Small nuclear ribonucleoprotein 70 kDa, another autoantigen in SLE, is a small protein conforming the spliceosome complex. This protein has a 7 aa similarity against *Bacillus cereus* (**Table 1**).

PubMed ref	Organism	Epitopes
NP_001035828.1	*H. sapiens*	DVSASM
WP_036854258.1	*P. denticola*	DVSASM
NP_003080.2	*H. sapiens*	GYAFIEY
WP_061130177.1	*B. cereus*	GYAFIEY

P. denticola and *B. cereus* present epitopes with high similarities of self-autoantigens Ro60KDa and small nuclear ribonucleoprotein 70 KDa.

Table 1. Oral microbiome epitopes with similarities against connective.

3.2.2. Trojans against nerves

Aquaporin 4 is an integral membrane protein that conducts water through cell membrane founded in nervous system. It is presented as autoantingen in neuromyelitis optica, an autoimmune disease consisting of a chronic inflammation and demyelination of optical nerve and spinal cord. This protein has similarities against glycerol uptake facilitator protein 2 of *Streptococcus pneumoniae*, MIP family channel protein of *Prevotella oralis* and MIP family channel protein of *Enterococcus faecalis* (**Table 2**). Glycerol uptake facilitator protein 2 is a putative nonselective transport channel in the inner membrane of bacterium [49] and MIP family channel is a transmembrane protein transporting small molecules [50]. Both proteins are in external side of microbial cell membrane been more efficient form antibody-epitope complex.

Glutamate decarboxylase 2 is an autoantigen related in Stiff-Person syndrome, an autoimmune disease that affects nervous system. Glutamate decarboxylase of *Enterococcus spp*. possess 7 aa similarities against autoantigen (**Table 2**).

3.2.3. Trojans against diabetes

Type 1 diabetes is an autoimmune disease in which the B-cells in the Langerhans islands of pancreas are destroyed by T and B reactive cells lacking the insulin production [28], affecting children and latent autoimmune disease of adults [51]. One of characteristics of this disease is the recognition of beta cell proteins as autoantigens such as preproinsulin GAD65, islet antigen 2 (IA-2), ZnT8, nonspecific islet cell autoantigens (ICAs), imogen 38, pancreatic duodenal homeobox factor 1, chromogranin A, islet specific glucose-6-phosphatase catalytic subunit-related protein, heat shock protein 60 and islet cell antigen 69. IA-2, possess 6 aa with similar

PubMed ref	Organism	Epitopes
AAB26958.1	*H. sapiens*	ISG-HINPA-T
WP_004369577.1	*P. oralis*	ISG-HINPA-T
AAB26958.1	*H. sapiens*	G-IIGA-ILY
WP_004369577.1	*P. oralis*	G-IIGA-ILY
AAB26958.1	*H. sapiens*	S-NPARS-GPA
WP_004369577.1	*P. oralis*	S-NPARS-GPA
AAB26958.1	*H. sapiens*	SVNPARS
EFM77965.1	*Enterococcus spp.*	SVNPARS
NP_000809.1	*H. sapiens*	HVDAA-GG
WP_086305260.1	*Enterococcus spp.*	HVDAA-GG

P. oralis and *Enterococcus spp*. present epitopes with high similarities of self-autoantingens against aquaporin 4 and glutamate decarboxylase 2.

Table 2. Oral microbiome epitopes with similarities against nerves.

PubMed ref	Organism	Epitopes
NP_001186692.1	*H. sapiens*	PKAE-PA
WP_033676705.1	*S. mitis*	PKAE-PA

S. mitis presents epitope with high similarities of self-autoantigen against islet antigen A.

Table 3. Oral microbiome epitopes with similarities against diabetes.

PubMed ref	Organism	Epitopes
pir||B54197	*H. sapiens*	SFENP
CDB05904.1	*Prevotella sp.*	SFENP
pir||B54197	*H. sapiens*	FTNEDNP
EPH90635.1	*E. faecalis*	FTNEDNP
pir||B54197	*H. sapiens*	FENPVL
WP_002676716.1	*T. denticola*	FENPVL

Prevotella sp., E. faecalis., T. denticola., present epitopes with high similarities of self-autoantigens against Ku autoantigen 70 k.

Table 4. Oral microbiome epitopes with similarities against thyroiditis.

characteristics with LysM peptidoglycan-binding domain-containing protein of *Streptococcus mitis* (**Table 3**) that is present to bind noncovalently to peptidoglycan and chitin in cell wall [52].

3.2.4. Trojans against thyroiditis

Thyroiditis is an autoimmune disease that destroys thyroid cells by reactive T and B cells. This disease is also known as chronic autoimmune thyroiditis and chronic lymphocytic thyroiditis. The pathology of thyroiditis involves the formation of antithyroid antibodies that attack thyroid tissue, causing progressive fibrosis [53]. One common autoantigen of many described in the literature is the thyroid autoantigen 70 k also known as Ku autoantigen [54]. Ku is an abundant protein in the body with multiple functions as replication, transcription and cell signaling [54]. Pilin isopeptide linkage domain protein of *E. faecalis*, ompA family protein of *Prevotella sp*. and aldo/keto reductase of *T. denticola* have small epitopes with high similarities with Ku autoantigen 70 k (**Table 4**).

3.2.5. Trojans against myasthenia gravis

Myasthenia gravis is an autoimmune disease that attacks neuromuscular junction where synapsis occurs between nerves and muscles causing muscle weakness in patients [55]. Autoantibodies such as muscle-specific tyrosine kinase (MUSK), acetylcholine, agrin and low-density lipoprotein receptor–related protein 4 (LPR4) have been described in the literature [56, 57]. MUSK is a transmembrane protein that contains three IgG domains and one cysteine-rich domain in the extracellular region and a kinase domain in the intracellular region [56]

and possesses 6 aa similarity to Stk1 family PASTA domain-containing Ser/Thr kinase of *Lactobacillus sp.* (**Table 5**). This lactobacillus protein is present in cell wall in gram positives and negatives associated to penicillin-binding proteins [58].

3.2.6. Trojans against chronic autoimmune gastritis

Autoimmune gastritis represents approximately 5% of the whole spectrum of chronic gastritis and must be differentiated from the one associated with chronic *Helicobacter pylori* infection [59]. Gastritis is a chronic inflammatory disease involving gastric body and fundus, with the progressive reduction and/or disappearance of gastric glands that are sometimes replaced by intestinal or pyloric epithelium [60]. Autoantigens for the autoimmune gastritis has been related as Gastric ATPase α subunit, Gastric ATPase β subunit and Gastric intrinsic factor [61]. Gastric ATPase α subunit have three epitopes in different position in the same protein with a 6 aa, 7aa and 15 aa similarity, to Ca2 + −transporting ATPase of *Streptococcus pneumoniae* (**Table 6**).

3.2.7. Trojans against liver

Autoimmune hepatitis is a chronic and progressive inflammation of the liver from an unknown cause, whose pathology is explained by the failure of immune tolerance in a genetically susceptible individual leading to a reactive T-cell mediated inflammation caused by various environmental triggers including infections, medications, and toxins [62]. Autoantigens for autoimmune hepatitis have been related such as O-phosphoseryl-tRNA(Sec) selenium transferase (SLA), cytochrome P450 2D6 isoform 1 (CYP2D6) and formimidoyltransferase-cyclodeaminase isoform C (FTCD) [61]. FTCD epitopes have similarities with glutamate

PubMed ref	Organism	Epitopes
NP_001159752.1	*H. sapiens*	KIADFG
WP_083289611.1	*Lactobacillus spp.*	KIADFG

Lactobacillus spp. presents epitope with high similarities of self-autoantigen MUSK.

Table 5. Oral microbiome epitopes with similarities against neuromuscular junctions.

PubMed ref	Organism	Epitopes
NP_000695.2	*H. sapiens*	ICSDKTGTLTQN-MTV
CKF15123.1	*S. pneumoniae*	ICSDKTGTLTQN-MTV
NP_000695.2	*H. sapiens*	MIDPPR
CKF15123.1	*S. pneumoniae*	MIDPPR
NP_000695.2	*H. sapiens*	TGDGVND
CKF15123.1	*S. pneumoniae*	TGDGVND

S. pneumoniae present epitope with high similarities of self-autoantigen against gastric ATPase α subunit.

Table 6. Oral microbiome epitopes with similarities against gastritis.

PubMed ref	Organism	Epitopes
NP_001307341.1	*H. sapiens*	ECVPNFSEG
WP_054191567.1	*P. gingivalis*	ECVPNFSEG
NP_001307341.1	*H. sapiens*	GEHPRMGA-DVCPF
WP_010922735.1	*Streptococcus spp.*	GEHPRMGA-DVCPF
NP_001307341.1	*H. sapiens*	APGGGSV
WP_088387656.1	*F. nucleatum*	APGGGSV
NP_001307341.1	*H. sapiens*	PNFSEG
WP_010922735.1	*Streptococcus spp.*	PNFSEG

P. gingivalis., Streptococcus spp., F. nucleatum., present epitopes with high similarities of self-autoantingens FTCD and PDC-E2.

Table 7. Oral microbiome epitopes with similarities against liver.

formimidoyltransferase of *Porphyromonas gingivalis*, formimidoyltetrahydrofolate cyclodeaminase of *Fusobacterium nucleatum* and glutamate formimidoyltransferase of *Streptococcus spp* (**Table 7**).

Primary biliary cirrhosis (PBC) is now known as primary biliary cholangitis [63]. It is an autoimmune disorder which leads to gradual destruction of intrahepatic bile ducts resulting into periportal inflammation, cholestasis [63]. This disease is common among women of middle age worldwide. Primary biliary cirrhosis is associated with highly specific autoantibody [64]. The anti-mitochondrial antibody is found in 85% of the cases, other antibodies associated with disease is an antinuclear antibody (ANA), anti-multiple nuclear dot antibody (anti-MND), anticentromere antibody, pyruvate dehydrogenase complex E2 (PDC-E2) and antinuclear envelop antibody [61, 63] . PDC-E2, possess 7 aa with similarities to dihydrolipoyllysine-residue acetyltransferase of *Enterococcus spp*.

3.2.8. Trojans against muscle

Myositis is an autoimmune disease that attack muscles [65]. There are three types of this disease: polymyositis, dermatomyositis, and juvenile myositis and possess and autoimmune origin, meaning the immune system is attacking the muscle [66]. This disease is not present in etiology. Although myositis is often treatable, these diseases are poorly understood and do not always completely respond to current medications [66]. Autoantigens has been related in the literature: histidine–tRNA ligase, cytoplasmic isoform 2, threonine–tRNA ligase, cytoplasmic isoform 1, exosome complex component RRP45 isoform 1, exosome component 10 isoform 1, chromodomain-helicase-DNA-binding protein 4 isoform 1, interferon-induced helicase C domain-containing protein 1, MORC family CW-type zinc finger protein 3 isoform 2, signal recognition particle 54 kDa protein isoform 2, E3 ubiquitin-protein ligase TRIM33 isoform alpha and 3-hydroxy-3-methylglutaryl-Coenzyme A reductase isoform 1 [61]. Threonine–tRNA ligase, cytoplasmic isoform 1 autoantigen, possess many epitopes with high similarities with threonine-tRNA ligase of *Aggregatibacter actinomycetemcomitans* and threonine–tRNA ligase of *Streptococcus spp.* (**Table 8**).

PubMed ref	Organism	Epitopes
NP_001245366.1	*H. sapiens*	TLPDG
WP_005555043.1	*A. actinomycetemcomitans*	TLPDG
NP_001245366.1	*H. sapiens*	NGFYYD
WP_005555043.1	*A. actinomycetemcomitans*	NGFYYD
NP_001245366.1	*H. sapiens*	CRGPHV
WP_005555043.1	*A. actinomycetemcomitans*	CRGPHV
NP_001245366.1	*H. sapiens*	RDHRKIG
WP_005555043.1	*A. actinomycetemcomitans*	RDHRKIG
NP_001245366.1	*H. sapiens*	KPMNCPGH
WP_005555043.1	*A. actinomycetemcomitans*	KPMNCPGH
NP_001245366.1	*H. sapiens*	QDDAHIFC
WP_005555043.1	*A. actinomycetemcomitans*	QDDAHIFC
NP_001245366.1	*H. sapiens*	LSTRPEK
WP_005555043.1	*A. actinomycetemcomitans*	LSTRPEK
NP_001245366.1	*H. sapiens*	GAFYGPK
WP_005555043.1	*A. actinomycetemcomitans*	GAFYGPK
NP_001245366.1	*H. sapiens*	TIQLDF
WP_005555043.1	*A. actinomycetemcomitans*	TIQLDF
NP_001245366.1	*H. sapiens*	HRAILGS
WP_005555043.1	*A. actinomycetemcomitans*	HRAILGS
NP_001245366.1	*H. sapiens*	GFYYD
WP_000591038.1	*Streptococcus spp.*	GFYYD
NP_001245366.1	*H. sapiens*	DLCRGPHV
WP_000591038.1	*Streptococcus spp.*	DLCRGPHV
NP_001245366.1	*H. sapiens*	RDHRK
WP_000591038.1	*Streptococcus spp.*	RDHRK
NP_001245366.1	*H. sapiens*	TSGHW
WP_000591038.1	*Streptococcus spp.*	TSGHW
NP_001245366.1	*H. sapiens*	SGALTGL
WP_000591038.1	*Streptococcus spp.*	SGALTGL
NP_001245366.1	*H. sapiens*	AFYGPK
WP_000591038.1	*Streptococcus spp.*	AFYGPK

A. actinomycetemcomitans, and *Streptococcus spp.,* present epitopes with high similarities of self-autoantingen Threonine—tRNA ligase, cytoplasmic isoform 1.

Table 8. Oral microbiome epitopes with similarities against gastritis.

PubMed ref	Organism	Epitopes
NP_110447.2	*H. sapiens*	GAKG-RGEKG
ZP_05918585.1	*Prevotella sp.*	GAKG-RGEKG

Prevotella sp. presents epitope with high similarities of self-autoantigen against collagen alpha-1(XXI) chain isoform a.

Table 9. Oral microbiome epitopes with similarities against collagen.

3.2.9. Trojans against collagen

Collagen is the most tissue presented in the body; it is associated with the skin, kidney, nerves, blood vessels and muscles protecting them against compressive forces [67, 68]. Rheumatoid arthritis (RA) is a progressive autoimmune disease that affects directly the collagen by the chronification of inflammation causing a tissue damage (specially cartilage and bone), functional impairment, severe disability and premature mortality [69, 70]. Periodontitis is a chronic disease by microbial multispecies insult. Microbiome of periodontal disease (PD) could be showed some bacteria such *P. gingivalis, P. intermedia, Tannerella forsythia. F. nucleatum* and *Aggregatibacter actinomycetemcomitans,* with epitopes that provokes autoreactivity against collagen [71]. Anti-citrullinated protein is an important autoantigen present in patients with RA having antibodies anti-Pg [72]. Obando-Pereda et al. showed that an epitope of *Prevotella sp.* has high similarity with human collagen report a positive antigen-antibody complex in RA and PD patient's sera [8] (**Table 9**).

4. Conclusion

The majority of autoimmune diseases possess an unknown etiology and can be explained from genetic factors to molecular mimicry. In silico, tools for biological purposes are important to determinate if external epitopes that possess similarities with epitopes from autoantigens. Epitopes for Systemic lupus erythematosus, Sjögren's syndrome, neuromyelitis optica, Stiff-Person syndrome, autoimmune diabetes, autoimmune thyroiditis, myasthenia gravis, autoimmune gastritis, autoimmune hepatitis, myositis and rheumatoid arthritis, possess microbial epitopes belong to oral microbiome with high similarities that can explain the possible etiology of autoimmune disease by molecular mimicry.

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Conflict of interest

The authors declare no conflict of interest in the present chapter.

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