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Alterations in the Oral Microbiome Leading to Inflammatory Periodontal Disease

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Abstract

The oral cavity contains a complex micro-ecosystem of flora inhabiting a variety of different niches. Some of these niches include saliva, shedding surfaces, and non-shedding surfaces. Biofilms in the oral microbiome, colloquially referred to as dental plaque, can accumulate on non-shedding surfaces, such as natural teeth and other artificial hard parts such as braces, dentures, fillings, and implants. These biofilms are made up of many mutualistic bacteria that stick to each other and adhere to hard surfaces. Scientists speculate that at least 700 different species of bacteria inhabit various parts of the mouth. These bacteria occupy different niches in varying amounts to help protect the teeth and body systems from invading pathogens. However, when plaque grows on non-shedding surfaces, the bacteria can over accumulate and cause inflammatory periodontal disease. Inflammatory periodontal disease includes both gingivitis and periodontitis. Gingivitis is an inflammatory disease caused by the overaccumulation of dental plaque in the supragingival area. When this plaque exists for extended periods of time without being removed, the biofilm can spread to the subgingival region of the gum and cause periodontitis. Periodontitis is characterized by tooth loss due to bone resorption and tissue damage. This review aims to discuss the community shifts in the oral microbiome that are associated with the onset of inflammatory periodontal disease, as well as the mechanisms in which these bacteria contribute to disease symptoms.

Section 1: Historical Context of the Human Oral Microbiome

The human oral microbiome consists of all the bacteria, fungi, viruses, and protozoa found in the oral cavity (Deo & Deshmukh, 2019). It is considered to be the second largest microbiome found in the body (Verma, Garg, & Dubey, 2018). The oral microbiome was first discovered by scientist Antony van Leewenhoek in 1674 as he observed tiny moving particles in his own dental plaque. He referred to these moving particles as "animalcules" and laid the foundation for the field of microbiology (Deo & Deshmukh, 2019). Around 200 years later, Jacob Henle was curious about these "animalcules" and hypothesized that they could be the causative agents of disease (*Robert Koch biography.*). Robert Koch, who was a student of Henle, was the first person to link bacteria to a specific disease by inoculating mice with *Bacillus anthracis*. His findings suggested that microbes are the causative agents of disease, and he published these results in 1876 (*Robert Koch biography.*). Dentist Willoughby D. Miller was inspired by the associations between microorganisms and disease and decided to further study these associations in the oral microbiome. He postulated that dental caries, otherwise known as dental cavities, were caused by bacteria in the mouth via the "chemico-parasitic" theory (Yamashita & Takeshita, 2017). He published his findings in a book called *The Microorganisms of the Human Mouth* (Ring, 2002). Research on the oral microbiome continued into the 20th century as James K. Clarke isolated *Streptococcus mutans* from a healthy oral cavity and learned that this bacterium contributes to tooth decay (Verma, Garg, & Dubey, 2018). The discovery of pathogenic bacteria in the mouth influenced many researchers to learn more about the human microbiome and its correlation with the onset of disease.

As research on bacteria in the oral microbiome became more popular, new sequencing techniques were developed in order to study it. In 1987, Carl Woese discovered a way to classify

bacteria using 16S rRNA sequencing. 16S rRNA sequencing uses hypervariable regions to determine the microbial diversity of bacteria in a sample (Verma et al., 2018). As researchers realized the importance of studying the human microbiome, the National Institute of Health launched the Human Microbiome Project in 2008. The Human Microbiome Project allowed for advances in sequencing the genomes of microorganisms. The project consisted of multiple sub projects involving 16S rRNA sequencing (Deo & Deshmukh, 2019). Two years later, the National Institute of Dental and Craniofacial Research launched the Human Oral Microbiome Database (HOMD) in order to classify the different taxa of the core bacteria existing in the mouth (Deo & Deshmukh, 2019). HOMD allowed for further research and classification of bacteria in the mouth and facilitated further research on diseases caused by alterations to the oral microbiome.

Section 2: The Human Oral Microbiome

The human oral microbiome is the second largest microbiome that exists in the human body with over 772 species of bacteria that have already been identified. Around 30% of these bacteria remain unculturable due to their specific nutrient requirements, oxygen sensitivities, and dependence on other microorganisms, which prevents full microbiome sequencing efforts (Arweiler & Netuschil, 2016). Around 96% of the bacteria found in the oral microbiome belong to six phyla including *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Bacteroidetes*, and *Spirochaetes* (Verma et al., 2018). These bacterial phylum make up the core microbiome, which is common among all individuals.

Firmicutes contribute the majority of bacteria found in the oral microbiome and include the genera *Streptococci* and *Granulicatella* (Zaura, Keijser, Huse, & Crielaard, 2009).

Streptococci, which contains Gram positive cocci, is considered the pioneer microbial colonizer of the oral microbiome and is associated with a healthy oral microbiome. Some of the species of *Streptococci* present in the oral microbiome include *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus gordonii*, and *Streptococcus salivarius* (Sedghi, DiMassa, Harrington, Lynch, & Kapila, 2021). The next most abundant phylum of bacteria found in the oral microbiome is *Proteobacteria*, which includes the genera *Neisseria* and *Haemophilus* (Zaura et al., 2009). There are five different types of proteobacteria which are all found in the oral microbiome, but only γ-proteobacteria have been cultivated and sequenced. *Actinobacteria* are also present in relatively high abundances and are usually Gram positive with a high guanine and cysteine content. *Spirochaetes* are the next most abundant phylum of bacteria found in the oral microbiome and include di-derm, helically coiled bacteria that are usually Gram negative. Among the six phyla listed, *Fusobacteria* are found in the lowest amount and include the late colonizers of healthy individuals. (Verma et al., 2018). However, *Fusobacteria* are also microbial colonizers and can be beneficial to the health of the oral microbiome (Sedghi et al., 2021). The remaining 4% of the bacteria found in the human oral microbiome are variable and may belong to the phyla *Euryarchaeota*, *Chlamydia*, *Chloroflexi*, *Synergistetes*, and *Tenericutes* (Verma et al., 2018*).* The exact amount of each bacterial phyla present in the oral cavity varies based on the individual.

Many of the bacteria found in the oral cavity have mutualistic relationships with each other and adhere to each other by producing various glycoproteins and polysaccharides to form biofilms on hard surfaces (Verma et al., 2018). Many of these biofilms consist of both oxygen dependent and anaerobic bacteria, which must work together to survive. Biofilms develop through three phases of succession. During the first phase, known as induction, the bacteria form

a linking film (Arweiler & Netuschil, 2016). During the second phase, more bacteria start to accumulate to this film through a process known as accumulation (Arweiler & Netuschil, 2016). The third phase is characterized as the existence phase, in which the biofilm is constantly growing and decomposing (Arweiler & Netuschil, 2016). In the existence phase, biofilms in the oral microbiome are colloquially referred to as dental plaque and can cause diseases and other issues if the bacteria are no longer in equilibrium (Arweiler & Netuschil, 2016). This disturbance of the regular equilibrium of the microbiome can be referred to as dysbiosis.

The microorganisms of the human microbiome may inhabit multiple different locations in the oral cavity. Primarily, bacteria can be found in the planktonic phase which occurs when they are free floating in the saliva (Arweiler & Netuschil, 2016). Since these bacteria are constantly being swallowed by humans at a rate of 10^9 microorganisms per liter, they do not contribute to the formation of plaque (Arweiler & Netuschil, 2016). In addition to the saliva, bacteria can be found in monolayers on shedding surfaces such as the lips, cheeks, palate, and tongue (Arweiler & Netuschil, 2016). Since these bacteria continuously shed from these surfaces, they also do not contribute to plaque formation (Arweiler & Netuschil, 2016). However, bacteria may form plaque on hard, non-shedding surfaces such as teeth or other artificial hard surfaces (Arweiler & Netuschil, 2016). Biofilms may form in tooth cavities as well as approach the dental pulp in fissure plaque. Fissure plaque can cause dental caries and other endodontic diseases (Arweiler & Netuschil, 2016). Plaque can form on supragingival surfaces, which consists of areas adjacent to the gingiva, which is the leading cause of gingivitis. If the plaque on supragingival surfaces exists for elongated periods of time, it may spread into the subgingival layer and lead to worsened gingivitis or periodontitis (Arweiler & Netuschil, 2016). The removal of plaque is an

important way to prevent the onset of periodontal disease, including both gingivitis and periodontitis. (Yamashita & Takeshita, 2017).

Disease in the microbiome is caused by the dysbiosis of the organisms inhabiting the oral microbiome. Many pathobionts exist in the oral microbiome in equilibrium with other microorganisms. However, when the equilibrium of the oral microbiome is disrupted, these pathobionts cause disease by stimulating immune responses (Sedghi et al., 2021). Since the oral cavity is constantly exposed to the external environment due to eating and breathing, the oral microbiome is highly susceptible to infection and dysbiosis (Sedghi et al., 2021). During periods of extended plaque formation and dysbiosis, the bacteria of the oral microbiome can instigate immune and inflammatory responses, leading to periodontal diseases (Sedghi et al., 2021). Gingivitis is the early form of periodontal disease and is characterized by various sites of inflammation at the gum level (Trombelli, Farina, Silva, & Tatakis, 2018). Gingivitis is reversible if it is treated. If gingivitis is left untreated, it can progress to periodontitis, which is characterized by long term, irreversible damage to the gingiva, which ultimately causes the tooth to detach from the alveolar bone (Sedghi et al., 2021).

Section 3: Gingivitis

3.1 Introduction to Gingivitis

Gingivitis is a plaque-induced disease caused by gingival inflammation along the gumline (Trombelli et al., 2018). The disease may cause bleeding at inflamed sites, but the inflammation and bleeding are not usually painful. Therefore, the disease is often left undiagnosed in patients (Huang et al., 2011). Gingivitis can be cured if it is treated properly by removing plaque build up (Trombelli et al., 2018). It often affects people of lower socioeconomic

class who may not have access to proper dental hygiene care (Fan et al., 2021). It is also frequently found in adolescents and teenagers due to high hormone levels during puberty (Fan et al., 2021). Although gingivitis is considered a mild and treatable disease, it is important for dentists to recognize and treat gingivitis because if it is left untreated, it can progress into chronic periodontitis, which is untreatable and irreversible.

In order to understand the cause of gingivitis, many researchers have studied the shift in the oral microbiome that may lead to gingival inflammation. Diagnosing gingivitis is difficult because there is no universal definition of how much gingival inflammation must be present in order to confirm the presence of the disease (Trombelli et al., 2018). Therefore, it is hard to distinguish how many individual sites of gingival inflammation warrant a true case of gingivitis (Trombelli et al., 2018). In order to determine whether a patient has gingivitis, a technique called Bleeding on Probing (BoP) is often used. During this technique, the dentist may prod the gumline to check for bleeding and inflammation. A BoP score can then be given based on the percentage of sites in which the gingival inflammation resulted in bleeding (Trombelli et al., 2018). In 2018, Trombelli *et al.* determined that a BoP score greater or equal to 10% would be classified as a gingivitis case, whereas a BoP score greater or equal to 30% could be considered a case of periodontitis if it was accompanied by radiographic bone loss. If the BoP score is less than 10%, then patients are considered healthy, even if they have some sites of gingival inflammation. The findings of Trombelli *et al.* have not been universally accepted, but many researchers use the BoP scores to determine gingivitis using the Mazza Gingival Index (MGI). The MGI is a clinically based index created to assess the severity of gingivitis by considering BoP scores (Nowicki et al., 2018). However, the exact percentages of bleeding sites that warrant a case of gingivitis are subjective. The following section of the review will discuss various

research techniques and findings that have contributed to the characterization of the oral microbiome during the onset of gingivitis. The onset of gingivitis in longitudinal studies can be ethically studied due to the reversibility of the disease.

3.2 Research on Gingivitis:

In 2011, after the establishment of the HOMD, Huang *et al.* were interested in understanding the bacteria present in the oral microbiome during gingivitis. In order to study the biomarkers relating to gingivitis, a study was conducted consisting of six patients: three with healthy gingiva and three with gingivitis (Huang et al., 2011). 16S rRNA sequencing was used to define the operational taxonomic units (OTUs) present in the oral microbiomes of diseased and healthy patients (Huang et al., 2011). The microorganisms present at five different sites of the gumline were studied for each patient (Huang et al., 2011). The results suggested that the phyla *Actinobacteria* and *Bacteroidetes* were less abundant in gingivitis, whereas the phyla *Fusobacteria* and TM7 (now known as *Saccharibacteria*) were more abundant in cases of gingivitis (Huang et al., 2011). On the genus level, *Streptococcus*, *Veillonella*, and *Prevotella* were less abundant in cases of gingivitis, whereas *Leptotrichia* and *Selenomonas* were more highly present in cases of gingivitis (Huang et al., 2011). All of these bacteria are normally found in the oral microbiome, but *Leptotrichia* and *Selenomonas* are Gram negative anaerobic bacteria that are found in higher amounts during gingivitis (Huang et al., 2011). Therefore, Huang *et al.* concluded that gingivitis is usually accompanied by a shift in the oral microbiome from Gram positive aerobes and facultative anaerobes to Gram negative anaerobes. This study represents one of the first times the bacteria associated with gingivitis were identified (Huang et al., 2011). However, due to the highly variable nature of the human oral microbiome and the small sample

size of the study, more research was necessary in order to correctly classify the bacteria that cause gingivitis in the oral cavity (Huang et al., 2011).

Shi Huang continued his research on gingivitis-associated bacteria with a new research team in 2014. Huang *et al.* used a retrogression-progression model to study the biomarkers of gingivitis (Huang et al., 2014). During this study, 50 adults who had gingivitis were treated and cured of their gingivitis (Huang et al., 2014). Then, the research team induced experimental gingivitis in the candidates by having them temporarily stop all oral hygiene care. The oral microbiomes of the patients were tested during the natural gingivitis stage, the healthy gingival stage, and then the experimental gingivitis stage. Huang *et al.* used the MGI based on BOP scores to monitor these transitions and observed a change in over 27 bacterial genera. The bacterial genera present during the natural gingivitis and experimental gingivitis stages remained similar to each other, whereas the microbiota present during the healthy stage remained distinct from the diseased stage.

The results of this study showed that elevated levels of *Actinobacteria* and *Firmicutes* were associated with health, whereas elevated levels of *TM7*, *Bacteroidetes*, and *Fusobacteria* were associated with gingivitis (Huang et al., 2014). These results are similar to the findings from the Huang *et al.* study in 2011, but in the 2014 study, *Fusobacteria* was associated with gingivitis. Specifically, Huang *et al.* determined that out of the 27 genera detected, the following phyla are associated with a healthy microbiome: *Streptococcus*, *Rothia*, *Actinomyces*, *Haemophilius*, and *Lautropia.* In contrast, Huang *et al.* determined 22 genera and families associated with gingivitis, which include: "*Leptotrichia*, *Prevotella*, *Fusobacterium,* TM7 genus*,* *Porphyromonas*, *Tannerella*, *Selenomonas*, uncultured *Lachnospiraceae*, unclassified *Comamonadaceae*, *Peptococcus, Aggregatibacter*, *Catonella, Treponema*, SR1 genus*,*

Campylobacter, *Eubacterium*, *Peptostreptococcus*, unclassified *Bacteroidaceae, Solobacterium, Johnsonella*, *Oribacterium*, and unclassified *Veillonellaceae."* Some of the bacteria that could not be resolved to the genus level are listed in the family level. In addition, Huang *et al.* found that there are two different types of hosts of gingivitis which he defined as type I and type II. The type I host displayed large changes in their individual microbiomes during the onset of gingivitis, whereas type II hosts displayed only acute changes in their microbial structure (Huang et al., 2014). In general, Huang *et al.* concluded that there is a gradual shift that occurs in the oral microbiome between a healthy individual and the onset of gingivitis. In addition, the results suggest that the different genera and families mentioned can help to identify whether a patient may have a healthy microbiome or have gingivitis (Huang et al., 2014). Regardless, it is difficult to attribute an exact bacterial species to gingivitis because species composition differs among patients.

Around the same time that Huang *et al.* conducted research on the oral microbiome associations with gingivitis, Kistler *et al.* also became interested in studying this topic. Kistler *et al.* used 454-pyrosequencing and 16S rRNA sequencing to determine the bacteria present in the oral microbiome during the onset of gingivitis. This study was also a longitudinal study in which the oral microbiome of 20 volunteers was monitored for two weeks during the transition from healthy gingiva to gingivitis. The research team analyzed the microbial composition at week 0, week 1, and week 2. After two weeks of abstaining from oral hygiene, all of the patients had visible plaque on their teeth and increased BoP scores.

After the 454-pyrosequencing and 16S rRNA sequencing, Kistler *et al.* determined the different genera corresponding with health and gingivitis. The results suggested that the genera *Actinomyces*, *Rothia*, and *Streptococcus* are associated with healthy gingiva. These genera

usually include species that are early colonizers, which contribute to healthy oral microbiomes. Specifically, Kister *et al.* determined that the species *Rothia dentocariosa* is associated with low BoP scores and healthy gingiva. The sequencing results also suggested that the genera *Campylobacter*, *Fusobacterium*, *Lautropia, Leptotrichia, Porphyromonas, Selenomonas,* and *Tannerella* are associated with disease. These taxa usually consist of Gram negative anaerobes. Therefore, Kistler *et al.* concluded that the presence of Gram negative cocci, rods, filaments, spirilla, and spirochetes are often associated with the onset of gingivitis.

Another study conducted by Nowicki *et al.* attempted to study the type of bacteria involved in gingivitis using 16S rRNA sequencing and metatranscriptome sequencing. A 3-week study was conducted on a cohort to determine the changes between a healthy microbiome and the bacteria present during gingivitis (Nowicki et al., 2018). Using 16S rRNA sequencing, Nowicki *et al.* observed changes in the genera associated with health and gingivitis. The results suggested that the genera *Streptococcus*, *Neisseria*, and *Lautropia* were associated with health, whereas *Oribacterium*, *Leptotrichia*, *Tannerella*, and *Lachnoanaerobaculum* were associated with gingivitis (Nowicki et al., 2018). The research team also used metatranscriptome sequencing to determine the genera of bacteria present in the plaque of healthy individuals versus those with gingivitis and found similar results (Nowicki et al., 2018). *Streptococcus*, *Neisseria*, and *Capnocytophaga* were associated with healthy individuals, while a large abundance of *Leptotrichia*, *Prevotella,* and *Fusobacterium* were found in individuals with gingivitis (Nowicki et al., 2018). Although the 16S rRNA sequencing and metatranscriptome sequencing results were generally in agreement, there were some discrepancies in the data; however, none of the genera detected with the two sequencing techniques directly contradict each other. The differences in the genera associated with healthy gums versus inflamed gingiva suggest that there are differences in

the microbiome that can cause gingivitis due to dysbiosis. In addition, the research team concluded that the bacterial diversity in the oral microbiome is positively correlated with gingivitis (Nowicki et al., 2018).

3.3 Summary of the Bacterial Genera Associated with Gingivitis and Health

Based on the research presented in this review thus far, it is evident that gingivitis is caused by microbial dysbiosis in the oral cavity due to an increase in bacterial diversity and richness. Table 1 summarizes the various genera detected in the oral microbiome in each research study discussed thusfar. The information from table 1 was used to create table 2, which displays the similarities and differences in genera detected in the oral microbiomes of healthy and diseased individuals. Every research team found associations between *Streptococcus* species and health and associations between *Leptotrichia* and gingivitis. In addition, three out of four research teams detected associations between the genera *Selenomonas* and *Tannerella* with gingivitis. Two out of the four research teams found associations between *Campylobacter*, *Porphyromonas*, and *Lachnoanaerobaculum* with gingivitis and *Rothia* and *Actinomyces* with health. The genera *Veillonella* and *Lautropia* were detected in both healthy individuals and diseased individuals, but this may be due to the fact that these genera include species that may be either beneficial or detrimental to the oral microbiome. All of the bacteria detected by more than one research team that were associated with gingivitis are Gram negative anaerobes, with the exception of *Lachnoanaerobaculum*, which is a Gram positive anaerobe. In contrast, all of the bacteria that were identified to be associated with health by more than one research team were Gram positive aerobes or facultative anaerobes. The only exception is that *Lautropia* is a Gram negative facultative anaerobe and was detected by two teams in healthy individuals, but detected by one team in diseased individuals.

Based on the associations detected, the data suggests that healthy gingiva is associated with Gram positive aerobes or facultative anaerobes, whereas gingivitis is primarily accompanied by Gram negative anaerobic bacteria. The data shows more variation between Gram positive and Gram negative bacteria in healthy individuals, which suggests that both are needed for a healthy microbiome. Gingivitis may be accompanied by the onset of Gram negative bacteria since Gram negative bacteria are usually more resistant to harsh living conditions due to their outer membrane. Therefore, these bacteria may be able to thrive in harsh environments and over accumulate, ultimately causing pathogenicity. In addition, bacteria associated with healthy plaque usually require oxygen, whereas bacteria associated with gingivitis do not require oxygen. Among the bacteria involved in gingivitis, the lack of oxygen use of the bacteria may be due to the fact that the bacteria can perturb the gingiva to areas of low oxygen concentration and become invasive and cause inflammation. Although the bacteria present during gingivitis are usually present in small amounts in healthy subgingival flora, it can be concluded that the disruption of the bacterial equilibrium can cause gingivitis.

Findings Based on 16S rRNA sequencing

Research Team	Genera of Families Associated with Health	Genera or Families Associated with Gingivitis
Huang *et al.* (2011)	*Streptococcus*, *Veillonella*, and *Prevotella*	*Leptotrichia* and *Selenomonas*
Huang *et al.* (2014)	*Streptococcus*, *Rothia*, *Actinomyces*, *Haemophilus*, and *Lautropia*	*Leptotrichia*, *Fusobacterium,* TM7 genus*, Porphyromonas*, *Tannerella*, *Selenomonas*, *Lachnospiraceae*, unclassified *Comamonadaceae*, *Peptococcus, Aggregatibacter*, *Catonella, Treponema*, SR1 genus*, Campylobacter*, *Eubacterium*, *Peptostreptococcus*, *Bacteroidaceae, Solobacterium, Johnsonella*, *Oribacterium*, *Veillonellaceae*
Kistler *et al.*	*Actinomyces*, *Rothia*, and *Streptococcus*	*Campylobacter*, *Fusobacterium*, *Lautropia, Leptotrichia, Porphyromonas, Selenomonas,* and *Tannerella*
Nowicki *et al.*	*Streptococcus*, *Neisseria*, and *Lautropia*	*Oribacterium*, *Leptotrichia*, *Tannerella*, and *Lachnoanaerobaculum*

Table 1: Summary of the genera and families detected in the plaque of both healthy individuals and individuals with gingivitis using 16S rRNA sequencing. This data is based on the four research studies discussed thus far.

Summary of the Bacterial Genera or Families Associated with Health or Gingivitis

Bacteria Genus or Family	Association with Health	Research Team	Association with Gingivitis	Research Team	Gram Stain	Oxygen Use
Streptococcus	X	1,2,3,4			+	Facultative anaerobe
Veillonella	X	1	X	2	-	Obligate anaerobe
Prevotella	X	1			-	Obligate anaerobe
Rothia	X	2,3			+	Aerobe
Actinomyces	X	2,3			+	Facultative anaerobe
Haemophilus	X	2			-	Aerobe
Lautropia	X	2,4	X	3	-	Facultative anaerobe
Neisseria	X	4			-	Aerobe
Selenomonas			X	1,2,3	-	Anaerobe
Leptotrichia			X	1,2,3,4	-	Anaerobe
Tannerella			X	2,3,4	-	Anaerobe
Comamonadaceae			X	2	-	Aerobe
Peptococcus			X	2	+	Anaerobe
Aggregatibacter			X	2	-	Facultative anaerobe
Catonella			X	2	-	Anaerobe
Treponema			X	2	-	Anaerobe
Campylobacter			X	2,3	-	Aerobe
Eubacterium			X	2	+	Obligate anaerobe
Peptostreptococcus			X	2	+	Anaerobe
Bacteroidaceae			X	2	-	Anaerobe

Solobacterium			X	2	+	Anaerobe
Johnsonella			X	2	-	Aerobe
Oribacterium			X	2, 4	+	Obligate anaerobe
Fusobacterium			X	2, 3	-	Obligate anaerobe
Porphyromonas			X	2, 3	-	Obligate anaerobe
Lachnoanaerobaculum			X	2,3	+	Obligate anaerobe
SR1			X	2	-	Aerobe
TM7			X	2	+	Facultative anaerobe

Table 2: This table shows all the bacterial genera and some bacterial families identified through 16S rRNA sequencing during the four studies discussed thus far. In addition, it shows whether the genera are associated with health or gingivitis. The Gram stain for each genera or family is displayed, in which Gram positive bacteria are indicated by a plus sign and Gram negative bacteria are indicated by a hyphen. The oxygen use of each species is listed showing either aerobic bacteria which use oxygen, facultative anaerobes which prefer to use oxygen, but can function in the absence of oxygen, anaerobes that do not use oxygen, or obligate anaerobes that cannot function in the presence of oxygen. Study 1 refers to the research conducted by Huang *et al.* in 2011, study 2 refers to the research conducted by Huang *et al.* in 2014, study 3 refers to the research conducted by Kistler *et al.* in 2013, and study 4 refers to the research conducted by Nowicki *et al.* in 2018.

3.4 Microbial Mechanisms contributing to the onset of Gingivitis

Nowicki *et al.* was also able to use the data from the metatranscriptome sequencing to determine the virulence factors of the various bacteria contributing to gingivitis. Out of all the bacteria tested in the pool, around 8.5% of the genes were upregulated in gingivitis, whereas 8.0% of the genes were downregulated during gingivitis (Nowicki et al., 2018). The genes that were upregulated during gingivitis were associated with virulence factors and destructive pathways causing proteolysis, nucleotide degradation, hydrolysis, and nucleolytic activities

(Nowicki et al., 2018). Specifically, proteolytic and nucleolytic activities can cause the destruction of the gum tissue and may be drivers of gingival disease progression (Nowicki et al., 2018). The genes that were downregulated during gingivitis were associated with inflammation alleviation pathways (Nowicki et al., 2018). The genes that were upregulated during the healthy state were associated with various metabolic pathways such as ascorbate and aldarate metabolism, as well as the pentose phosphate pathway, pyruvate metabolism, and carbon fixation (Nowicki et al., 2018). Overall, these findings show that the bacteria associated with gingivitis are also associated with destructive pathways, whereas the bacteria associated with health are associated with metabolism and inflammation reduction pathways. Therefore, the inflammation and tissue destruction present during gingivitis may be accompanied by the bacteria exhibiting these negative virulence factors, ultimately contributing to the progression of the disease.

Section 4: Periodontitis

4.1 Introduction to Periodontitis:

Periodontitis is an inflammatory disease that may occur if gingivitis is left untreated for an extended period of time. Periodontitis is characterized by the loss of alveolar bone and gum tissue, which can cause deep periodontal pockets that harbor bacteria (Abusleme et al., 2013). The loss of gum tissue and alveolar bone cause the roots of a tooth to detach and fall out. Periodontitis can be diagnosed by measuring the level of the tooth root attachment to periodontal tissue and alveolar bone (Abusleme et al., 2013). BoP techniques can also be used to determine the severity of periodontal inflammation and the depth of periodontal pockets (Abusleme et al., 2013). During periodontitis, the bacteria present in periodontal pockets may enter the bloodstream and lead to a phenomena called bacteremia (Lee et al., 2020). Bacteremia can lead

to other systemic diseases such as heart disease, diabetes, Alzehimer's disease, and arthritis (Lundmark et al., 2019).

There are some similarities and differences between gingivitis and periodontitis. Similar to gingivitis, periodontitis also has a polymicrobial etiology, which means that it is caused by bacteria (Griffen et al., 2012). However, it is dissimilar from gingivitis because it causes irreversible damage that can negatively affect dental health. The main differentiation between gingivitis and periodontitis is whether bone resorption or tooth loss is present. There are also a few periodontitis-specific biomarkers.

The specific pathogenesis of periodontitis is not fully understood due to limitations in culture colonization, yet researchers have started to gain a general understanding of how the disease is caused over time using advanced sequencing techniques such as 16S rRNA sequencing and 454-pyrosequencing. Many researchers agree that periodontitis is caused by the dysbiosis of the oral microbiome. In addition, periodontitis is generally accompanied by an increase in microbial diversity in the oral microbiome (Abusleme et al., 2013). This phenomena is dissimilar to many bacterial related mucosal diseases because they are usually caused by a decrease in microbial diversity when one bacterial species overwhelms the microbiome (Abusleme et al., 2013). In order to understand the cause of periodontitis, many researchers have tried to pinpoint the bacteria present in periodontitis and discern the mechanisms by which these bacteria may cause disease symptoms. This section aims to discuss the various microbial causes of periodontitis and mechanisms in which these microbes contribute to the onset of this disease.

4.2: Research and Bacteria Associated with Periodontitis

In 1998, Socransky *et al.* conducted preliminary research on the causes of periodontitis by using checkerboard DNA-DNA hybridization techniques to determine the bacterial species

present in health and periodontitis. At the time, it was evident that many species that cause dental plaque have mutualistic relationships with each other. Therefore, the research team was interested in discovering the different mutualistic species complexes that may be present during periodontitis and health (Socransky, Haffajee, Cugini, Smith, & Kent, 1998). The research study consisted of 185 different subjects in which 160 subjects had periodontitis and 25 subjects were considered healthy (Socransky et al., 1998). Six different sites were tested for each patient in order to sequence the bacteria found at each site (Socransky et al., 1998). Five different clusters of bacterial species were identified and randomly named based on colors (Socransky et al., 1998). The purple and yellow complexes were associated with health, whereas the red and orange complexes were associated with periodontitis (Pérez-Chaparro et al., 2014). Among the complexes found, the red complex was most frequently detected in periodontal pockets. The species that make up the red complex include *Porphyromonas gingivalis*, *Treponema denticola*, and *Bacteriodetes forsythus* (which has now been renamed *Tannerella forsythia*) (Socransky et al., 1998).

Among the bacteria of the red complex, *P. gingivalis* was never found without the presence of *T. forsythia* (Socransky et al., 1998). In addition, *P. gingivalis* was often found in deep periodontal pockets, greater than 4mm deep (Socransky et al., 1998). These two bacteria also have strong associations with *T. denticola*, which was found in both deep periodontal pockets and the surface layers of the gumline (Socransky et al., 1998). The strong associations among the bacteria in these pockets suggest that the periodontitis-causing bacteria may work together as commensals in order to cause inflammatory effects (Socransky et al., 1998). Socrasky *et al.* also found more bacterial species in deeper pockets, suggesting that periodontitis can worsen with an increase in bacterial richness. Many of the bacteria involved in periodontitis

produce proteolytic enzymes, which could explain how tissue lesions and bone resorption occur (Socransky et al., 1998). In general, this study shows the initial research efforts that linked the species of the red complex to periodontitis. In addition, this article shows that the bacteria found in subgingival plaque may operate as commensals and cause more bacterial aggregation, further worsening the disease.

In 2012, Griffen *et al.* revisited the relationship between the oral microbiome and its role in causing periodontitis. Griffen *et al.* used 16S rRNA sequencing as well as 454-pyrosequencing in order to determine the types of bacteria that may cause periodontitis. During this study, the oral microbiome of 29 healthy patients and 29 patients with periodontitis were assessed. Based on previous research, the team knew that microbial community diversity is higher in diseased patients in comparison to healthy patients. However, Griffen *et al.* wanted to know the types of bacteria that caused these differences in diversity and microbial load. Based on the results, Griffen *et al.*, was able to confirm the presence of the bacteria in the red complex as well as expand upon the list of bacteria present in periodontitis in general. The results indicated that *P. gingivalis* and *T. denticola* were two of the three main contributors to periodontitis, while a newly discovered bacteria, *Filifactor alocis*, was found to be the third main contributor. Associations among *T. forsythia* and periodontitis were also detected, but they were detected in lower amounts in comparison to *F. alcosis*.

Griffen *et al.* discovered associations between health and the phyla *Proteobacteria* and *Bacilli.* In contrast, the bacterial species belonging to the phyla *Spirochetes*, *Syngergistes*, and *Bacteroidetes* were often associated with periodontitis. Using the phyla and species detected, Griffen *et al.* concluded that there was a general shift from Gram positive bacteria in healthy patients to Gram negative bacteria in patients with periodontitis. However, there was only a

small increase in Gram negative bacteria in periodontitis, which may have been caused by a decrease in the proportion of *Streptococcus* species available. In general, the bacteria associated with health were not lost during periodontitis, but they were present in lower proportions. Therefore, Griffen *et al.* found an increase in species diversity and richness in periodontitis in comparison to health. In addition, there was a positive correlation between diversity and the depth of periodontal pockets, showing that more species existed as periodontal pockets became deeper. Many of the disease causing bacteria were found in deep pockets, but some of the disease causing bacteria were also found in shallow layers along the gumline. This shows that the disease causing bacteria were not completely dependent on varying levels of oxygen concentration.

At the same time that Griffen *et al.* researched the relationship between the human microbiome and periodontitis, Absuleme *et al.* also examined this topic using 16S rRNA sequencing and 454-pyrosequencing. During this study, the oral microbiome of 22 subjects with periodontitis and 10 healthy individuals was assessed at two different sampling sites (Abusleme et al., 2013). The researchers concluded that periodontitis was caused by a shift in the composition of the microbes present in subgingival plaque (Abusleme et al., 2013). The results of the study suggest that the main contributors to periodontitis belonged to the phyla *Firmicutes*, *Spirochetes*, *Synergistetes*, and *Chloroflexi* (Abusleme et al., 2013). In addition, the main OTU's associated with periodontitis belong to the genus *Treponema* (Abusleme et al., 2013). Absuleme *et al.* also found associations among the bacteria of the red complex with periodontitis, but periodontitis biomarkers are not limited to these bacteria. Furthermore, the results suggested that *Actinobacteria* was the main phylum associated with health (Abusleme et al., 2013). In addition, the researchers found two genera to be present in healthy individuals including *Rothia* and *Actinomyces* (Abusleme et al., 2013). The bacterial species belonging to these two genera were

still present in lower proportions in diseased patients (Abusleme et al., 2013). In general, the research team discovered that over 32 genera were associated with an increase in microbial load during periodontitis and only 4 genera increased in healthy individuals (Abusleme et al., 2013). This result suggests that the increase in microbial diversity can contribute to the onset of periodontitis due to the dysbiosis of the oral microbiome (Abusleme et al., 2013).

After analyzing the results, Absuleme *et al.* concluded that the onset of periodontitis positively correlated with an increase in bacterial diversity among subgingival communities. The results also showed that there was no significant difference in OTUs in periodontitis patients at bleeding and non-bleeding sites (Abusleme et al., 2013). This suggests that the diversity of bacteria in patients with periodontitis is generally higher throughout the entirety of the oral microbiome and exists beyond inflamed sights. This information is crucial because it suggests that periodontitis is caused by dysbiosis in the oral microbiome at both inflamed and healthy sites.

4.3 Summary of the Bacteria involved in Periodontitis:

Based on the studies conducted by Socransky *et al.*, Griffen *et al.*, and Abusleme *et al.*, some conclusions may be drawn on the types of bacteria that cause periodontitis. Firstly, all three researchers agreed that periodontitis was accompanied by an increase in bacterial diversity and species richness. Therefore, it is safe to assume that the dysbiosis of the oral microbiome and increase in microbial diversity facilitates the onset of periodontitis. In addition, all of the researchers concluded that the three species involved in the red complex, including *P. gingivalis*, *T. denticola*, and *T. forsythia* are associated with periodontitis. However, both Griffen *et al.* and Abusleme *et al.* mentioned that these bacteria are not the only species indicative of periodontitis and other bacterial species may have stronger associations with periodontitis.

In order to further understand the bacteria that contribute to periodontitis, it is important to consider the phyla and genera mentioned by both Griffen *et al.* and Abusleme *et al.*. Both research teams used 16S rRNA and 454-pyrosequencing in order to determine the bacteria present in the oral microbiome of both diseased and healthy patients. A summary of the results found by each research team are displayed on table 3. Based on table 3, it is clear that both research teams found associations between the genus *Treponema* and periodontitis. In addition, both teams discovered associations between the phyla *Spirochetes* and *Synergistes* with periodontitis. Since these associations were determined by both research teams, they are likely indicative of periodontitis. The main discrepancy between the two researchers is that they found differences in the phyla associated with health. This discrepancy may be due to the geographic location of each study. The Griffen *et al.* study took place in the USA, whereas the Abusleme *et al.* study took place in Chile (Abusleme et al., 2013). Since the oral microbiome is highly individual and varies among populations, the geographical differences among the two studies could contribute to some of the differences in the phyla and genera detected.

Table 4 shows the various genera studied by each research team and whether these genera contribute to periodontitis, their oxygen use, and their Gram stain status. All the bacteria associated with health were Gram positive facultative anaerobes, whereas all the bacteria associated with disease were Gram negative anaerobes or obligate anaerobes. This result is likely due to the fact that Gram negative bacteria may be more resistant to environmental changes and stress due to their outer membrane. This resistance to changing environmental conditions may allow them to over accumulate leading to pathogenicity. In addition, the results suggest that the onset of periodontitis is accompanied by a shift in bacteria from facultative anaerobes to anaerobes. It is likely that the low oxygen use of the bacteria in periodontitis may allude to the

invasive nature of the bacteria which inhabit deep periodontal pockets where oxygen concentration is limited.

The Findings of Griffen *et al.* and Absuleme *et al.*

Researcher	Phyla Associated with Health	Phyla Associated with Disease	Genera Associated with Health	Genera Associated with Disease	Species Associated with Disease
Griffen *et al.*	*Proteobacteria*	*Spirochetes* *Synergistes* *Bacteroidetes*	*Streptococcus*	*Prevotella,* *Treptonoma* *Fusobacterium*	*P. gingivalis* *T. denticola* *F. alcosis* *T. forsythia*
Abusleme *et al.*	*Actinobacteria*	*Firmicutes* *Spirochetes* *Synergistes* *Chloroflexi*	*Rothia* *Actinomyces*	*Treponema*	*P. gingivalis* *T. denticola* *T. forsythia*

Table 3: A summary of the findings of the specific phyla, genera, and species associated with periodontitis and health as discovered by Griffen *et al.* and Absuleme *et al*.

Summary of the Bacterial Genera Associated with Health or Periodontitis

Bacteria Genus	**Association with Health**	**Research Team**	**Association with Periodontitis**	**Research Team**	**Gram Stain**	**Oxygen Use**
Streptococcus	X	Griffen *et al.*			+	Facultative anaerobe
Rothia	X	Absuleme *et al.*			+	Facultative anaerobe
Actinomyces	X	Absuleme *et al.*			+	Facultative anaerobe
Prevotella			X	Griffen *et al.*	-	anaerobe
Treptonoma			X	Both	-	anaerobe
Fusobacterium			X	Griffen *et al.*	-	Obligate anaerobe

Table 4: This table summarizes the bacteria genera associated with health or periodontitis, their Gram stain status, and their oxygen use as found by Griffen *et al.*, Absuleme *et al*., or both.

4.4 Microbial Mechanisms contributing to the onset of Periodontitis:

Based on the various types of bacterial phyla, genera, and species that have been found to contribute to periodontitis, researchers have started to gain a better understanding of the mechanisms by which these bacteria contribute to periodontitis. Among the bacterial species involved in the red complex, *P. gingivalis* has strong associations with periodontitis and is considered one of the top contributors to the disease (Griffen et al., 2012). *P. gingivalis* is a Gram negative obligate anaerobe and a pathobiont (Mysak et al., 2014). Therefore, it is found in healthy individuals, yet it can become destructive if it is present in high amounts (Lee et al., 2020). This bacteria contributes to the onset of periodontitis by perturbing the epithelial cells and releasing a variety of pro-inflammatory virulence factors (Mysak et al., 2014). During the onset of periodontitis, periodontal pockets form which allows for bacterial colonization. When harmful bacteria aggregate in these pockets in large amounts, they can cause severe inflammation by releasing virulence factors (Mysak et al., 2014). The virulence factors released can contribute to bone resorption and tissue loss, which causes periodontitis (Mysak et al., 2014). It has been discovered that *P. gingivalis* contributes to bone resorption by releasing osteoclastogenesis factors and causes tissue destruction by releasing liposaccharides and gingipains, which act as a vehicle for antigens and active proteases (Mysak et al., 2014). The virulence factors associated with *P. gingivalis* show how the presence of the bacteria in high amounts causes symptoms of periodontitis.

In addition to *P. gingivalis*, *F. alcosis* has also been considered one of the most abundant pathogens associated with periodontitis. *F. alcosis* is a Gram positive obligate anaerobe that is found in indiscernible amounts in healthy individuals and found in large amounts in patients with periodontitis (Lee et al., 2020). The bacteria has a strong capability to evade deep periodontal

pockets in patients and worsen the symptoms of periodontitis by strongly associating with *P. gingivalis* (Lee et al., 2020). *F. alcosis* can promote the inflammatory response caused by *P. gingivalis* by downregulating the release of anti-inflammatory cytokines (Lundmark et al., 2019). Therefore, these two bacteria work together to promote inflammation, bone resorption, and tissue degradation. Although *P. gingivalis* and *F. alcosis* are only two of the main contributing factors to periodontitis, their relationship to the onset of the disease can allude to how bacteria in the oral microbiome cause this disease.

Section 5: Conclusion

Based on the information presented on gingivitis and periodontitis, a few general assumptions can be concluded for both diseases. Firstly, the research presented indicates that both gingivitis and periodontitis are accompanied by an increase in microbial diversity and richness. In general, the increase in microbial diversity is accompanied by a shift from Gram positive aerobes to Gram negative anaerobes. However, it is important to note that the Gram positive anaerobes do not decrease in microbial load during disease, but the ratio of Gram positive aerobes to Gram negative anaerobes decreases as microbial richness increases. The various bacteria associated with these diseases often produce a variety of virulence factors such as enzymes that contribute to inflammation and suppress immunity. In gingivitis, the virulence factors usually include enzymes that cause proteolysis, nucleotide degradation, hydrolysis, and nucleolytic activities. In periodontitis, the virulence factors include osteoclastogenesis factors and proteases, which can cause bone resorption and tissue damage.

The results of the studies discussed suggest that the main bacterial genera that contribute to the onset of gingivitis include *Selemonas, Leptotrichia*, and *Tanerella*. These various genera

are all Gram negative anaerobic bacteria that accompany the shift discussed above. The research discussed did not pinpoint any specific bacterial species to serve as biomarkers for gingivitis. However, research on the bacteria causing gingivitis is limited due to the fact that there is no widely accepted definition of gingivitis. In the future, a more widely accepted diagnosis of gingivitis must be created in order to conduct more research on the causes of gingivitis.

In contrast to gingivitis, there are several bacterial species that can serve as biomarkers of periodontitis including *F. alcosis* and the bacteria belonging to the red complex: *P. gingivalis*, *T. denticola*, and *T. forsythia*. These bacteria are often found in clustered complexes and work together to cause the symptoms of periodontitis. New research efforts are aiming to find more biomarkers to predict the presence of periodontitis using updated sequencing techniques. If this research is conducted successfully, the associations between the red complex and periodontitis may become outdated as more specific microbial biomarkers are discovered.

References:

Abusleme, L., Dupuy, A. K., Dutzan, N., Silva, N., Burleson, J. A., Strausbaugh, L. D., et al. (2013). The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *The ISME Journal, 7*(5), 1016-1025. doi:10.1038/ismej.2012.174

Arweiler, N. B., & Netuschil, L. (2016). The oral microbiota. *Advances in Experimental Medicine and Biology, 902*, 45-60. doi:10.1007/978-3-319-31248-4_4 [doi]

Deo, P. N., & Deshmukh, R. (2019). Oral microbiome: Unveiling the fundamentals. *Journal of Oral and Maxillofacial Pathology : JOMFP, 23*(1), 122-128. doi:10.4103/jomfp.JOMFP_304_18 [doi]

Fan, W., Liu, C., Zhang, Y., Yang, Z., Li, J., & Huang, S. (2021). Epidemiology and associated factors of gingivitis in adolescents in Guangdong province, southern china: A cross-sectional study. *BMC Oral Health, 21*(1), 311-1. doi:10.1186/s12903-021-01666-1

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., et al. (2012). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *The ISME Journal, 6*(6), 1176-1185. doi:10.1038/ismej.2011.191

Huang, S., Li, R., Zeng, X., He, T., Zhao, H., Chang, A., et al. (2014). Predictive modeling of gingivitis severity and susceptibility via oral microbiota. *The ISME Journal, 8*(9), 1768-1780. doi:10.1038/ismej.2014.32

Huang, S., Yang, F., Zeng, X., Chen, J., Li, R., Wen, T., et al. (2011). Preliminary characterization of the oral microbiota of Chinese adults with and without gingivitis. *BMC Oral Health, 11*, 33-33. doi:10.1186/1472-6831-11-33

Lee, J. S., Spooner, R., Chowdhury, N., Pandey, V., Wellslager, B., Atanasova, K. R., et al.

(2020). In situ intraepithelial localizations of opportunistic pathogens, porphyromonas gingivalis and filifactor alocis, in human gingiva. *Current Research in Microbial Sciences, 1*, 7-17. doi:10.1016/j.crmicr.2020.05.001

Lundmark, A., Hu, Y. O. O., Huss, M., Johannsen, G., Andersson, A. F., & Yucel-Lindberg, T. (2019). Identification of salivary microbiota and its association with host inflammatory mediators in periodontitis. *Frontiers in Cellular and Infection Microbiology, 9*, 216. doi:10.3389/fcimb.2019.00216

Mysak, J., Podzimek, S., Sommerova, P., Lyuya-Mi, Y., Bartova, J., Janatova, T., et al. (2014). Porphyromonas gingivalis: Major periodontopathic pathogen overview. *Journal of Immunology Research, 2014*, 476068. doi:10.1155/2014/476068

Nowicki, E. M., Shroff, R., Singleton, J. A., Renaud, D. E., Wallace, D., Drury, J., et al. (2018). Microbiota and metatranscriptome changes accompanying the onset of gingivitis. *mBio, 9*(2), e00575-18. doi:10.1128/mBio.00575-18

Pérez-Chaparro, P. J., Gonçalves, C., Figueiredo, L. C., Faveri, M., Lobão, E., Tamashiro, N., et al. (2014). Newly identified pathogens associated with periodontitis: A systematic review. *Journal of Dental Research, 93*(9), 846-858. doi:10.1177/0022034514542468

Ring, M. E. (2002). W. D. miller. the pioneer who laid the foundation for modern dental research. *The New York State Dental Journal, 68*(2), 34-37.

Robert koch biography. Retrieved 10-26-22https://www.nobelprize.org/prizes/medicine/1905/koch/biographical/

Sedghi, L., DiMassa, V., Harrington, A., Lynch, S. V., & Kapila, Y. L. (2021). The oral microbiome: Role of key organisms and complex networks in oral health and disease. *Periodontology 2000, 87*(1), 107-131. doi:10.1111/prd.12393

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., & Kent, R. L. J. (1998). Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology, 25*(2), 134-144. doi:10.1111/j.1600-051x.1998.tb02419.x

Trombelli, L., Farina, R., Silva, C. O., & Tatakis, D. N. (2018). Plaque-induced gingivitis: Case definition and diagnostic considerations. *Journal of Periodontology, 89 Suppl 1*, S46-S73. doi:10.1002/JPER.17-0576

Verma, D., Garg, P. K., & Dubey, A. K. (2018). Insights into the human oral microbiome. *Archives of Microbiology, 200*(4), 525-540. doi:10.1007/s00203-018-1505-3

Yamashita, Y., & Takeshita, T. (2017). The oral microbiome and human health. *Journal of Oral Science, 59*(2), 201-206. doi:10.2334/josnusd.16-0856 [doi]

Zaura, E., Keijser, B. J. F., Huse, S. M., & Crielaard, W. (2009). Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiology, 9*(1), 259. doi:10.1186/1471-2180-9-259