

# Gram-positive anaerobes in periodontal pathogenesis: new kids on the block? - a mini review

## Abstract

Periodontal diseases are a group of chronic inflammatory, polymicrobial infections, which result in gradual loss of tooth attachment to the bone and eventual loss of the tooth. The microbial etiology of periodontitis is defined by the sub gingival plaque biofilm in which resides an interdependent microbial community containing numerous species of bacteria. Many Gram-negative anaerobic bacilli such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, were considered as keystone pathogens and a pre-requisite for periodontal disease activity and progression. However, with the advent of open-ended molecular techniques such as 16s rRNA sequencing and cloning, organisms that were not considered pathogenic to periodontal infections are now emerging as possible contributors to the microbial etiology of periodontitis. Among these, the Gram-positive anaerobic bacteria are in the forefront. Over the past decade, various studies have showed the association of GPA with periodontal disease conditions. However, their absolute role in the same has not been clearly defined. This mini review aims at a comprehensive analysis of the literature available on the association of GPA (Gram Positive Anaerobes) with various periodontal disease conditions.

**Keywords:** chronic periodontitis, keystone pathogens, polymicrobial synergy dysbiosis, gram positive anaerobes, diabetes mellitus, cardiovascular diseases

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**Abbreviations:** PSD Model, polymicrobial synergy dysbiosis model; GPA, gram positive anaerobes; DNA, deoxyribonucleic acid

## Introduction

Periodontitis is a polymicrobial infection leading to chronic inflammation of the supporting structures of the teeth that leads to pocket formation and breakdown of alveolar bone around the teeth, resulting in tooth loss.<sup>1</sup> Besides its local impact, periodontal infections are also associated with various far-reaching systemic effects, like diabetes mellitus and cardiovascular diseases.<sup>2</sup>

Advances in molecular research and the oral microbiome<sup>3</sup> study have depicted the number of oral bacteria (cultivable and not) to a mind-boggling 700 species! The subgingival biofilm is the primary etiology of periodontal disease initiation and progression and for this reason periodontal microbiology has been the focus of research over centuries. It has gone through paradigm shifts of ideologies over the past century such as the non-specific/plaque hypotheses, the ecological plaque hypothesis and recently the PSD model. The “Non-Specific Plaque Hypothesis” proposed by Black and Miller in the late 1800s, believed that the total microflora could lead to disease. The “Specific Plaque Hypothesis”, which followed in 1976 concluded that only a few species of the total microflora are actively involved in disease taking into account differences in virulence among bacteria. The “Ecological Plaque Hypothesis” put forward by Marsh<sup>4</sup> in 1994, emphasized that periodontal disease is the result of an imbalance in the microflora by ecological stress resulting in an enrichment of certain disease-related micro-organisms. “Keystone-Pathogen Hypothesis” in 2012<sup>5</sup> proposes that certain low-abundance microbial pathogens can cause inflammatory disease by interfering with the host immune system and remodeling the microbiota.

## Current scenario of periodontal microbiology

The past few decades of periodontal microbial research have been spent primarily on certain keystone pathogens (mostly Gram-negative anaerobes), which were believed to be positively associated with periodontal disease. The currently accepted model of periodontal pathogenesis, however, believes that periodontitis is initiated by a synergistic and dysbiotic microbial community rather than by certain select keystone pathogens. Virulent pathogens such as *Porphyromonas gingivalis*, elevate the virulence of the entire biofilm community following interactive communication with accessory pathogens such as mitis group streptococci. This impairs the host immune surveillance and creates a dysbiotic environment eventually disrupting tissue homeostasis and causing destruction of periodontal tissues. This led to the currently believed model of periodontal pathogenesis, that is, polymicrobial synergy and dysbiosis or PSD model.<sup>6</sup>

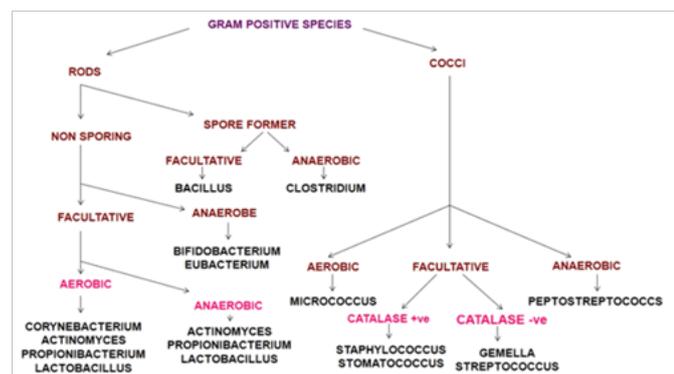
This advancement in our understanding has made been possible, due to open-ended molecular techniques such as DNA-DNA checkerboard hybridization techniques and 16s rRNA cloning and the revolutionary oral microbiome study in 2010. Newer microorganisms which were previously associated with periodontal health have now been found to be contributing to the periodontal disease process.<sup>7-11</sup>

Of these, Gram-positive anaerobes such as *Filifactor alocis*, *Peptostreptococcus micros* and *Eubacterium nodatum* which have been recently isolated from patients with periodontitis, are emerging to be considered as important contributors to the bacterial etiology of periodontitis. However, the literature evidence is controversial and inconsistent across various studies. This discrepancy could be explained by geographic variability,<sup>12</sup> or by difference in the depths of the pockets sampled,<sup>13</sup> as well as the sample size and the DNA analytic bias.<sup>14</sup>

This review article aims to search all the available literature associating GPA and periodontal disease and draw a conclusion about the validity of GPA contributing significantly to periodontal pathology.

## Gram-positive bacteria in periodontal disease

Classification system of bacteria discovered by Gram in 1884, allows a large proportion of clinically important bacteria to be classified as either Gram positive or negative. The following flow chart depicts the classification system of Gram +ve species in detail (Figure 1).



**Figure 1** Classification system of gram positive species.

The potentially pathogenic role of bacteria, which were not considered as primary keystone pathogens in periodontal disease, has been implicated in periodontal literature time and again. Paul Keyes, way back in 1970, said - "I am convinced that although many clinicians and investigators do not exclude the role of bacteria in periodontal lesions, at this point interest in microorganisms often dissipates and attention shifts to other areas".

Gram-positive anaerobes have been isolated way back in 1990s from periodontal biofilms.<sup>15</sup> Kumar et al.<sup>8</sup> also identified the dominance of Gram positive anaerobic species in periodontally diseased sites compared to healthy individuals.<sup>8</sup> In another study supporting this evidence, Haffajee et al.<sup>16</sup> examined supragingival biofilm samples to understand the nature of the microbial complexes that exist in supragingival plaque. An interesting observation was that *Eubacterium nodatum*, a Gram-positive anaerobe was found both in the mature and the long-term redevelopment biofilms along with the red complex species, *P. gingivalis*, *T. forsythia*, and *T. denticola* usually observed in subgingival plaque. Not surprisingly the same research group while studying the subgingival samples found merit in including *E. nodatum* as a part of the red complex.

## Characteristics of a few gram-positive anaerobes commonly identified in periodontal disease and evidence linking them to periodontal disease

*Eubacterium nodatum* is an obligate anaerobe, filamentous or club-shaped asaccharolytic, Gram-positive rod. They grow slowly in culture and share cultural, biochemical, or morphological characteristics with other well-known species of anaerobic bacteria. They elaborate virulence factors such as esterases, acid phosphatases and aminopeptidases and have been isolated from a significant proportion of the subgingival microbiota of chronic periodontitis ranging from 10.8 to 54%.<sup>17</sup> Three species, *E. nodatum*, *Eubacterium timidum*, and *Eubacterium brachy*, have been described, primarily

from subgingival samples taken from patients with moderate and severe periodontitis.<sup>18</sup>

*Parvimonas micra* is a species of the orange microbial complex put forward by Socransky et al.<sup>13</sup> The presence of *P. micra* has been positively associated with periodontitis over the past 2 decades. Previously known as *Peptostreptococcus micros*, this Gram-positive, micro-*aerophilicococcus* is usually associated with polymicrobial infections such as intracranial abscesses, sinus infections, and periodontitis. *Peptostreptococcus sp.* are colonisers of the oral cavity, vagina, skin, GI tract and urinary tract. They are also found to cause systemic infections such as abscesses, necrotizing tissue infections, and infections of GIT and urinary tract in immunocompromised individuals. *P. micra* possesses several virulence factors that contribute to its pathogenic potential. The cell wall of *P. micra* has been shown to induce a potent inflammatory response in macrophages.<sup>19,20</sup> They elaborate proteases that enable it to penetrate the basement membrane.<sup>21</sup> It also makes a carbohydrate-mediated co-aggregation with *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.<sup>19</sup> These data suggest that *peptostreptococci* may play a role in preventing wound healing in chronic disease and may be important in the physical structure of a disease-associated biofilm.

*Filifactor alocis* is a fastidious, Gram-positive, obligatory anaerobic rod possessing trypsin-like enzymatic activity similar to *P. gingivalis* and *T. denticola*. It has the ability to survive in the periodontal pocket and shares common virulence properties with *Fusobacterium*. *Filifactor alocis* (ATCC 35896T) was first isolated in 1985 from the human gingival crevice as *Fusobacterium alocis* and later reclassified as *Filifactor alocis*. Oxidative stress resistance-Sialidase activity exhibited by *F. alocis* results in release of sialic acids that scavenge oxidative stress in the periodontal pocket.<sup>22</sup> The fastidious nature of this organism has contributed to its low detection in culture-based methods. The organism is associated to cause endodontic infection and periodontal destruction. This organism has been found in elevated numbers in Aggressive Periodontitis (77.8%) and Chronic Periodontitis (76.7%) compared with periodontally healthy individuals due to its potential to withstand oxidative stress and inflammatory microenvironment provided by periodontal pocket.<sup>17</sup>

A study by Dahlen & Leonhardt<sup>18</sup> concluded that *F. alocis* should be added to the 12 species used for routine diagnostics of periodontitis-associated bacterial flora. This is one of the marker organisms and is considered an important periodontal pathogen. The organism is now identified to be significant to the pathogenic structure of biofilms associated with periodontal inflammation.<sup>23-25</sup> In comparison with the other traditional periodontal pathogens, the high incidence of *F. alocis* in the periodontal pocket compared with its absence in healthy individuals or those who are periodontitis-resistant has highlighted its importance in the infectious disease process.<sup>17,23,24</sup> *Streptococcus sanguinis* are Gram-positive cocci are non-motile and non-spore forming Pili of *S. sanguinis* bind to salivary  $\alpha$ -amylase, and contribute to the formation of biofilm on saliva-coated surfaces. They initiate aggregation of other oral bacteria and maturation of dental plaque. Sortase A (SrtA) of *S. sanguinis* have an influence on the expression of various cell surface virulence factors.<sup>26</sup>

*E. Streptococcus parasanguinis* are Gram-positive, non-motile, non-spore forming cocci. They are facultative anaerobes. The long peritrichous fimbriae of *S. parasanguinis* are critical for the formation of biofilms on solid surfaces.<sup>27</sup> It is one of the major early colonizers of dental surfaces in the human oral cavity. Fim A protein is a potential virulence factor (Table 1).

**Table 1** A description of the various studies associating Gram-positive anaerobes and periodontal disease is elaborated

| Author  | Technique/method of bacterial identification         | Organism  | Observations  |
|---|--|---|---|
| Chi-Ying Tsai et al. <sup>28</sup>                  | 16S rRNA metagenomic approach and (qPCR)             | <i>Peptostreptococcus Filifactor alocis</i>   | Six genera, including Porphyromonas, Treponema, Tannerella, <i>Aggregatibacter</i> , <i>Peptostreptococcus</i> , and <i>Filifactor</i> , were significantly enriched in the diseased group  |
| Feres et al. <sup>29</sup>                          | Checker-board DNA DNA hybridisation                  | <i>Eubacterium nodatum</i> and <i>Parvimonas micra</i>  | Studied subgingival recolonisation in smokers before and after treatment. Two species from the orange complex ( <i>Eubacterium nodatum</i> and <i>Parvimonas micra</i> ) showed reduction at day 0 and at 63 days post- therapy (p<0.05).   |
| Anna Paula Vierra Colombo et al. <sup>30</sup>      | Checkerboard DNA-DNA hybridization                   | <i>Peptostreptococcus anaerobius</i>  | <i>Peptostreptococcus anaerobius</i> were prevalent in the CP group in the highest mean counts (>4x 10 <sup>5</sup> ). It was also detected in higher counts in areas where PD/CAL were greater than or equal to 4mm.   |
| Matthew Mason et al. <sup>31</sup>                  | 16S pyrotag sequencing                               | <i>F. alocis</i>  | High levels of <i>F. alocis</i> in periodontally healthy smokers.   |
| Al Hebshi et al. <sup>32</sup>                      | Taqman q-PCR assay                                   | <i>Parvimonas micra</i>   | <i>P. micra</i> showed the strongest association with the disease being present at significantly higher absolute and relative counts in periodontitis sites in all study subjects.  |
| Moon et al. <sup>33</sup>                           | 16S rRNA gene-based pyrosequencing                   | <i>Filifactor alocis</i>  | Among species-level taxa occupying > 1% of whole subgingival microbiome of smokers, higher abundance (≥ 2.0-fold compared to non-smokers) of seven species or operational taxonomic units (OTUs) was found: <i>Fusobacterium nucleatum</i> , <i>Neisseria sicca</i> , <i>Neisseria oralis</i> , <i>Corynebacterium matruchotii</i> , <i>Veillonella dispar</i> , <i>Filifactor alocis</i> , and <i>Fretibacterium AY349371</i>                  |
| Talita Gomes Baeta Lourenco et al. <sup>34</sup>    | Human Oral Microbe Identification Microarray (HOMIM) | <i>Peptostreptococcaceae</i> sp   | Presence of <i>Aggregatibacter actinomycetemcomitans</i> , <i>Cardiobacterium hominis</i> , <i>Peptostreptococcaceae</i> sp., <i>P. alactolyticus</i> were associated with aggressive periodontitis.  |
| Pérez-Chaparro <sup>35</sup><br>A systematic review |  | <i>Filifactor alocis</i>  | Four microorganisms of the 17 taxa included in the moderate evidence category are not-yet-cultivable, and 13 have been cultivated before. Five of the cultivable species are Gram positive ( <i>Eubacterium saphenum</i> , <i>Mogibacterium timidum</i> , <i>Peptostreptococcus stomatis</i> , <i>Filifactor alocis</i> and <i>Enterococcus faecalis</i> ), characteristics of most of the microorganisms involved in polymicrobial infections. |
| Fine and co-workers <sup>36</sup>                   | HOMIM  | <i>P. micra</i> , <i>F. alocis</i> , <i>Peptostreptococcus</i> sp., <i>Streptococcus parasanguinis</i>      | <i>A. actinomycetemcomitans</i> positive adolescents who presented bone loss had also high prevalence of <i>P. micra</i> , <i>F. alocis</i> , and <i>Peptostreptococcus</i> sp. At vulnerable sites, <i>A. actinomycetemcomitans</i> , <i>Streptococcus parasanguinis</i> , and <i>F. alocis</i> levels were elevated prior to bone loss.   |
| Wilson Aruni et al. <sup>37</sup>                   | 16s rRNA gene sequencing                             | <i>F. alocis</i>  | During the invasion of HeLa cells, there was increased expression of several of the genes encoding these proteins in the potentially more virulent <i>F. alocis</i> D-62D compared to <i>F. alocis</i> ATCC 35896, the type strain.   |
| Ann Griffen et al. <sup>38</sup>                    | 16S rRNA sequencing                                  | <i>Filifactor alocis</i>  | <i>Filifactor alocis</i> and many Spirochetes were represented by a large fraction of sequences as compared with previously identified targets.   |
| Ana Paula Colombo et al. <sup>39</sup>              | Human Oral Microbe Identification Microarray (HOMIM) | <i>Filifactor alocis</i> , <i>Eubacterium</i> spp, <i>Parvimonas micra</i> , <i>Peptostreptococcus</i> spp. | Most species/cluster decreased significantly in prevalence after treatment (p<0.05, Chi-square test), <i>Filifactor alocis</i> , <i>Eubacterium</i> spp., <i>Parvimonas micra</i> , <i>Peptostreptococcus</i> spp.,   |
| Shaddox et al. <sup>40</sup>                        | 16S rRNA-based microarrays                           | <i>P. micra</i> , <i>F. alocis</i>  | In addition to <i>A. actinomycetemcomitans</i> , the species <i>P. micra</i> , <i>F. alocis</i> , were more prevalent in localized AgP than in healthy children.  |
| Catherine Moffatt et al. <sup>41</sup>              | Routine culture                                      | <i>F. alocis</i>  | <i>F. alocis</i> has characteristics in common with established periodontal pathogens and has the potential to contribute to periodontal tissue destruction.  |
| Wilson Aruni et al. <sup>42</sup>                   | 16s rRNA gene sequencing                             | <i>F. alocis</i>  | <i>F. alocis</i> has virulence properties that may enhance its ability to survive and persist in the periodontal pocket and may play an important role in infection-induced periodontal disease.  |

Table Continued...

| Author                                  | Technique/method of bacterial identification   | Organism   | Observations   |
|---|--|--|--|
| Abusleme et al. <sup>43</sup>           | 454-pyrosequencing of 16S rRNA gene libraries and q-PCR                              | <i>Eubacterium</i> species                         | Increase of <i>Eubacterium</i> species was associated with periodontal destruction along with a shift in composition of the subgingival microbial communities.   |
| Sebastian Schlafer et al. <sup>45</sup> | PCR and dot blot hybridization   | <i>F. alocis</i>                                   | While the majority of patients suffering from GAP or CP harboured <i>F. alocis</i> , it was rarely detected in the control group.  |
| Urban et al. <sup>44</sup>              | Culture and commercial PCR based hybridization methods                               | <i>Parvimonas micra</i>                            | <i>Parvimonas micra</i> was cultured and identified only in 38% of the samples and only in 14% of the specimens were found in high number (105 CFU/ml). Fewer than 20% of the samples contained in detectable numbers are <i>E. nodatum</i> .  |
| Shchipkova et al. <sup>45</sup>         | 16s cloning and sequencing   | <i>Parvimonas micra</i>                            | <i>Parvimonas micra</i> formed a large fraction of the subgingival microbial community in smokers  |
| Haffajee et al. <sup>46</sup>           | Checkerboard DNA-DNA hybridization   | <i>Eubacterium nodatum</i>                         | For the 824 subjects the consensus pathogens <i>P. gingivalis</i> and <i>T. forsythia</i> as well as <i>Eubacterium nodatum</i> and <i>Treponema denticola</i> had significantly higher mean counts, proportions and percentage of sites colonized in samples from subjects with periodontitis than from periodontally healthy subjects. |
| Kumar et al. <sup>24</sup>              | Ribosomal 16S cloning and sequencing   | <i>F. alocis</i>                                   | <i>F. alocis</i> accounted for 1.5% of all clones, and higher levels of <i>F. alocis</i> were seen in the group whose periodontal health worsened. <i>Peptostreptococci</i> did not show any statistically significant association with disease in this study  |
| Kumar et al. <sup>24</sup>              | Ribosomal 16S cloning and sequencing   | <i>Peptostreptococcus</i> Filifactor <i>alocis</i> | Several genera, many of them uncultivated, were associated with periodontitis, the most numerous of which were Gram positive, including <i>Peptostreptococcus</i> and Filifactor   |
| Booth et al. <sup>47</sup>              | Oligonucleotide probe for <i>E. nodatum</i> detected by chemiluminescent method.     | <i>E. nodatum</i>                                  | <i>E. nodatum</i> and <i>S. exigua</i> were associated with clinical indicators of periodontal disease   |
| Kumar et al. <sup>23</sup>              | PCR amplification of the 16S rDNA and the downstream intergenic spacer region (ISR). | <i>Peptostreptococcus micros</i>                   | <i>Peptostreptococcus micros</i> commonly found in subjects with chronic periodontitis than in healthy subjects, $P < 0.05$ . <i>Eubacterium saphenum</i> , Filifactor <i>alocis</i> were detected ( $P < 0.002$ ).  |
| Van Winkelhoff et al. <sup>48</sup>     | Culture  | <i>P. micra</i> and <i>Fusobacterium nucleatum</i> | The mean percentage of total count of <i>P. micra</i> and <i>Fusobacterium nucleatum</i> were higher in both untreated and treated smokers.  |
| Tanner et al. <sup>49</sup>             |  | Filifactor <i>alocis</i>                           | Filifactor <i>alocis</i> has been seen more commonly in sites with periodontitis than in healthy sites   |
| Rams et al. <sup>15</sup>               |  | <i>P. micros</i>                                   | In a cross-sectional study involving 907 people reported prevalence of <i>P. micros</i> in 58-63% of periodontitis subjects. In culture-positive patients, <i>P. micros</i> averaged 12-15% of total viable counts and it was concluded to be potential pathogen in adult periodontitis.   |

## Conclusion

As evident from the above literature review, a number of Gram-positive anaerobes such as Filifactor *alocis*, *Parvimonas micra*, *Eubacterium nodatum* and *Streptococcus parasanguinis* are now considered as potential periodontal pathogens.<sup>50-52</sup> But these findings are not consistent across all studies. The possible contributory factors to this variability are study design, the population studied and the methods of detection of microorganisms. Also, we need to keep in mind while interpreting the results of association studies, the “causal versus casual” concept. Analysis of the associations based on the Hill’s criteria of causality can give us an insight into this aspect.

The paradigm shift in the understanding of periodontal pathogenesis is attributed to the introduction of novel theories about

the ecological events associated with periodontal destruction. The Polymicrobial Synergy and Dysbiosis model makes us question the individual role of these Gram-positive anaerobes implicated in periodontal pathogenesis.<sup>53-55</sup> This has to be confirmed by future studies. Further, whether they fulfill all of Koch’s postulates in being an infectious organism is yet to be studied.

This mini review clearly highlights that the etiology of periodontitis is more complex than a previous model associating Gram-positive bacteria with health and implicating Gram-negative bacteria as the causative agents in disease. Whether Gram Positive Anaerobes contribute independently to the periodontal pathogenesis is to be decided by further uniformly designed studies and future systematic reviews.<sup>56-59</sup>

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## Conflict of interest

The author declares no conflict of interest.

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