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Overview of Gram-Negative Anaerobic Pathogens Associated with Periodontal Disease: A Review

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Abstract
Periodontal disease is a polymicrobial disease initiated by the presence and propagation of bacterial infections in dental biofilm. Gram-negative anaerobic bacteria were reported as the most prevalent oral bacteria associated with periodontitis. The periodontopathogens may destroy the periodontal tissue of the host directly or indirectly via inflammatory reaction. *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum, Tanerella forsythia*, *Prevotella intermedia*, *Treponema denticola* and *Campylobacter rectus* are Gram-negative anaerobic bacteria which are highly associated with periodontitis. This group of pathogens plays a vital role in the onset and subsequent development of periodontal disease. This review article highlights the general overview of Gram-negative anaerobic pathogens associated with periodontitis.

Keywords: Gram-Negative, Anaerobic Pathogens, Periodontal Disease, Periodontitis

Introduction

Periodontal disease is a significant oral health problem characterised by the inflammation of the gum (gingivitis) and the destruction of tooth-supporting tissues (periodontitis). The disease affects approximately 20-50% of the global population [1]. Recently, the National Oral Health Survey of Adults (NOHSA) 2020, conducted in Malaysia, showed that 95.4% of dentate adults presented with unhealthy periodontium in which 38.2 % had periodontitis [2]. The primary cause of periodontal disease is bacterial growth in dental biofilm, with multifactorial risk factors contributing to the disease such as microbial component, physiological, genetic and behaviour [3]. However, several studies found that the combination of specific bacteria species overgrowth such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and *Fusobacterium nucleatum*, and the susceptible host like genetic predisposition, immune system status, smoking, hormonal fluctuations, poor dental hygiene, stress, and systemic disorders were the most significant factors in the initiation of periodontal disease [4-6]. Furthermore, the interaction between oral bacteria in dental biofilm and the host's immune response is also an important factor in determining the progression and clinical manifestations of periodontal disease.

In normal conditions, bacteria are natural members of the oral microbiome. However, the increased accumulation of dental biofilm can be directly associated with increased severity and prevalence of periodontal disease [1]. Studies have reported that most periodontal pathogens, as shown in Figure 1, are anaerobic bacteria that develop biofilm in periodontal pockets. However, it has been shown that facultative aerobes, capnophiles and microaerophilic bacteria can also be isolated [7, 8]. It is known that the Gram-negative anaerobic bacteria in the subgingival plaque biofilm play a major role in the development of periodontitis [9, 10]. Moreover, these organisms become more pathogenic compared to Gram-positive bacteria in periodontal disease due to the ability to colonise deeper areas of the periodontium and trigger the release of inflammatory mediators [7]. On the other hand, Gram-negative anaerobic bacteria can produce endotoxins, which directly contribute to the occurrence or progression of systemic disease [10, 11].

Studies have reported that the main anaerobic pathogens causing periodontitis are *Porphyromonas gingivalis*, *Prevotella intermedia, Aggregatibacter actinomycetemcomitans*, *Tanerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, and *Campylobacter rectus* [7, 12]. These Gram-negative anaerobic bacteria possess different types of bacterial characteristics, morphology, and virulence factors which can affect host tissues as shown in Table 1. Additionally, several review articles on periodontal pathogenic bacteria have been published. However, most of these review articles focus on specific Gram-negative anaerobic bacteria and their particular roles and functions [13-15]. Therefore, this presents a greater challenge for readers, particularly if they lack fundamental knowledge of oral bacteria, which is required for comprehension. The purpose of this review is to provide an overview of the Gram-negative anaerobic bacteria associated with periodontitis, summarising their profiles and effects on the host for easy reference by researchers studying this field.

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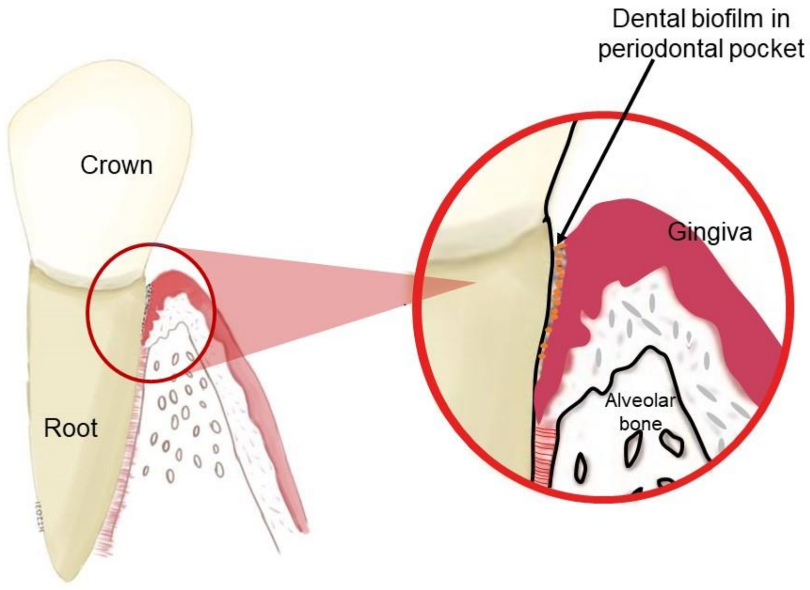

Figure 1: The illustration of periodontal tissues showing dental biofilm in the periodontal pocket

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Table1: Main characteristic of Gram-negative anaerobic periodontopathogens involve in periodontitis

Name of bacteria	Bacteria characteristic	Morphology on agar	Virulence factors	Effect on host
P. gingivalis	Gram-negative anaerobic rod [16]	Small opaque round in morphology and showed convex colonies after 48 hours of incubation. black pigmentation on blood agar after 7 to 10 days of incubation [17]	Lipopolysaccharide Gingipains Pili [18]	Causing perturbation of epithelial host cells and stimulating the inflammatory and immune process which leads to the destruction of the tooth-supporting tissue [19].
A. actinomycetemcomitans	Gram-negative facultative anaerobic spherical, oval or rod [20, 21]	Small colonies with a rough texture and strongly adhere to the agar surface. The diameter of colonies with ≤0.5 mm after 24 hours and possibly exceed 2 mm after 48 hours on chocolate agar [20].	Leukotoxin Bacteriocin Chemotaxis inhibiting Fc binding protein Lipopolysaccharide Collagenase Antibiotic resistant determinants [20, 22]	Virulence factors of this bacterium will interact with host cells which initiate inflammatory response in the gingival tissue [23].
P. intermedia	Gram-negative, obligate anaerobic, nonmotile, short and round-ended [7, 24]	Dark pigmented colony morphology on Brucella blood agar [25]	Lipopolysaccharide Protease Interpain A [26, 27]	Lipopolysaccharide promoted the production of TNF-α from the THP-1 cell of a human monocytic host cell line which led to osseous destruction [26].
T. forsythia	Gram-negative anaerobic rod [28, 29]	Colony morphology presence with circular, slightly convex and pale speckled-pink on blood agar plate [30]	S-layer oligosaccharideSerpin protein [29, 31]	S-layer facilitates adhesion and invasion to gingival epithelial host cells with suppression of pro-inflammatory cytokine and also inhibits monospecies biofilm formation [32].

T. denticola	Gram-negative anaerobic spirochete [33]	Colonies were small, dense and pinpoint-shaped on agar plate [34].	Outer-sheath-associated peptidases Chymotrypsin-like and trypsin-like proteinases Haemolytic and hemagglutinating activities, Adhesins that bind to matrix proteins and cells, Outer-sheath protein [35]	Rapid motility and chemotaxis aided this bacterium in colonising new sites, penetrating epithelial layers and entering into the deep periodontal pockets [36].
C. rectus	Gram-negative motile bacteria with a small, straight rod and possesses a single polar flagellum [37]	Appear as a convex-shaped and spread or corrode on blood and chocolate agar plates after 7 days of incubation in anaerobic conditions [37, 38]	S-layer protein Lipopolysaccharide Toxin *ciaB* gene [39, 40]	S layer and toxin facilitate bacterial invasion and adhesion of the epithelium pocket wall [39].
F. nucleatum	Obligate anaerobic Gram-negative bacterium with unique spindle shape with tapered ends [41]	Colonies appeared with white, speckled or crumb-like on agar [41]	Proteolytic FadA Protein [7]	*F. nucleatum* able to adhesion and invasion of the host cell [42].

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Porphyromonas gingivalis

Porphyromonas gingivalis (*P. gingivalis*) is a Gram-negative anaerobic rod and non-motile bacteria belonging to the phylum Bacteroidetes [16]. When cultured on agar plates, this bacterium appeared small, opaque, and round in morphology and showed convex colonies after 48 hours of incubation. After 7 to 10 days of incubation, black pigmentation can be observed on the blood agar due to the ability of the bacteria hemagglutinin to lyse the blood, which causes it to clump together [17]. A previous study found that this bacterium was able to metabolize carbohydrates for their energy source only in the presence of carbon. Some studies reported that *P. gingivalis* can survive in the presence of less than 6% O_2 [43]. The ability of *P. gingivalis* to withstand low levels of O_2 is due to altering gene expression. It upregulates genes involved in protecting against oxidative stress, such as alkyl hydroperoxide reductase, superoxide dismutase, and thiol peroxidase. It also enhances formate metabolism while downregulating the expression of genes involved in lactate consumption and protease activity. Furthermore, it regulates the transportation of metals by downregulating genes responsible for iron intake and upregulating genes involved in manganese transport. These modifications facilitate the survival of *P. gingivalis* in low oxygen conditions [43]. The main surface structures of this bacterium were fimbriae, capsules, lipopolysaccharides, and peptidoglycan, as illustrated in Figure 2(a). Each component has a specific function, mainly during pathogenesis, defence mechanisms and the production of virulence factors. It has been shown that lipopolysaccharide, gingipains and pili/fimbria were the important pathogenic factors of *P. gingivalis* in the determination of periodontitis progression [18, 44]. *P. gingivalis*, together with *T. forsythia* and *T. denticola* were classified as red complex bacteria group which are highly associated with the pathogenesis and severity of periodontitis and is one of the most influential aetiological agents for periodontitis [17, 19, 45].

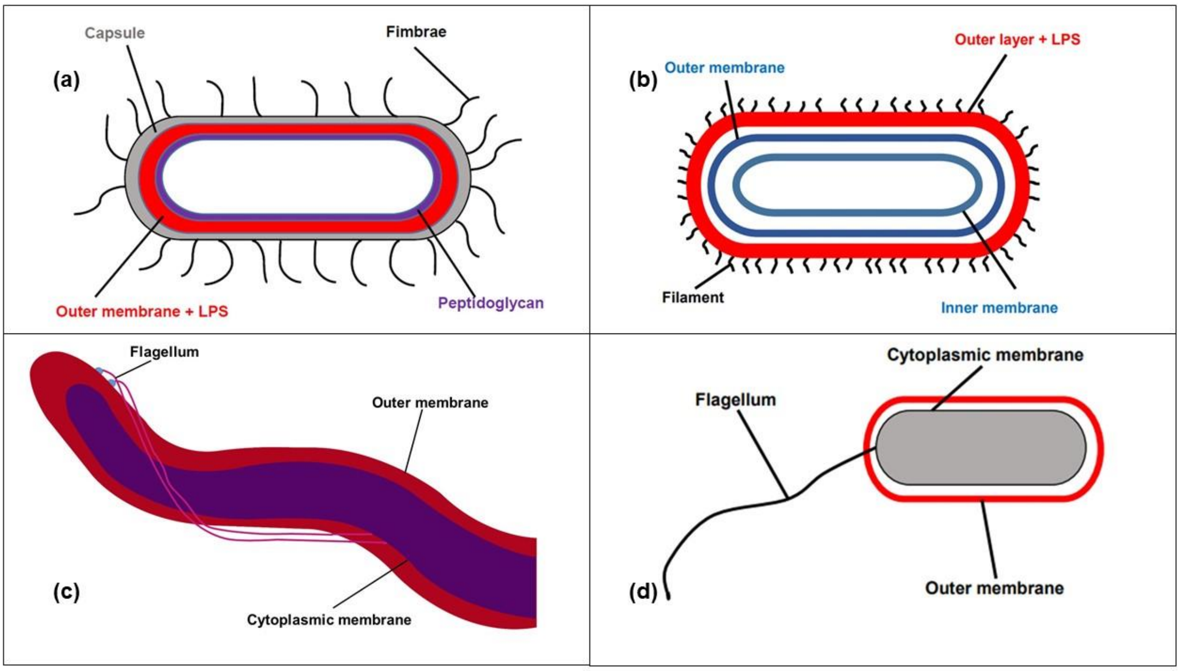

Figure 2: Schematic diagram of Gram-negative anaerobic pathogens, (a) *P. gingivalis*, (b) *T. forsythia,*(c) *T. denticola* and (d) *C. rectus s*urface structures with flagella.

Aggregatibacter actinomycetemcomitans

Aggregatibacter actinomcetemocomintas (*A. actinomycetemcomitans*) is a Gram-negative facultative anaerobic spherical, oval or rod-shape, nonmotile bacterium that is part of the family *Pasteurellaceae* and grows in blood and chocolate agar [20, 21]. It is a small, rounded and irregular edge with sticky colonies present on the surface of the agar plate [20]. The basic structures of *A actinomycetemcomitans* were fimbriae or afimbriae, vesicles and extracellular amorphous materials [46, 47]. This periodontopathogen produces many virulence factors such as leukotoxin, bacteriocin, chemotaxis inhibiting, Fc binding protein, lipopolysaccharide collagenase and antibiotic resistant determinants [20, 22]. The genetic diversity of *A. actinomycetemcomitans* allows it to produce a wide array of virulence factors, resist antibiotic treatment, evade the immune system, adapt to various host environments, and exchange genetic material, all of which contribute to its potential to cause significant harm and severe infections [20, 22]. One of the most important virulence factors of this bacterium was leukotoxin which has the potential to kill polymorphonuclear leukocytes, peripheral blood monocyte, macrophage and other types of cells such as epithelial, endothelia, erythrocytes and platelets [20]. This nonmotile bacteria are often associated with severe forms (localized aggressive) periodontitis [48]. On the other hand, this bacterium is also commonly found in young individuals who suffer from periodontitis. About 90% of cases were found in localised severe periodontitis and 30-50% in generalised severe periodontitis [46]. A study also reported that the DNA of this bacterium was not only isolated from periodontitis, but also from atherosclerotic plaques of patients, indicating systemic involvement [49].

Tanerella forsythia

Tannerella forsythia (*T. forsythia*) is a Gram-negative anaerobic rod bacterium within the *Cytophaga-Bacteroidetes* family [28, 29, 50, 51]. It is a filamentous, nonpigmenting and nonmotile bacterium [29, 52, 53]. This bacterium contained a unique cell envelope with a glycosylated surface layer attached to the lipopolysaccharide protein of the outer bacterial layer. This S-layer is also important as a virulence factor to delay the bacterium's recognition by the host immune system [29]. The presence of outer membrane vesicles which are built-up from glycoproteins were a new additional virulence factor for this bacterium [31]. The schematic diagram of *T. forsythia* structures is illustrated in Figure 2(b). In addition, the S-layer of *T. forsythia* provided serum resistance and influence coaggregation with other bacteria [54]. A study on mice which were orally infected by *T. forsythia* showed that the alveolar bone resorption response to this bacterium increased along with serum markers of inflammation. This condition altered the serum lipid profile, which increased the risk factors for atherosclerosis progression [55]. Serpin protein also known as miropin, is an important virulence factor in inhibiting serine proteases from neutrophils which protects this bacterium from proteolytic effects. Moreover, the presence of both *P. gingivalis* and *T. forsythia* has been found to be associated with an increased risk of oesophageal cancer [5].

Prevotella intermedia

Prevotella intermedia (*P. intermedia*) is a Gram-negative, obligate anaerobic, nonmotile, short and round-ended [7, 24]. It presents dark pigmented colony morphology on Brucella blood agar, brick-red autofluorescence under ultraviolet light and a negative fluorescence test for lactose production [25]. The main structures of this bacterium were a cell envelope which comprised lipopolysaccharide constituents and filaments [26, 56]. It is classified as an orange complex group bacterium together with *Campylobacter rectus*, *F. nucleatum*, and *P. micros* [7]. This bacterium possesses haemolytic activity due to the proteases enzyme which is capable of breaking down collagen and fibronectin proteins [7, 12]. This bacterium is also capable of producing extracellular viscous material for biofilm formation [57]. Study also found that *P. intermedia* possesses specific genes functioning in adhesion and secreting bacteriocin [58]. A recent study also reported that the *P. intermedia* is not only a pathogenic bacterium involved in periodontal disease but also associated with severe asthma [59].

Treponema denticola

Treponema denticola (*T. denticola*) is a Gram-negative anaerobic spirochete with high motility [33]. Figure 2(c) shows the schematic diagram of the *T. denticola* surface. The presence of the outer membrane, cytoplasmic membrane and periplasmic flagella assists this bacterium to be actively motile even in a highly viscous environment [60]. This bacterium was distinctive compared with other Gram-negative bacteria. A study reported that the outer membrane of lipopolysaccharide (LPS) contained lipoteichoic acid, which is similarly found in Gram-positive bacteria. In addition, research found that Msp was the major antigenic protein contained in the outer membrane, while TmpC protein enclosed the inner membrane of *T. denticola* [33]. Other virulence factors, such as the activity of proteolytic exotoxins, complex anaerobic fermentation of certain amino acids, and the production of toxic metabolites and outer membrane vesicles, facilitated the development of periodontitis by this pathogenic bacterium [61]. Rapid motility and chemotaxis aided this bacterium to colonise new sites, penetrate epithelial layers and enter deep periodontal pockets [36]. Furthermore, this bacterium is also capable of interacting synergistically with other periodontal pathogens to form biofilm. A study proved that *T. denticola* and *P. gingivalis* displayed synergetic interaction during biofilm formation. In addition, the presence of various types of cell-surface proteins in *T. denticol*a were important to dysregulate the host defence and to protect the subgingival biofilm which led to host tissue destruction [34, 36].

Campylobacter rectus

Campylobacter rectus (*C. re*ctus) is one of the *Campylobacte*r species of bacteria. It is an anaerobic Gram-negative motile bacterium with a small, straight rod and possesses a single polar flagellum as shown in Figure 2(d) [37, 62, 63]. The colonies of *C. rectus* appear in a convex shape and spread or corrode on blood and chocolate agar plates after 7 days of incubation in anaerobic conditions [37, 38]. *C. re*ctus was classified as an intermediate oral pathogen and is labelled in the "orange complex" [64]. A study also found the appropriate anaerobic conditions for this bacteria's growth were 30, 35 and 42^{o}C [38]. *C. rectus* is commonly isolated from subgingival sites of healthy oral humans and periodontitis patients [65]. There were several important virulence factors of this bacterium in the determination of disease pathogenesis, for example, the S-layer on this bacterium cell's surface. A previous study found that *C. rectus* contained *ciaB* gene, which is an important virulence factor for host cell invasion [66]. Another study also reported *C. rectus* not only contained *ciaB*, but also *peb4* for adhesion, *cad*F protein for cell binding and *cdtB* for cytolethal toxin production to arrest mammalian cells during the cell cycle process [39]. In addition, *C. rectus* was also reported as one of the oral species implicated in extra-oral infections and inflammation associated with cardiovascular diseases, adverse pregnancy outcomes, meningitis or brain abscesses and cerebral microbleeds in acute stroke patients [67, 68].

Fusobacterium nucleatum

Fusobacterium nucleatum (*F. nucleatum*) is an obligate anaerobic Gram-negative bacterium that belongs to the Bacteroidaceae family and the phylum *Fusobacteria.* The microscopic examination shows its unique spindle shape with tapered ends as shown in Figure 3 [41, 69]. It possesses an outer and inner-membrane cell envelope which are separated by periplasmic space made by the peptidoglycan layer. The inner membrane of this bacterium contained a symmetrical phospholipid bilayer with proteins and phospholipids in equal amounts. In addition, the outer membrane is an asymmetric membrane constructed from phospholipids, lipopolysaccharides, lipoproteins and proteins [70]. A study also reported that this bacterium was negative for lipase testing and susceptible to kanamycin and colistin while resistant to vancomycin [41]. The virulence factor of *F. nucleatum* is classified into two groups: colonisation and dissemination and induction host responses [42]. Fap2, RadD and Aid1 were the adhesin proteins involved in controlling coaggregation processes with various oral bacteria species during dental biofilm formation [71]. On the other hand, the FDA protein of this bacterium plays an important role in the invasion of epithelial and endothelial cells for dissemination. In addition, this bacterium elicits a variety of host responses including TLR4-mediated inflammatory responses in the placenta of pregnant mice, which cause fetal demise [71]. On the other hand, a synergistic relationship between fusiform bacteria, such as *F. nucleatum*, and spirochetes, specifically *Treponema* species, known as the fusospirochetal complex, establish a microbial consortium commonly found in periodontal and endodontic infections. This complex relationship enhances the pathogenic capabilities of periodontal disease through cooperative interactions among the bacterial species involved [72]. Furthermore, this bacterium species is one of the most abundant bacteria species found in healthy and unhealthy oral cavity individuals [71].

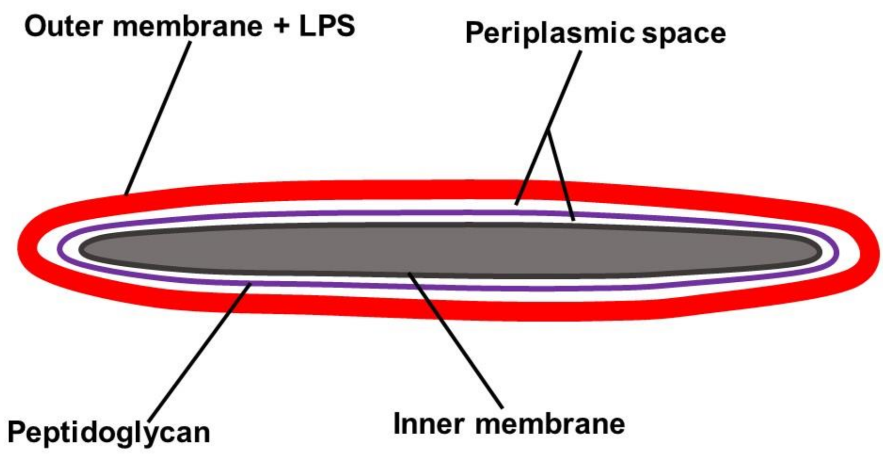

Figure 3: Schematic diagram of spindle shape form with tapered ends of *F. nucleatum*

Conclusions

Gram-negative anaerobic periodontal pathogens possess a variety of virulence factors that are able to trigger a host inflammatory response that leads to periodontitis. It is critical for researchers to correctly recognize the characteristics of periodontopathogens, particularly when conducting studies that require basic identification. It is challenging if we are unable to distinguish the basic features of these bacteria's properties. It is hoped that this review provides guidance and knowledge to the researchers about the specific properties, structures and functions of Gram-negative anaerobic pathogens associated with periodontitis. Moreover, identifying the significant characteristics of these microorganisms may help in the development of effective therapeutic strategies for the management of periodontitis.

Conflict of Interest

The authors declare that they have no conflicts of interest relevant to this study

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