

CHAPTER 2

LITERATURE REVIEW

2.1 Periodontal Disease

Periodontal disease is a chronic dental problem that affects 20 to 50% of the world's population (Nazir, 2017). Periodontal disease is a polymicrobial biofilm-related disease that occurs in the subgingival tissue and develops into the deep pocket underneath the teeth bone. The virulent factors from the bacteria induce inflammation, swelling and bleeding of gum tissue and deepen the pockets between gum tissue and tooth bone (Michaud et al., 2017). Clinical observation of gingivitis includes the inflammation and bleeding of gum tissue due to inflammatory immune responses. Severe cases of gingivitis advance to periodontal disease are marked by the formation of deep pockets, resulting in the detachment of gum tissue surrounding the tooth. Periodontal disease can develop into a more dangerous condition that affects the host's immune system and compromise a person's systemic health (Frencken et al., 2017).

Management of periodontal disease includes screening, prevention, correct diagnosis, and adequate interventions that can improve tissue attachments and reduce biofilm adhesion. Screening and prevention rely on individual awareness of oral condition and actions taken to improve oral health. Clinical procedures by scaling and root debridement are commonly employed to remove the calcified plaque or calculus on the supragingival and subgingival lines of the gum tissue. Other than that, surgical procedures are conducted for the more severe periodontal disease where the healthy neighbouring teeth are compromised (Graziani et al., 2017). In addition, antimicrobial treatments are administered as adjunct treatment for periodontal disease management (Graziani et al., 2017). Due to the complexity of the periodontal pathogens dynamic, supportive treatment is required to eradicate the putative pathogens (Hajishengallis et al., 2020). Residual putative pathogens are often detected at the diseased site during reassessment thus requiring supportive therapy to eliminate the disease-related pathogens (Mombelli, 2018).

In microbiological aspects, prominent periodontal disease-associated pathogens were identified from previous studies (Bale et al., 2017). Periodontal disease is marked by the balance shift between periodontal pathogens and healthy bacteria. In diseased conditions, periodontal pathogens possess larger microbial loads (Curtis et al., 2020). The dominant bacteria are mostly Gram-negative, facultative, or obligate anaerobes that survive by utilising the decayed food debris and blood from the affected gingival tissue. These antagonistic bacteria also promote biofilm formation and possess multiple virulent factors that weaken the immune system (Oliveira et al., 2016). A few most common periodontal pathogens are *Fusobacterium nucleatum*, *T. forsythia* sp., *T. denticola* sp., *A. actinomycetemcomitans* sp. and the keystone pathogen, *P. gingivalis* (Bhardwaj & Alwaeli, 2018). These bacteria are found in the periodontal pockets that are difficult to reach by common oral cleaning procedures.

2.2 Management of Periodontal Biofilm and Recolonization

Non-surgical procedures involve removal of dental plaque and calculus from supragingival and subgingival area by instruments. The common non-surgical procedures are scaling and root planing at the affected areas (Kinane et al., 2017). Scaling refers to the removal of plaque and calculus from subgingival areas by ultrasonic instruments while root planing implies the removal of plaque on the affected root surfaces to preserve the teeth attachment (Graziani et al., 2017). However, there is limitation of non-surgical therapy and it is commonly detected at the reassessment visit. Examples of limitations are residual inflammation, residual pockets, inability of equipment to reach plaque at deeper site and the recolonization of periodontal pathogens (Graziani et al., 2017; Kinane et al., 2017; Mombelli, 2018). Further treatments are required in order to overcome the limitations such as surgical procedures and adjunct antimicrobial treatments.

2.2.1 Antimicrobial Treatments

Antimicrobial treatments are administered as adjunct therapy for periodontal disease managements (Graziani et al., 2017). Due to the complexity of periodontitis aetiology especially the microbiological dynamic for periodontal disease, supportive periodontal therapy is required to eradicate the putative pathogens (Mombelli, 2018). A

consensus report revealed six randomized controlled trials with systemic antibiotics administration such as amoxicillin and metronidazole were proven to improve the subjects' condition clinically (Pretzl et al., 2019). Another option of adjunct antimicrobial treatment is the application of oral mouthwash to reduce periodontal pathogens in the oral cavity especially in the affected area. Extensive researches have been done to assess the efficacy of local antimicrobial agents in the form of mouthwash and gel on reducing the microbial loads and improving the clinical condition of periodontal patients (Jepsen & Jepsen, 2016).

Despite the positive outcomes, the administration of systemic antibiotics and antimicrobials are risky and supposed to be given after careful consideration. Antibiotics are risky not only because of the adverse effects such as nausea and hypersensitivity, but the main concern is the emergence of multi drug resistance bacteria (Jepsen & Jepsen, 2016). Moreover, antimicrobial mouthwash administration should be weighed properly due to its drawback such as palatal sensation changes, dry mouth, oral ulcers, gingival numbing and teeth staining (Zhao et al., 2020). Hence, new strategies are required to eradicate putative pathogens, prevent biofilm formation, counter recolonization of periodontal pathogens and mediate excessive inflammatory responses (Hajishengallis et al., 2020; Mombelli, 2018). Due to these limitations with the existing methods to manage periodontal disease, various researches were carried to identify the novel or supportive method to assist periodontal disease treatments.

2.2.2 Recent Strategies for Periodontal Disease Management

Management of periodontal disease begins by preventing the evasion and virulent activity of periodontal pathogens. New strategies are required to overcome the dysbiosis of oral pathogens, formation of oral biofilm at the early stage and subsequently prevent the recolonization of periodontal pathogens (Mombelli, 2018). Prevention of biofilm formation on supragingival and subgingival lines is important to ensure that there are no residual periodontal pathogens that can recolonize and cause diseased conditions (Graziani et al., 2017). A few suggested approaches to assist periodontal disease management includes, antimicrobial dental materials, antibacterial photodynamic therapy (APDT), bioactive compounds from various sources, and probiotics (Gerits et al., 2017; Jiao et al., 2019).

Antimicrobial dental materials are suggested as one of the approach to combat recolonization of pathogens after clinical treatment (Jiao et al., 2019). There are a few categories of well studied antimicrobial dental materials including drug releasing antimicrobial materials, antimicrobial nano-particles, antimicrobial and anti-fouling polymers, and antimicrobial peptides incorporated polymers (Ramburrun et al., 2021). In example of drug releasing antimicrobial materials, the incorporation of chlorhexidine in dental cements, dental adhesives, dental resins and nanoparticles had been studied which shown the potential of this method to overcome oral pathogens recolonization and biofilm formation (Boutsiouki et al., 2019; Campos et al., 2020; Carvalho et al., 2021; Kalagi et al., 2020; Takahashi et al., 2006). Other than that, studies related to antimicrobial dental nanoparticles showed promising outcomes in overcoming several dental pathogens. A study reported that synthesized antimicrobial material containing silver nanoparticles (AgNP) exhibited inhibition activities against *Streptococcus* and *Candida* species (Kachoei et al., 2021). In another study, AgNP synthesized from green tea extract and AgNP coated with silicon dioxide showed antimicrobial and anti-biofilm effect against *Streptococcus mutans* (Rodrigues et al., 2020). The stated examples of studies involving antibacterial dental materials proved its development and potential to overcome recolonization issue.

Other than that, antibacterial photodynamic therapy (APDT) was suggested as one of the new physical strategies to kill periodontal pathogens by exposing the affected site to low-energy laser light to interact with photosensitive agents (Chi et al., 2019). This method has garnered interest to be applied as adjunct treatment for periodontal disease due to its non-invasive procedures and localized action (Moro et al., 2021). A study reported the reduction of probing depth and bleeding in diabetic periodontal patients that were given multiple APDT session as adjunct treatment after a non-surgical treatment (Cláudio et al., 2021). In another study, it was discovered that the group of patients receiving APDT as adjunct treatment reported the absence inflammatory cells, moderate fibrogenesis and neoangiogenesis which indicated that the inflammation on the affected sites are reduced with the APDT adjunct treatment (Silva et al., 2012). Generally, addition of APDT as adjunct treatment plays synergistic roles to improve the periodontal patients' condition which includes eradication of bacteria, modulation of inflammatory activity which manifested as a better condition of the diseased sites. However, as stated

in the review, APDT treatment needed to be given repeatedly over a period of time and it is a costly management method (Vohra et al., 2016).

Aside from photodynamic and polymeric approaches, bioactive compounds from various sources were also proposed as a strategy to manage periodontal disease. Bioactive compounds have multiple benefits such as antimicrobial, antioxidants, anti-inflammatory, anti-tumor, and analgesics properties which boost its potential as an adjunct approach to manage periodontal pathogens and periodontal disease (Cicalău et al., 2021). In example, a study highlighted significant antimicrobial activities of honokiol and magnolol isolated from *Magnolia officinalis* plant against several periodontal pathogens of several stages of disease such as *A. actinomycetemcomitans*, *P. gingivalis*, *P.intermedia*, *Micrococcus luteus* and *Bacillus subtilis* (Ho et al., 2001). Additionally, *Salvadora persica*, a well known plant in oral health were reported to exhibit multiple actions to improve the periodontal condition such as anti-inflammatory action by suppression of inflammatory cytokines, antimicrobial activity against periodontal pathogens, and regenerative activity to heal the affected gingival tissue (Mekhemar et al., 2021). Regardless of the efficacy, deeper studies are required to understand the efficacy and risks of plant bioactive compounds to oral condition.

Additionally, bioactive compounds from marine algae also gained a lot of research interest as one supportive approach to manage periodontal disease due to their multiple actions including anti-bacterial and anti-inflammatory activity (Huang et al., 2021). A study reported that sulphated polysaccharides from marine algae *Gracilariacaudata* prevent periodontal tissue damage in rat model induced with periodontal disease. The efficacy were indicated by reduction of oxidative stress as observed in the rats blood serum and lowered concentration of inflammatory related proteins (Da Silva et al., 2019). Another study observed that *Eklonia cava* ethanolic extract reduced inflammation in periodontal disease induced rats and murine macrophage model induced with *P. gingivalis* LPS. The extract was reported to reduce nitrous oxide, prostaglandins, and expression of inflammatory cytokines in murine macrophage tissue model. Administration of *E. cava* extract in rat induced with periodontal disease resulted to significant reduction of gingival index, alveolar bone loss, matrix metalloproteinases, and expression of pathway related to alveolar bone degeneration (Kim et al., 2019). However, marine algae are hard to source due to their location, expensive extraction

process and a lot of study are required to assess the extraction procedure that gives a large yield from the algae (Bleakley & Hayes, 2017).

Onto the other potential management strategy, probiotics were suggested in a few reviews as a novel supportive treatment to improve periodontal condition by inhibition of periodontal pathogens colonization, biofilm formation, anti-inflammatory activity, and regenerative properties (Coenye et al., 2020; Gerits et al., 2017; Mahasneh & Mahasneh, 2017).

2.3 Probiotics in General Health

Probiotics, by the definition of the World Health Organization (WHO), are "live microorganisms that, when administered in adequate amount confer a health benefit on the host" (Martín & Langella, 2019). In recent years, probiotics efficacy for human health had been proven in various studies. Probiotics consist of various types of microorganisms such as bacteria, fungi and yeast (Santacroce et al., 2019). Bacterial probiotics are the most common probiotics that have been studied throughout the years since the discovery of probiotics in the last century. The most common bacteria studied are Lactic Acid Bacteria (LAB) from the *Lactobacillus* and *Bifidobacterium* genus (Chalas et al., 2016). There are a lot of other identified probiotics aside from the two-mentioned genus.

Nowadays, it has been studied that probiotics come from various sources, including fermented foods, dairy products, and human breast milk. Foods such as kimchi, fermented soy grain, kefir milk, cheese, and yoghurt are the most known sources of these beneficial life bacteria (Kok & Hutkins, 2018). Other than that, probiotics also come from certain fruits, vegetables and aquaculture sources (Roobab et al., 2020). A few studies have shown that there are few strains of probiotics available in human breast milk (Riaz Rajoka et al., 2017; Zacarías et al., 2019).

Since the discovery of probiotics, researchers have been focusing on the efficacy of probiotics for health benefits (Pujia et al., 2017). The most prominent studies are the benefits of probiotics for gut health and the balance of microbiota in the stomach, the ability to modulate the immune system, alleviate topical problems and studies for the prevention of urogenital infections (Abatenh, 2018; Pujia et al., 2017). There are also rare strain-specific studies of probiotics for health benefits including neurological benefits,

endocrine disease management and the production of specific bioactive for medicinal purposes (Sanders et al., 2018).

Probiotics' benefit for gut health includes the ability of probiotics to manage inflammation in the intestinal tract. Probiotics have been studied for their efficacy against inflammatory bowel disease (IBD) such as Crohn's disease and ulcerative colitis (Palumbo et al., 2016). Other than that, probiotics are also known for their benefits towards immune system regulation. Probiotics established the communication with intestinal epithelial cells to regulate macrophages and dendritic cells with a lower inflammation effect (Maldonado Galdeano et al., 2019).

Besides, probiotics are beneficial in the modulation of allergic asthma (Mennini et al., 2017). Studies found that the administration of probiotics, namely *L. rhamnosus* had shown a significant effect in the restoration of lung condition. The results from mice models showed that probiotics suppress certain immune components including eosinophils, neutrophils, IL-1 β and IgE which cause airway inflammation (Spacova et al., 2020; J. Zhang et al., 2018).

Probiotics are also studied for their ability to reduce the symptoms of eczema and atopic dermatitis. The presence of probiotics in gut flora is proven to be beneficial in the regulation of several immune components that cause hypersensitivity (Maldonado Galdeano et al., 2019). A few studies investigated the association between the intake of probiotics and its effect on atopic dermatitis symptoms in mice models. The study showed suppressed activity of Th2 and Th17 cells that are responsible for the inflammatory activity for atopic dermatitis. Other than that, IL-10 works to inhibit inflammatory responses that are upregulated when treated with probiotics (Holowacz et al., 2018)

2.3.1 Probiotics for Oral Health

The benefits of probiotics were also studied in the oral health field as an adjunct therapy to maintain oral health and to assist the healing process for compromised oral health conditions such as dental caries, gingivitis, halitosis, and periodontal disease (Pujia et al., 2017). Antimicrobial properties of probiotics against *S. mutans*, cariogenic bacteria had been reported in a study where probiotics isolated from kefir exhibit excellent

antimicrobial and anti-biofilm activity. The study investigated the mechanism of inhibition in the *S. mutans* gene and reported that probiotics downregulated the expression of carbohydrate metabolism and regulatory protein genes that are important for biofilm formation and stress responses (Jeong et al., 2018).

Aside from that, probiotics also exhibit antimicrobial activity against candidiasis pathogens, including *Candida albicans*, *Candida glabrata*, *Candida krusei*, and *Candida tropicalis* (Jørgensen et al., 2017). Probiotics-based oral care containing species such as *L. rhamnosus*, *L. reuteri* Prodentis, *L. plantarum* and many other species were tested in clinical settings and several positive outcomes were reported after the administration of probiotics. Probiotics administration reduced pathogenic species bacterial counts and improved gingival tissue conditions that were determined based on bleeding on probing percentage, probing depth and gingival inflammation index (Seminario-Amez et al., 2017). Although the effects of probiotics might not be able to counter all factors of oral disease, probiotics might become a new approach in oral healthcare. Consequently, researchers are moving towards the application of probiotics as an alternative or adjunct treatment for periodontal health improvements.

2.3.2 Probiotics for Periodontal Health

Probiotics have been researched in dental health as one of the sources to support the prevention of pathogens colonization and biofilm formation. In periodontal health, probiotics were deduced to be beneficial in two major ways that are direct antagonism towards periodontal pathogens and indirect effect on periodontal health that includes modulation of inflammatory components or supportive roles in gingival epithelial cells restoration (Mahasneh&Mahasneh, 2017; Pujia et al., 2017).

The direct antagonism of probiotics against periodontal pathogens involves two modes that are competition for attachment sites on gingival tissue and nutrient requirements, besides the production of antimicrobial agents by the probiotics as secondary metabolites. It is well studied that probiotics secrete a wide range of antimicrobial agents such as hydrogen peroxide, antimicrobial peptides, bioactive compounds and various organic acids (Allaker& Stephen, 2017). A few studies have investigated the direct effect of probiotics against periodontal pathogens.

In a study, the direct effect of probiotics against periodontal pathogens was investigated based on the viability of periodontal pathogens *P. gingivalis* and *F. nucleatum* when being co-cultured with *Streptococcus dentisani* cell-free supernatant over a time course. The result showed a reduction of *P. gingivalis* and *F. nucleatum* growth by 35% to 38%. The study deduced that the result might be related to the disruption of the pathogen's cell walls as observed by scanning electron microscope (SEM) and competition for nutrients available in the culture media (Esteban-Fernández et al., 2019). Another study highlighted the direct effect of probiotics in preventing the co-aggregation of periodontal pathogens and biofilm attachment by periodontal pathogens. The study findings showed that *Lactobacillus brevis* BBE - Y25 autoaggregation with *P. gingivalis* and *F. nucleatum* which prevents the two pathogens from forming a synergistic actions to exhibit virulent activity. The result also highlighted that *L. brevis* BBE - Y25 had better adherence to the human oral epithelial cell line compared to the stated pathogens that prove the idea of probiotics' role in competing for attachment to human epithelial tissue (Z. Fang et al., 2020).

Hydrogen peroxide, antimicrobial peptides, organic acids, and fatty acids are a few common antimicrobials agents secreted by probiotics (Chugh & Kamal-Eldin, 2020). Antimicrobial activity of commercial probiotics oral care OralCMU containing *Weissella cibaria* strain from Cheonnam Medical University (*Weissella cibaria* CMU) was investigated and characterized in a recent study. The antimicrobial activity of the Oral CMU supernatant against periodontal pathogens was characterised to determine the possible component of the supernatant that has antimicrobial properties. The gas chromatography (GC) analysis revealed that the cell-free supernatant of OralCMU contains 37 different fatty acids and high-performance liquid chromatography (HPLC) analysis showed that lactic acid was present in the supernatant at the highest concentration, followed by acetic acid and citric acid. Organic acid and fatty acid produced by probiotics provide a suitable environment for low-pH bacteriocin (Shokri et al., 2018). Other than organic acids and fatty acids, antimicrobial peptide of the lysozyme group, N- acetylmuramidase that can cleave the peptidoglycan layer is also an active component of the antimicrobial activity. Apart from that, OralCMU supernatant also secreted hydrogen peroxide under aerobic conditions. The stated antimicrobial agents might be the underlying component that exhibits antimicrobial activity against periodontal pathogens (Lim et al., 2018).

The indirect effects of probiotics on periodontal health were investigated including the modulation of inflammatory responses. The ability of probiotics to modulate inflammatory responses was recorded in a study where the supplementations of multi-strain probiotics to male rats with apical periodontal disease were investigated. The probiotics dietary supplements showed downregulation of IL - 6, IL -1 β , receptor activator of NF- κ B ligand (RANKL) and reduction of mature osteoclasts cells. Aside from the inflammatory interleukins, RANKL plays an important role in periodontal inflammation due to its ability to induce uncontrolled bone resorption which is not favourable for normal bone function (Weitzmann, 2013). The effect of the stated inflammatory interleukins and RANKL downregulation was observed in the histological study of the rats' tissue that showed that rats with probiotics treatment presented less intense inflammation on the tissue when being compared to the non-treatment groups (Cosme-Silva et al., 2021).

Single-strain probiotics, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) HN019 were administered to male Wistar rats in the form of irrigation suspension. The results showed downregulation of IL - 1 β and RANKL and upregulation of IL - 10 that plays a protective role in periodontal inflammation. Additionally, the regulation of stated components assists osteoprotegerin (OPG) as a natural inhibitor of the RANKL pathway. The modulations of inflammatory responses were observed in the rats' tissue and bone structure. The treatment groups presented less extensive inflammatory infiltrates, numerous fibroblasts, only slight interstitial oedema, and intact collagen fibres. The bone structure study reveals that the non-treatment groups showed higher bone porosity and a lower percentage of the area filled with bone tissue that agreed with the pathological findings. The immunochemical study reported a significantly greater expression of beta defensin which is innate protective peptide for gingival epithelial tissue health (Oliveira et al., 2017).

The other mode of probiotic action to improve periodontal health is the supportive role in gingival epithelial tissue regeneration. It is well known that probiotics can assist tissue repair and have been widely tested on various sites (Allaker & Stephen, 2017). A study investigated the balance of pathogenic bacteria *P. gingivalis*, and probiotics *Lactobacillus reuteri* on wound healing activity. The study deduced that imbalance of pathogenic species in the oral condition might impair the ability of

mesenchymal stem cell (MSC) homeostasis which is important in the wound healing process. The results showed that the balanced condition of pathogenic and probiotic species selected for this study improves gingival MSC migration, function, and restoration of osteogenic factors function. The wound healing improvement with probiotics culture in the oral cavity was also observed physically where the mice with *P. gingivalis* and *L. reuteri* showed accelerated wound healing on gingival tissue. The underlying mechanism was revealed with the molecular study on *P. gingivalis* and it was proven that reuterin from *L. reuteri* inhibits LPS actions on gingival mesenchymal stem cell (GMSC) activity and osteogenic factors (Han et al., 2020).

In a clinical study, probiotics *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) HN019 in lozenges form was given as an adjunct to the scaling and root planing procedures for chronic periodontal disease patients. The study reported the improvement in clinical observations such as the reduction of pocket probing depth (PPD) from ≥ 7 mm to ≤ 3 mm after 90 days in the test group. In addition, the study reported that administration of the lozenges reduced the recolonization of red-complex bacteria and lowered the level of inflammatory agents (Invernici et al., 2020). In another study, reduction of PPD was also reported in the group administered with adjunct probiotics *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289 lozenges after clinical procedures to remove calculus. Additionally, the risks of disease progression based on clinical parameters were also lower in the probiotics group compared to the placebo group (Laleman et al., 2020).

2.4 *Lactobacillus rhamnosus*

Lactobacillus rhamnosus is a Gram-positive, rod-shaped lactic acid bacteria that have the potential to be screened for antimicrobial activity against *P. gingivalis*. This bacteria is aerobic bacteria that appeared as thick white colonies on MRS agar (Collins et al., 1989). *L. rhamnosus* exhibits multiple benefits on general health especially on the intestinal health, allergic reaction modulation, urogenital health, and oral health (Segers & Lebeer, 2014). A study reported the benefit of *L. rhamnosus* CNCM I-3690 for the intestinal health where the administration of *L. rhamnosus* CNCM I-3690 on mice model stimulated mucus production and cytoprotective response which subsequently protect the intestinal wall from inflammation (Martín et al., 2019). Additionally, the benefits of *L.*

L. rhamnosus was also prominent in the alleviation of atopic dermatitis in children, where a study reported the children administered with probiotics supplementation recorded significant reduction of atopic dermatitis after three months of intervention. In addition, the study also reported the reduction of allergy scoring in the group administered with probiotics supplementation (Cukrowska et al., 2021). Other than that, a study reported the antimicrobial activity of *L. rhamnosus* GG isolated from vaginal area against urogenital pathogens which showed that *L. rhamnosus* is important for the regulation of urogenital microbial balance (Stivala et al., 2021). *L. rhamnosus* is also studied in oral health for multiple purposes especially in the disease related to pathogenic invasion.

L. rhamnosus was studied for its benefits on oral health improvement such as improvement of dental caries and oral candidiasis. A clinical study reported that healthy individuals administered with milk containing *L. rhamnosus* supplementation showed reduction of cariogenic bacteria (Rungsri et al., 2017). As for *L. rhamnosus* benefits on oral candidiasis improvement, administration of *L. rhamnosus* L8020 reduced the pseudomembranous candidiasis on tongue region in the *in-vivo* model and the effect of *L. rhamnosus* L8020 protective action against *Candida sp.* was revealed in the gene expression of mice treated with *L. rhamnosus* L8020 (Ito et al., 2021).

L. rhamnosus has been investigated as one of the potential probiotics to be developed as periodontal disease adjunct therapy. The probiotics were found to exhibit antimicrobial activity in a few studies and the clinical significance of *L. rhamnosus* was reported in a few clinical trials (Jeong et al., 2018; Morales et al., 2016). The excellent antimicrobial activity and its manifestation in clinical evidence required an in-depth study regarding the molecular effect of *L. rhamnosus* exposure on the periodontal pathogens and its effect on bacterial gene expression that affected the pathogens' colonization and biofilm formation. This in-depth study might give insight into developing an adjunct treatment that can directly inhibit periodontal pathogens and prevent biofilm formation.

2.5 Microbiology of Periodontal Disease

Pathogenic dental bacteria growths are usually encouraged by the remains of food debris resulting from poor oral hygiene practices or high sugar intakes (Peres et al., 2016). The pathogens break down nutrients in food, such as carbohydrates and proteins, as a nutrient source (Scannapieco & Gershovich, 2020). Some periodontal pathogens like *P. gingivalis* and *T. denticola* can utilize blood and protein in the lesions from the inflamed gum tissue as a source of nutrition (Brown et al., 2019; Listyarifah et al., 2017).

A vast number of microbial species have inhabited the oral cavity since birth consisting of the beneficial, core, and pathogenic species colonising the oral cavity (Curtis et al., 2020). The shift of microbial load in the oral cavity from normal to diseased condition clearly indicates periodontal disease development (Deo & Deshmukh, 2019). Initially, the healthy oral microbiota contained more beneficial and core species bacteria, then the balance of the three groups shifted where pathogenic species became a dominant colonizer in the oral cavity. The shift of the microbial balance is as demonstrated in Figure 2.1.

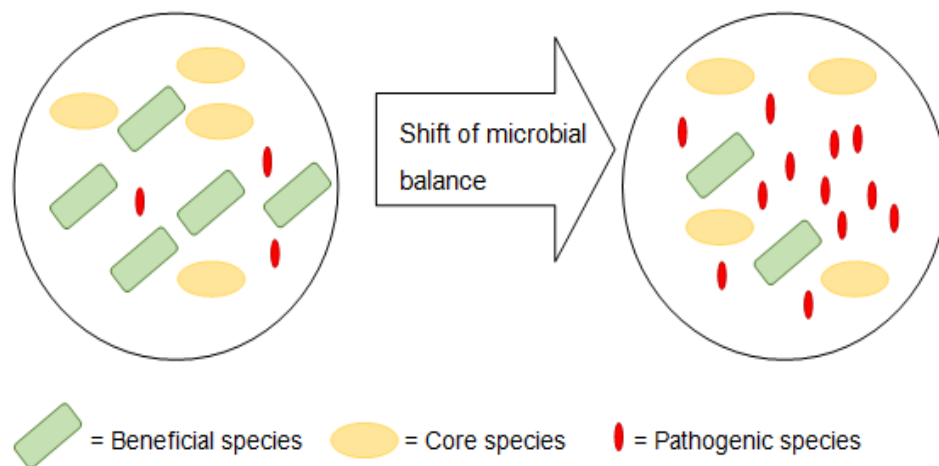


Figure 2.1: The shifting of microbial balance in oral microbiota from normal condition to diseased condition.

The establishment of periodontal-related pathogens began with the early colonization of *Streptococcus* and *Actinomyces* species among the health-related microbiota. These early colonizers contributed to the growth of late colonizers by providing the substrates and creating a favourable environment for the late colonizers' activities (Valm, 2019). An example of this relationship is shown in the interaction between *Streptococcus gordonii* and *P. gingivalis*, where the haemolytic activity of *S. gordonii* lysed the oxidised haemoglobin in blood cells into the form that *P. gingivalis* can utilize. Other than that, *S. gordonii* also encourages the signalling activity of *P. gingivalis* in periodontal biofilm (Brown et al., 2018). After the colonization of early colonizers and involvements of important periodontal pathogens such as *A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum*, *T. forsythia*, and *T. denticola*, a stable biofilm is formed, and it encourages the synergistic actions by pathogens within the biofilm layer.

2.5.1 Biofilm Formation

The aggregation of periodontal disease bacteria and complex microbial communication leads to the formation of stable polymicrobial biofilm and presents itself as plaque. Polymicrobial biofilm is the community of various bacteria gathered together by several extracellular matrixes formed between the soft gum tissue and the hard teeth. The plaques are usually present on the surfaces between the teeth and the subgingival line between the gum tissue and teeth (Akcalı & Lang, 2018).

Biofilm in the oral cavity begins with the attachment of early colonizers to the pellicles on tooth surfaces. The early colonizers, such as *Streptococcus mutans* and *Actinomyces* species, will form exopolysaccharides (EPS) matrix that encourages the maturation of biofilm and the inclusion of other periodontal pathogens into the biofilm at the later stage (Krzysciak et al., 2016). Then, the inclusion of putative species, *F. nucleatum* into the biofilm community acted as the bridging species that promoted the colonization of late antagonistic colonizers such as *P. gingivalis*, *T. forsythia*, and *T. denticola*. The stated putative pathogens damage the host tissue by disrupting gingival tissue as a source of nutrients and interfering with the host's immune responses (Jiao et al., 2019). After biofilm maturation, the plaque form calcified into calculus, which appears as a hard layer on the subgingival line. The calcification of

plaque into calculus is also detrimental because it provides oxygen scarce area where anaerobic Gram-negative antagonistic pathogens can thrive and affect the host immunity regulation (Gedif Meseret, 2021).

The calcification of biofilm will cause difficulties in removing it by salivary movement, teeth brushing or interdental cleaning. This condition requires professional non-surgical procedures by scaling or root planing (Balaji et al., 2019; Kamath & Umesh Nayak, 2014). Pathogenic bacteria in the biofilm possess multiple virulent factors that exhibit destructive effects on the gum tissue and teeth attachments beside affecting the host's immune responses and causing irregularities in epithelial cell proliferation. In some severe cases, the virulent factors of periodontal pathogens might affect systemic circulation (Jiao et al., 2014). A few key pathogens in the periodontal biofilm are termed 'Socransky's red-complex bacteria', including *T. forsythia*, *T. denticola*, and *P. gingivalis* (Socransky et al., 1998). Other than 'red complex bacteria', antagonistic species such as *F. nucleatum*, *A. actinomycetemcomitans*, *Prevotella intermedia* and *Eikenella corrodens* were also found in dysbiotic biofilm from periodontal disease patients (Reinhardt et al., 2019). The similarities of all the stated species are that these pathogens are Gram-negative bacteria with lipopolysaccharide (LPS) layer that secrete various types of endotoxins that trigger higher inflammatory responses (Nakayama & Ohara, 2017).

2.5.2 Synergistic Actions of Periodontal Pathogens

In dysbiotic conditions, the aggregation of certain species exhibit synergistic upregulation of virulence genes. In the presence of *Prevotella intermedia* dead cells, the upregulation of *kgP*, *rgPA* and *rgPB* genes were expressed by *P. gingivalis* where all three genes are prominent in *P. gingivalis* virulent activities (Herrero et al., 2018). In the presence of *P. gingivalis* dead cells, some virulence genes in *P. intermedia* were upregulated such as *kpsD*, *ecf*, *pgh* and *inpA*. The *kpsD* and *ecf* genes are involved in the development of resistant polysaccharides on the cell surfaces. The other two virulent genes, *pgh* and *inpA* are responsible for the haemolytic activity of *P. intermedia* (Rodriguez Herrero et al., 2017).

The aforementioned 'red-complex bacteria' also exhibit the mutual interaction where *T. denticola* was found to bind only with *P. gingivalis* and *T. forsythia*. *T.*

denticola acted with *P. gingivalis* in fibrinogen hydrolysis-induced bleeding and interfered with tissue repair. On the other hand, *P. gingivalis* and *T. forsythia* were found to upregulate type II *fimA* and *bspA* genes when aggregated together (Mahalakshmi et al., 2017). The synergistic relationship between the 'red complex bacteria' was found to cause cell death by various factors on macrophages that triggered the secretion of endogenous molecules that facilitate Toll-Like Receptors (TLRs). These endogenous molecules also triggered the release of the proteolytic components from *T. denticola* which caused the degradation of cytokines. The continuous trigger and stressor action of these bacteria also brought detrimental effects on the cardiovascular system (Jun et al., 2017).

2.6 *Porphyromonas gingivalis*

P. gingivalis is the most significant species among the pathogens present in the periodontal biofilm. *P. gingivalis* is a Gram-negative, non-motile rod-shaped bacteria that colonize as black-pigmented colonies on the blood agar as shown in Figure 2.2, due to haemolytic activity (Chu et al., 1991). These periodontal pathogens are commonly found in the biofilm that forms on the deep subgingival line and the deep pocket between the gum tissue and teeth surface where oxygen is scarce.

P. gingivalis is identified as a 'keystone pathogen' in the microbiology of periodontal disease. *P. gingivalis* is involved in most synergistic actions with other pathogens as well as amplification of virulence factors in the biofilm community. Other than that, *P. gingivalis* is also included in the 'red-complex bacteria' group, where this group of bacteria is detrimental to gingival tissue and causes inflammatory interference. Besides, the presence of *P. gingivalis* in the oral microbiome marked the shift of the microbial community from a healthy microbial load to a dysbiosis condition where the abundance of pathogenic bacteria is higher than the core species and healthy bacteria (Olsen et al., 2017; Reinhardt et al., 2019).

The presence of *P. gingivalis* in the oral microbiota indicated that periodontal destruction is at the irreversible phase, where all the damage done by the periodontal pathogens and inflammatory actions will require clinical treatments. Even without the amplification through synergistic actions, *P. gingivalis* already possess multiple virulence

factors that are detrimental not only to the gum tissues but also to the innate immune responses and systemic circulation.

2.6.1 Virulence Factors

P. gingivalis virulent factors are functional for a lot of things, including colonization and recolonization inside the oral cavity, evasion of the immune responses, subduing the host immunity, intercellular entry, nutrient exploitation, and secretion of antagonistic factors. Multiple virulence factors that are expressed by *P. gingivalis* include fimbriae, gingipains, lipopolysaccharides (LPS), proteases, capsule formation and adhesin components (Jia et al., 2019; Jun et al., 2017).

2.6.1.1 Fimbriae

One of the most well studied *P. gingivalis* virulent factors is fimbrial protein or fimbriae. Fimbriae usually play synergistic action to manipulate the immune responses and encourage attachments to the host cells and biofilm formation (Nakayama & Ohara, 2017). Fimbriae are protein filaments that are present on the surface of a bacterial cell that promotes adhesion and invasion of the host cells. Fimbriae promote the adhesion of *P. gingivalis* to the early colonizer pathogens and encourage the formation of stable polymicrobial biofilm. There are two types of fimbrial protein that are long fimbriae encoded by the *FimA* gene and short fimbriae encoded by the *mfaI* gene where both fimbriae have the function for invasion and pro-inflammatory properties. In addition, the gene expressions of fimbrial protein are supported by a few accessory genes in the groups of *Fim* and *mfa* gene clusters (Bostanci & Belibasakis, 2012; Hasegawa & Nagano, 2021).

Long fimbriae encoded by the *FimA* gene together with its accessory genes, *FimB*, *FimC*, *FimD* and *FimE* initiate *P. gingivalis* inclusion in the biofilm of early colonizers by exploiting the host oral substrates such as lactoferrin, proline-rich protein, glycoproteins and fibrinogen proteins (Hasegawa & Nagano, 2021). These substrates were exploited to promote the adhesion of pathogens to the host tissue. Long fimbriae mediate the co-aggregation of *P. gingivalis* with other pathogens in the process of biofilm stabilisation. An example of this function is the mediation of *P. gingivalis* co-

aggregation with another putative pathogen, *T. denticola*, via specific signalling to its receptor (Enersen et al., 2013).

On the other hand, short fimbriae are encoded by the *mfa1* gene, which is supported by the accessory proteins encoded by *mfa2*, *mfa3*, *mfa4* and *mfa5* genes. The accessory proteins are essential in the regulation of fimbriae formation and functions (Hasegawa & Nagano, 2021). Whilst long fimbriae are involved in the early phase of biofilm formation, short fimbriae are said to be involved in the regulatory activity as the poly-microbial biofilm develops. Additionally, short fimbriae can induce attachment of *P. gingivalis* surface proteins with most streptococcal adhesin receptors and then suppress the mechanism as the biofilm developed to adapt to the necessity of adhesin requirements (Enersen et al., 2013; Xu et al., 2020).

Fimbriae also play an important role in the induction of inflammatory activities and manipulation of immunity responses. Long and short fimbriae are involved in the induction of inflammatory responses by employing different Toll-like Receptors (TLR) to trigger severe inflammatory actions (Bostanci & Belibasakis, 2012). The long fimbriae exploit Toll-like Receptor 2 (TLR2) in the induction of inflammatory responses. The stimulation of TLR2 together with a cluster of decay-accelerating factors, specifically CD14 stimulates the upregulation of inflammatory cytokines such as IL-8, IL-6, IL-1 β and tumour necrosis factor- α (TNF- α) that promotes early cell apoptosis and increase bone resorption activity (Jia et al., 2019). Meanwhile, short fimbriae were less studied but assumed to exploit another TLR which is the TLR4 pathway that results in a similar inflammatory expression as the TLR2 pathway (Hajishengallis & Diaz, 2020). Other than that, similar to gingipains, fimbriae can manipulate neutrophil signalling to evade phagocytosis and simultaneously increase the expression of inflammatory responses that are favourable for *P. gingivalis* (Sochalska & Potempa, 2017).

2.6.1.2 Gingipains

Gingipains are surface cysteine proteinases which is categorised into two main groups. One group of gingipain is Arginine-gingipain (RgP) encoded by *rgpA* and *rgpB* gene while the other one is Lysine-gingipain (KgP) encoded by *kgp*. Almost 85% of proteolytic activity by *P. gingivalis* is caused by gingipains (Bostanci & Belibasakis, 2012). The important modes of gingipain action are by degradation of the epithelial adhesion complex and ability to cleave extracellular proteins. Arginine-

gingipain (RgP) mostly involved in this action. Degradation of the epithelial barrier caused penetration of *P. gingivalis* LPS, peptidoglycan and gingipains itself into the host tissue. Physically, the degradation of the epithelial barrier manifests as the detachment of periodontal tissue from the teeth' surface (Takeuchi et al., 2019).

After the penetration of gingipains into the host cell, gingipains interfere with the regulation of immune responses and exhibit proteolytic activity to evade the immune responses. Then, gingipains interrupt the regulation of polymorphonuclear (PMN) myeloid cells to evade bacterial elimination by neutrophil chemotaxis (Sochalska & Potempa, 2017). Other than that, inactive gingipains skew the differentiation of T cells into Th17 cells, which is important to fight bacterial invasion but causes bone degeneration when being in excess (Glowczyk et al., 2017). Gingipains also affect the regulation of macrophage, IgG dependent opsonisation, interfere with TNF - α signalling, and exploit IL -8 signalling to cause continuous inflammation and evade bacterial phagocytosis (Castro et al., 2017; Hou et al., 2017; Sochalska & Potempa, 2017). Interestingly, gingipains are also involved in the coordination, regulation, and maturation of fimbrial protein gene expression. The selectiveness of gingipains action on protein contributes to the invasion and manipulation of immune responses by *P. gingivalis* (Hočevár et al., 2018).

2.6.1.3 Lipopolysaccharides

The LPS layer present on *P. gingivalis* outer membrane has been studied for its antagonistic effect on the periodontal tissues. LPS was studied to induce severe inflammatory responses by interrupting immune signalling (Nakayama & Ohara, 2017). The heterogeneity of LPS on *P. gingivalis* surfaces also caused arrays of unnecessary inflammatory responses. One isomeric form of LPS was studied to dominantly exploit one of the Toll-like Receptors (TLRs) that is TLR2, which induced the over secretion of TNF - α , IL-1, IL- 6, IL - 8, IL - 18 that caused inflammation (Bostanci & Belibasakis, 2012). On the other hand, the less dominant form of *P. gingivalis* LPS stimulates TLR4 and mediator MD2 complex that induces interferon responses factor (IRF) 1,5 and 7 (Jia et al., 2019). The heterogeneity of LPS and its differential responses support the theory of manipulation of host immune responses by *P. gingivalis*.

2.6.1.4 Capsules, Adhesins and Hemagglutinins

Aside from the stated virulence factors, *P. gingivalis* also possess surface capsules that are the external structure made up of polysaccharides enveloping the bacterial cell and protecting it from external stress and bactericidal actions. It is stated that encapsulated *P. gingivalis* showed higher resistance towards phagocytosis compared to the non-encapsulated *P. gingivalis*. However, non-encapsulated *P. gingivalis* showed better adhesion on host tissue compared to the encapsulated strain (Xu et al., 2020). This is proven by the comparison of two *P. gingivalis* strains, *P. gingivalis* W83 with capsules and *P. gingivalis* ATCC 33277 without capsules showing different levels of adhesion and resistance (Mendez et al., 2019). The presence of capsules also assists the co-aggregation of *P. gingivalis* with another pathogen such as *F. nucleatum* where the mixed infection with encapsulated *P. gingivalis* has augmented virulence factors that caused higher disease severity compared to the mixed infection with non-encapsulated *P. gingivalis* (Polak et al., 2017).

Adhesins component in *P. gingivalis* also contributed to the virulence factors of the pathogens. The adhesins component encourages the maturation of the surface protein such as gingipains and fimbrial protein. Aside from that, the adhesins domain also contributes to the formation of adhesin-gingipains complexes that trigger different TLRs pathways. Adhesins such as hemagglutinin are involved in hemagglutination and hemolysis of erythrocytes to facilitate heme acquisition (Nakayama & Ohara, 2017). Hemagglutinin encoded with *hagA*, *hagB* and *hagC* were found to be higher in *P. gingivalis* isolated from periodontal patients compared to isolates from healthy persons. Hemagglutinins are also identified as one of the contributing elements to the adhesion of *P. gingivalis* to the gingival epithelial tissue besides from its hemagglutination activity (Mendez et al., 2019).

Various types of *P. gingivalis* virulent factors and its multi-faceted actions depending on its environment, surrounding pathogens, nutrition acquisition, and external stressor showed the capability of *P. gingivalis* to subvert the host's immune responses to cause damage instead of causing a bactericidal effect on the pathogens. The mediation of different pathways to trigger various inflammatory responses and evades phagocytosis also plays a beneficial role in the invasion of *P. gingivalis* and inflammatory actions that manifests as plaque, bleeding gum, and tissue degeneration that is shown clinically as

deep pocket and alveolar bone degradation. Various disease managements are practised currently to combat periodontal disease as a whole and *P. gingivalis* invasion specifically.

