INTRA-SUBJECT INVESTIGATION INTO THE ROLE OF DENTAL PLAQUE INDUCED IL-8, IL-6 AND IL-1α EXPRESSION AND ITS ASSOCIATION WITH THE SUBGINGIVAL PLAQUE MICROBIOME

MAHEEN TARIQ

UNIVERSITI SAINS MALAYSIA

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by

MAHEEN TARIQ

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LIST OF SYMBOLS

α	Alpha
β	Beta
μ	Micro
γ	Gamma
r_s	Spearman Coefficient
$\leq$	Less than or equal to
$\geq$	Greater than or equal to
°C	Degree Celsius
$>$	Greater than
$<$	Less than
*	Asterisk
κ	Kappa

LIST OF ABBREVIATIONS

16S rRNA	16S Ribosomal Ribonucleic Acid
AP-1	Activator Protein-1
ATP	Adenosine Triphosphate
B cells	Bursa-Derived Lymphocyte Cells
BIOM	Biological Observation Matrix
BMI	Body Mass Index
BOP	Bleeding On Probing
bp	Base Pair
BSA	Bovine Serum Albumin
CAL	Clinical Attachment Loss
Cathelicidin LL-37	Active Form Of Cathelicidin Antimicrobial Peptide
CD-14	Cluster Of Differentiation 14
CD4	Cluster Of Differentiation 4
CLC	Cardiotrophin-Like Cytokine
CNTF	Ciliary Neurotrophic Factor
CT-1	Cardiotrophin 1
CXCL-8	C-X-C Motif Ligand 8 (IL-8)
DNA	Deoxyribonucleic Acid
dNTP	Deoxy Nucleoside Triphosphates
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELIspot	Enzyme-Linked Immunosorbent Spot
EPS	Extracellular Polymeric Matrix
ERK	Extracellular Signal-Regulated Kinase
FASTA	Fast-All
FASTQ	Fast-quality
g	gravitational force
GCF	Gingival Crevicular Fluid
GDP	Gross Domestic Product
gp	Glycoprotein
H	Healthy

H_2SO_4	Sulfuric Acid
HCl	Hydrochloric Acid
IFN-α	Interferon Alpha
IFN-γ	Interferon-Gamma
i.e.	that is (Latin = id est)
Ig	Immunoglobulin
IL	Interleukin
IL-1ra	Interleukin-1 Receptor Antagonist
IL-6R	Interleukin 6 Receptor
IL-R	Interleukin Receptor
IQR	Inter-Quartile Range
IRAKs	IL-1R-Associated Kinases
JNK	C-Jun N-Terminal Kinase
kDa	Kilodaltons
LIF	Leukaemia Inhibitory Factor
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinases
ml	Milliliter
mm	Millimeter
MMP	Matrix Metalloproteinase (Such As MMP-8)
MPC	Milk Protein Concentrate
MspTL	Major Surface Protein of Treponema lecithinolyticum
Myd88	Myeloid Differentiation Factor-88
MYR	Malaysia Ringgit
NF-Kb	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NK cells	Natural Killer Cells
ODF	Osteoclast Differentiation Factor
OPG	Osteoprotegerin
OPGL	Osteoprotegerin Ligand
OSM	Oncostatin M
OTU	Operational Taxonomic Unit
P	Periodontitis
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffer Saline

PC	Principal Component
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PDL	Periodontal Ligament
pg	Picogram
PGE_2	Prostaglandin E_2
PMN	Polymorphonuclear Leukocyte
PPD	Periodontal Pocket Depth
PPi	Inorganic Pyrophosphate
PRRs	Pattern Recognition Receptors
QIIME	Quantitative Insights into Microbial Ecology
R & D	Research and Development
RANKL	Receptor Activator of Nuclear Factor-Kb Ligand
RBCs	Red Blood Cells
RDP	Ribosomal Database Project
RNA	Ribonucleic Acid
RDP	Ribosomal Database Project
RNAase	Ribonucleases
ROS	Reactive Oxygen Species
SD	Standard Deviation
SEM	Standard Error Of Mean
SYBRsafe	Synergy Brands, Inc
T cells	Thymus Cells
TAK-1	Transforming Growth Factorb-Activated Kinase-1
TBE	Tris/Borate/Edta
TGF-β	Transforming Growth Factor-B
Th	T Helper Cell (Such As TH1, TH2)
Th17	T Helper Type 17
TIR	Toll-IL-1 Resistance
TNF-α	Tumor Necrosis Factor- α
TRAF6	Tumour Necrosis Factor Receptor-Associated Factor 6
TRANCE	TNF Related Activation-Induced Cytokine
UniFrac metric	Unique Fraction Metric
UK	United Kingdom

µl	Microlitre
USA	United States of America
USM	Universiti Sains Malaysia
V_4	16S Fourth Hypervariable Region
V_6	16S Sixth Hypervariable Region
w/v	Weight/Volume

LIST OF APPENDICES

PENYELIDIKAN INTRA-SUBJEK MENGENAI PERANAN EKSPRESI IL-8, IL-6 DAN IL-1α YANG DISEBABKAN PLUG GIGI DAN KAITANNYA DENGAN MIKROBIOMA PLUG SUBGINGIVAL

ABSTRAK

Matlamat: Kajian ini bertujuan untuk menilai komposisi mikrobioma plak subgingival dan mengukur tahap GCF IL-8, IL-6, dan IL-1α dari kawasan yang sihat dan periodontitis pada subjek yang berpenyakit periodontitis. Kajian ini juga meneroka hubungan antara komposisi plak subgingival dan tahap GCF IL-8, IL-6, dan IL-1α di tempat yang berpenyakit dan sihat. **Bahan dan kaedah:** Kajian ini merangkumi 34 subjek periodontitis untuk mengumpulkan plak subgingival dan sampel GCF dari kawasan yang sihat dan periodontitis. DNA yang diekstrak dari plak gigi dihantar ke makmal Illumina untuk analisa metagenomik 16s rRNA. Kawasan V_3 dan V_4 RNA 16s diperkembangkan. Perpustakaan diurutkan dengan Sistem MiSeq, dan data dianalisis menggunakan MiSeq Reporter. Selepas itu, 313480 urutan penyaringan kualiti diperoleh dan disusun dalam OTU berdasarkan ambang 97%. Sampel GCF dianalisis menggunakan ELISA untuk mengukur tahap IL-8, IL-6 dan IL-1α. Ketumpatan optik ditentukan, dan lengkung standard dihasilkan menggunakan lengkung logistik empat-parameter yang sesuai. **Hasil:** Analisis rRNA 16s menunjukkan bahawa kawasan periodontitis menunjukkan kepelbagaian spesies yang lebih tinggi daripada kawasan sihat. Di samping itu, tidak ada corak perkelompokan spesies yang jelas dalam sampel periodontitis dan sihat. Sebanyak 19 filum, 142 genera, dan 56 spesies dikesan. Hasil ELISA menunjukkan kecenderungan untuk tahap IL-8 dan IL-6 yang lebih tinggi di tempat periodontitis dan tahap IL-1 yang lebih tinggi di tempat yang sihat. Walau bagaimanapun, perbezaan antara kawasan sihat dan periodontitis tidak signifikan secara statistik. Di samping itu, terdapat juga kecenderungan hubungan positif dan negatif antara parameter periodontal (PPD, CAL,

dan BOP) dengan IL-8, IL-6, dan IL-1α. Walau bagaimanapun, penemuan ini tidak signifikan secara statistik. Hubungan antara spesies bakteria dan IL-8, IL-6, dan IL-1α juga disiasat. Hasil kajian kami menunjukkan korelasi yang signifikan secara statistik (gabungan korelasi negatif dan positif) antara beberapa spesies bakteria dan sitokin (IL-8, IL-6 dan IL-1α). Sebagai contoh, di lokasi periodontitis, IL-8 menunjukkan korelasi positif yang kuat secara statistik dengan *Bacillus thermoamylovorans*. **Kesimpulannya:** Penemuan ini menunjukkan bahawa mikrobioma tertentu boleh menyebabkan sistem imun perumah mengeluarkan sitokin pro-radang seperti IL-8, IL-6, dan IL-1α. Lebih-lebih lagi, bakteria yang berkaitan dengan penyakit seperti Phlyum (Spirochaetes, TM7 dan Synergestetes), genus (*Porphyromonas, Filifactor,* dan *Peptococcus*) dan spesies (*Prevotella intermedia, Prevotella copri, Prevotella nanceiensis,* dan *Treponema socranskii*) mungkin tinggi dalam kawasan yang sihat dan mungkin dapat membantu peralihan dari keadaan sihat kepada penyakit periodontal.

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ABSTRACT

Aims: The study aims to evaluate the subgingival plaque microbiome composition and measure the GCF level of the IL-8, IL-6, and IL-1α of healthy and periodontitis sites in the same periodontitis subject. The study also explored the association between subgingival plaque composition and GCF levels of IL-8, IL-6, and IL-1α at periodontitis and healthy sites. **Materials and methods:** This study included 34 periodontitis subjects to collect the subgingival plaque and GCF samples from the periodontitis and healthy sites. The extracted DNA from dental plaque was sent to Illumina lab for 16s rRNA metagenomics. The V_3 and V_4 region of 16s RNA were amplified. The library was sequenced with MiSeq System, and data was analysed using MiSeq Reporter software (Illumina, San Diego, CA). After, quality filtering 313480 sequences were obtained and arranged in OTU based on 97% threshold. The GCF samples were analysed using ELISA to measure the level of IL-8, IL-6 and IL-1α. The optical density was determined, and standard curve was generated using a four-parameter logistic curve fit. **Results:** The analysis of 16s rRNA revealed that periodontitis sites showed a higher species diversity than healthy sites. Moreover, there was no apparent clustering of healthy and periodontitis samples. Total 19 phylum, 142 genera, and 56 species were detected. The ELISA results show a trend of higher IL-8 and IL-6 concentrations in periodontitis sites and higher IL-1 concentrations in healthy sites. However, the differences between healthy and periodontitis sites were not statistically significant. Moreover, there was also a trend of positive and a negative correlation between periodontal parameters (PPD, CAL, and BOP) with IL-8, IL-6, and IL-1α. However, these findings were not statistically significant. The correlation

between IL-8, IL-6, and IL-1α and bacterial species was also investigated. Our results show a statistically significant correlation (a mix of negative and positive correlation) between some bacteria species and cytokines (IL-8, IL-6 and IL-1α). For example, at the periodontitis site, IL-8 showed a statistically significant strong positive correlation with *Bacillus thermoamylovorans*. **Conclusion:** These findings suggests that certain microbiome may possibly cause the host immune system to secrete pro-inflammatory cytokines such as IL-8, IL-6, and IL-1α. Moreover, disease-related bacteria such as Phlyum (Spirochaetes, TM7 and Synergestetes), genus (Porphyromonas, Filifactor, and Peptococcus) and species (*Prevotella intermedia, Prevotella copri, Prevotella nanceiensis,* and *Treponema socranskii)* may be found abundantly in healthy sites and may help in the shift from healthy to periodontal disease.

CHAPTER 1

INTRODUCTION

1.1 Periodontal disease

Periodontitis is described as chronic inflammatory disease of periodontium caused by bacteria. It is the most frequent reason of tooth loss (Richard P Darveau, 2010; Kinane *et al.*, 2017). The periodontium consists of supporting structure of teeth. It encompasses and supports the maxillary and mandibular teeth within the bone. Accumulation of plaque initiates gingival inflammation. Gingivitis is the reversible inflammation of gingiva in addition to bleeding, redness, and swelling. It is the early sign of the disease, and it is not associated with the loss of supporting structure of the tooth. Gingivitis, if left untreated, often leads to periodontitis that is irreversible. Periodontitis affects gingiva, periodontal ligaments, alveolar bone leading to gingival bleeding, increased pocket depth, tooth mobility, and eventually tooth loss (Ericsson *et al.*, 2009; Könönen *et al.*, 2019; Page *et al.*, 1978), which leaves the patient unable to eat and function properly.

1.2 Epidemiology

In the US, 53.1% of the adult population aged 30 to 60 years had been found to have ≥3mm of attachment loss, and 35% of the adults suffered from periodontitis. It was reported that males have a high prevalence of periodontal disease than females (Albandar *et al.*, 1999). National Health and Nutrition Examination Survey of the US of 2009 and 2010 revealed that 64.7 million adults of US had periodontitis, and a considerable amount of the money spent on treatment and prevention, which also put pressure on the economy (P.I. Eke *et al.*, 2012). While the combined report for 2009-2014 revealed that 42% of the US population had periodontitis (P.I. Eke *et al.*, 2012;

Paul I. Eke *et al.*, 2018b). Rise in advanced periodontitis prevalence in Malaysian population was observed from 6% in 1990 to 18.2% in 2010 (Jaafar *et al.*, 2021; Mohd Dom *et al.*, 2016). While a study reported that the average cost of managing periodontitis patients was MYR 2,820 (€705) for a patient per year, and MYR 376 (€94) per outpatient visit in the dental specialist clinic (Mohd-Dom *et al.*, 2014; Mohd Dom *et al.*, 2016). The periodontal disease eventually precedes tooth loss. Early prediction of the biomarkers and factors leading to the progression of the periodontal disease can save the patients periodontal health and money spent on the treatment.

1.3 Pathogenesis of the periodontal disease

1.3.1 Risk factors

Pathogenesis of the periodontal disease is multifactorial, and it includes the host reaction to biofilm, microorganisms, and other risk factors, for instance, obesity, poor oral hygiene, smoking, and other psychological factors. Obesity is interlinked with an increased probability of cardiovascular and other diseases that increase the mortality rate (AlRahimi *et al.*, 2020; Falagas and Kompoti, 2006; Pi-Sunyer, 2002). High BMI is interlinked with enhanced release of Inflammatory markers such as C-reactive protein and IL-6 by adipose tissue endocrine activity (Ellulu *et al.*, 2017; Fantuzzi, 2005; Suvan *et al.*, 2011). Inadequate brushing technique and not frequent visits to dental health care provider results in plaque and calculus build-up. Individuals with poor oral hygiene practices are two to five times more likely to have periodontitis than the person who practices good oral hygiene (Lertpimonchai *et al.*, 2017). Flossing and tooth brushing helped reduce gingival inflammation instead of only tooth brushing (Sambunjak *et al.*, 2011; Worthington *et al.*, 2019).

Several studies have reported higher risk of periodontal disease in smoker as compared to non-smoker individuals. Moreover, the increased risk is proportional to duration and rate of smoking (Bergstrom, 2014; Do *et al.*, 2008; Paul I. Eke *et al.*, 2018a; Jiang *et al.*, 2020). Smokers exhibit deeper periodontal pockets, severe attachment loss and higher risk of tooth loss (Baljoon *et al.*, 2005; Jiang *et al.*, 2020; G. K. Johnson and Guthmiller, 2007; Ylöstalo *et al.*, 2004). Smoking increases the host susceptibility and risk of infection by inducing immune dysfunction (Jiang *et al.*, 2020; J. Lee *et al.*, 2011; Ryder, 2007). Stressful events like the death of loved ones or unemployment, or unsatisfactory life increase the person's susceptibility to periodontitis. The way the person perceives and reacts to the stress also affects their tendency to have periodontal disease (Hugoson *et al.*, 2002; Vasiliou *et al.*, 2016). The reason could be a high level of stress hormone like cortisol in the saliva, which is also related to bone loss.

1.3.2 Biofilm

"Biofilms have been defined as matrix-embedded microbial populations adherent to each other or surfaces or interfaces" (Jiao *et al.*, 2019; Marsh, 2005). Plaque is formed when there is pellicle formation. In few hours, bacteria attach to the pellicle, and a slime layer is formed around the attached bacteria (Gibbons and Nygaard, 1970; Rosan and Lamont, 2000). More than 700 bacterial species can be found in the oral cavity (Kilian *et al.*, 2016). The Gram-negative and anaerobic bacteria increases in the mature subgingival dental plaque associated with periodontal disease (Lazar *et al.*, 2017; Pihlstrom *et al.*, 2005; Ramberg *et al.*, 2003).

1.3.3 Microorganisms of the biofilm

The microorganisms like *Porphyromonas gingivalis, Tannerella forsythensis*, and the oral spirochete *Treponema denticola* are associated with periodontal disease.

Treponema denticola and *Porphyromonas gingivalis* are strongly associated with clinical measurement of severe periodontal disease such as periodontal pocket depth and bleeding on probing (Holt and Ebersole, 2005; Lamont and Jenkinson, 1998; H. M. Ng *et al.*, 2015, 2019; S S Socransky *et al.*, 1998). They release bacterial leucotoxins, collagenases, fibrinolysins, and other proteases (Mohanty *et al.*, 2019; Syed and Loesche, 1978). The bacteria release the proteolytic enzymes, which destroy the tissues. The release of microbial antigen causes humoral antibody-mediated and cell-mediated immune responses, which cause the breakdown of both soft and hard tissues (Reddy, 1997). The rapid renewal and shedding of junctional epithelial cells together with gingival crevicular fluid flow are efficient to inhibit colonisation by the bacteria (Dale and Fredericks, 2005; Pöllänen *et al.*, 2012; Subbarao *et al.*, 2019). The expression of proteases activated receptors and toll-like receptors by oral epithelial cells lead to the secretion of pro-inflammatory cytokines such as IL-6, IL8, and IL-1β, in response to *Porphyromonas gingivalis* (Jia *et al.*, 2019; Ren *et al.*, 2005). Defensins recruits immune cells in healthy and disease, thus it assists in host defence and homeostasis (Greer *et al.*, 2013; D. Xu and Lu, 2020).

In 1965, Loe suggested that dental plaque causes gingivitis. He conducted an experiment on periodontally healthy participants and induced experimental gingivitis with no oral hygiene measures. He clinically examined the participant's plaque in three phases. In the first phase (healthy phase), cocci flora was increased. Also, leukocytes on the gingival margin were observed. The second phase showed bacterial proliferation started two to four days after the oral hygiene measure was stopped. Filamentous forms (*Leptotrichia* and *Fusobacteria*) and slender rods were increased in strength and cocci. In the third and last phase of transition, vibrios and Spirochetes were seen. Cocci, rods and filamentous organisms were also present in large quantities. The transition from the

second to the third phase took six to ten days after oral hygiene was stopped (Löe *et al.*, 1965).

In 1998, Socransky first classified the bacteria from subgingival dental plaque in red, orange, green, purple, and yellow complex based on cluster analysis. The red cluster complex consists of *Porphyromonas gingivalis. Bacteroides forsythus* and *Tannerella. denticola.* They are found to be more associated with periodontal diseases. The orange complex consists of *Fusobacterium nueleatum* subspecies, *Provetalla intermedia, Provetalla nigrescens, Peptostreptoeoccus micros, Campylobacter rectus, Campvlobacter showae, Campylobacter gracilis, Eubacterum nodatum* and *Streptococcus constetllatus.* The red and orange complex is found to be closely related to each other. Even to this extent that without orange complex, it is hard to find red complex. The increased occurrence of the red complex occurs with an increase in pocket depth and bleeding on probing. He also concluded that the sites which have none of the bacteria from the red complex have shallow pocket depth while the sites which contained all three bacteria from red complex had deep pocket depth. Also, sites with *Porphyromonas gingivalis* had the deepest mean deep pocket depth. The green cluster complex consists of *Catnpylobacter conclsus, Eikenella corrodens* and *Actinobacillus actinomycetetncomitans* serotype. *Streptococcus mitis*. *Streptococcus sanguis* and *Streptococcus oralis* formed the yellow cluster. The *Actinotnvces odontolyticus* and *Veillonella parvula* are members of the purple cluster. Members of the group yellow and green complex were not as much similar to one another. While the species in the purple complex were more similar to one another, and they were least similar to the members of orange, green, and yellow complexes (Boutin *et al.*, 2017; S S Socransky *et al.*, 1998).

Several researchers have reported the difference in the healthy and periodontal bacterial composition. The composition of healthy subgingival plaque shows the dominance of streptococci. In comparison, the predominance of *Actinomyces spp*., anaerobic bacteria such as *Capnocytophaga*, *Fusobacterium*, and *Prevotella* species are seen in periodontitis (Curtis *et al.*, 2020; H. J. Lee *et al.*, 1995; W. E. C. Moore, 1987). The microbes such as *Porphyromonas gingivalis, Tannerella forsythia,* and *Actinobacillus actinomycetemcomitans are* suggested as causative agents in periodontitis (Curtis *et al.*, 2020; Timmerman and Weijden, 2006). The subgingival plaque at the periodontal pocket depth of more than 4mm contains *Porphyromonas gingivalis* and *Treponema denticola*. Moreover, *Treponema denticola* was observed in a surface layer of subgingival plaque and *Porphyromonas gingivalis* were found mainly in the deeper layer (How *et al.*, 2016; Kigure *et al.*, 1995).

The two most common Enterococci species are *Enterococcus faecalis* and *Enterococcus faecium* (Asfaw, 2019; Jakubovics and Grant Burgess, 2015). *Enterococcus faecalis* was reported more in a patient with refractory periodontitis than the successively treated periodontically healthy patient (Asfaw, 2019; Balaei-Gajan *et al.*, 2010). *Enterococcus faecalis* was found to be responsible for the induction of T-cell proliferation, the release of tumor necrosis factor-beta (TNF-β), and gamma interferon (IFN-γ). *Enterococcus faecalis* is also reported to cause tissue damage by producing gelatinase, hyaluronidase, and cytolysin (C. V. de Almeida *et al.*, 2018; Hill *et al.*, 1994; Kayaoglu and Ørstavik, 2004; Sigmund S. Socransky and Haffajee, 2005; Takao *et al.*, 1997).

1.4 Role of biofilm in periodontal disease

Oral biofilms can be classified into supragingival dental plaque and subgingival dental plaque. Supragingival dental plaque is described as biofilm which forms on the surface above the gum line, while subgingival dental plaque forms below the gum line (Jakubovics and Grant Burgess, 2015; Lazar *et al.*, 2017). The number of microorganisms in most biofilms is 10%, remaining 90% volume consists of a matrix secreted mainly by the microorganism in the biofilm. Biofilms consist of the extracellular polymeric matrix (EPS), which comprises polysaccharides, proteins, lipids, and nucleic acid. EPS keeps cells in proximity and allows interaction among cells. The biofilm contains different enzymes to degrade nutrients from the food, recycling, and it also provides a protective barrier from different antimicrobial agents. Protein, DNA, and polysaccharides make biofilm architecture (Bowen *et al.*, 2018; Flemming and Wingender, 2010; Xavier and Foster, 2007). Dental biofilm can contain up to 10^{11} microorganisms per mg of dental biofilm. Many organisms found in the biofilm have links with other systemic diseases, such as *Pseudomonas aeruginosa*, reported in the dental biofilm and observed in hospitalized patients (Brzozowski *et al.*, 2020; R. Souto *et al.*, 2006).

1.4.1 Biofilm formation

The steps involved in the biofilm formation are, i) Pellicle formation, ii) initial adhesion, iii) maturation, and iv) dispersion. The individual single cells gather together to form biofilm and encompass by the small amount of exopolymeric substance. Cells produce the extracellular polymeric matrix, which forms a more irreversible attachment of the cells to the surface. Type IV pili have also been responsible for the attachment from reversible to irreversible. In the next step, microorganisms make the architecture of the biofilm. During this process, microorganisms living in this biofilm can change

their physiological processes such as that they can grow anaerobically depending on their niche. Then, the microcolonies can scatter from the biofilm and give rise to the individual or planktonic microorganisms (D. Allison, 1998; O'Toole and Kolter, 1998; Stoodley *et al.*, 2002; Verderosa *et al.*, 2019). Mature biofilm has a three-dimensional structure. In a biofilm, bacteria are covered and connected by an extracellular matrix (Karygianni *et al.*, 2020; Kobayashi, 2007). Factors such as twitching motility, growth rate, cell signalling, and Extracellular polymeric substances (EPS) production play an essential role in determining biofilm structure (Karygianni *et al.*, 2020; Stoodley *et al.*, 2002). Bacteria present in the biofilm converts sugars into acid and facilitate dental caries. Oral streptococci produce an extracellular matrix by converting sucrose to glucose or fructose (Du *et al.*, 2020; Koo *et al.*, 2013).

1.4.2 Host response

1.4.2(a) Introduction

The host immune response consists of innate immune response and adaptive immune response. Both are responsible for maintaining a normal healthy state. Anaerobic Gram-negative bacteria present in subgingival plaque are mainly responsible for causing periodontitis. Periodontitis is caused by direct and indirect effects. The direct effects are due to the release of bacterial pathogens products. The indirect effects are due to the immune response initiated by the pathogenic products released by bacteria which includes lipopolysaccharide, lipoteichoic acid, extracellular vesicles, enzymes such as hyaluronidase, proteinase, and collagenase, various toxins (Kachlany, 2010; Sell *et al.*, 2017; Sorsa *et al.*, 2011). The host immune response and bacterial interactions maintain the progression of periodontitis (Song *et al.*, 2017).

1.4.2(b) Innate immunity

In the innate immune response, pattern recognition receptors (PRRs) can identify bacterial pathogens. Toll-like receptor (TLR) is a type of PRRs that can recognize periodontal pathogens and regulates the innate response to pathogenic bacteria (Amarante-Mendes *et al.*, 2018; Y Zhang and Li, 2015). The specific immune cells and antimicrobial proteins perform the innate immune response in the periodontitis, and response is termed the complement system (Meyle *et al.*, 2017). The host immune response releases inflammatory mediators and cytokines in the periodontal tissues, trigged by the periodontal bacteria. The cytokines play an essential role in the pathogenesis of periodontal disease, which causes soft tissue destruction and bone resorption (Ramadan *et al.*, 2020; Ramberg *et al.*, 2003). The oral mucosa has epithelial cells which act as the first line of defence against bacterial pathogens. They are also responsible for expressing different PRRs like proteinase-activated receptors and TLRs by attaching to pathogen-associated molecular patterns (Giacaman *et al.*, 2009; Günther and Seyfert, 2018).

Fibroblasts and neutrophils also play significant role in periodontal disease. Fibroblasts are the primary immune cells that produce collagen and proteins for the extracellular matrix of the tissues. During inflammation, fibroblasts play an important role in pathogenesis. They produce tissue degrading enzymes, matrix metalloproteinases, and pro-inflammatory mediators such as IL-8, IL-6, chemokine ligand 8 (CXCL-8), and prostaglandin E2 by responding to bacterial invasion (Baek *et al.*, 2013; Frangogiannis, 2016). In bacterial invasion, fibroblasts respond by expressing protease-activated receptors, extracellular TLRs, and intracellular TLRs (Hosokawa *et al.*, 2010; Ramadan *et al.*, 2020). Neutrophils migrate to the infected tissues in response to bacterial peptides or other pro-inflammatory chemotactic mediators like IL-1, IL-8,

IL-6, and tumor necrosis factor-α (TNF-α) (Ramadan *et al.*, 2020; Trevani *et al.*, 2003). These cytokines cause periodontal destruction by stimulating bone resorption. Neutrophils can also phagocyte the bacteria and create the reactive oxygen species (ROS), which is essential for periodontitis (Ramadan *et al.*, 2020; White *et al.*, 2014). Dendritic cells also activate an immune response by acting as antigen-presenting cells or produces IL-12 and IL-18 that thus promote interferon-γ (IFN-γ) (Mbongue *et al.*, 2017; Tew *et al.*, 2012). Innate immunity provides the initial response to the pathogen.

1.4.2(c) Adaptive immunity

In adaptive immunity, the immune system distinguishes the cells different from other cells of the body. The receptors recognize the microbial pathogens on dendritic cells or macrophages. Dendritic cells capture the antigen and present it to B and T cells of the acquired immune system (Cutler and Jotwani, 2004; Marshall *et al.*, 2018). Activated CD4 T-helper produces cytokine. Cytokines such as IL-12 and IL-4 induce the Th-1 and Th-2 cells, respectively, responsible for the cell-mediated and humoral immune responses (H.-G. Lee *et al.*, 2020; Murphy and Reiner, 2002). T-cells are the main cause for the shift of gingivitis lesions. The balance between subsets of T cells (Th1 and Th2) facilitates the shift, while Th2 cells mediates periodontitis (Figueredo *et al.*, 2019; Ohlrich *et al.*, 2009). In the adaptive immune response, the serum antibodies, including immunoglobulin IgM, IgG, and IgA, are primarily produced and helps to protect against bacterial and viral infections (Ramadan *et al.*, 2020).

1.5 GCF

Gingival crevicular fluid (GCF) is serum transudate or exudate in the gingival crevice formed by the periodontal tissues. GCF contents are derived from serum, connective tissue, and epithelium (Bibi *et al.*, 2021; Lamster, 1997).

1.5.1 GCF contribution in health and disease of the periodontium

GCF is crucial for the structure of the junctional epithelium and the defence mechanism of the periodontium. The healthy sulcus contains a minimal amount of GCF. However, in inflammation, the GCF flow increases. It flushes bacterial colonies and their metabolites away from the sulcus, which helps host defence (Subbarao *et al.*, 2019).

Prostaglandin E_2 in GCF is found to be pro-inflammatory in many studies. Moreover, Prostaglandin E_2 promotes periodontal diseases by recruiting inflammatory cells, collagenase production, and osteoclast activation. Studies show that Elevated interstitial collagenase or Matrix metalloproteinase-1 (MMP-1) activities have been detected in GCF of aggressive periodontitis and localized juvenile periodontitis (Al-Majid *et al.*, 2018; Lamster, 1997). The production of reactive oxygen species in the gingival sulcus probably initiates the mechanism of periodontitis (Katsuragi *et al.*, 2003; Waddington *et al.*, 2008; Y. Wang *et al.*, 2017). The oxidative stress levels were reported to be more in the saliva of the periodontal disease group in comparison with gingivitis and periodontally healthy groups (Almerich-Silla *et al.*, 2015).

1.5.2 GCF as a diagnostic tool

GCF contains various inflammatory mediators, cytokines, leucocytes, enzymes, organic ions, tissue breakdown products, and proteins. These give the idea about the periodontium condition (Armitage, 2004; Bibi *et al.*, 2021). The different disease markers such as IL-Iα, IL-1β, TNF-α, enzymes such as acid phosphatase, alkaline phosphatase, matrix metalloproteinases, collagenases, elastase are widely used in periodontal research to assess the resolution of periodontal disease (Bibi *et al.*, 2021; Oswal and Dwarakanath, 2010). GCF has been used as a diagnostic tool in many studies. The benefits of using GCF as a constituent of analysis as diagnosis of

periodontal disease are that because GCF is present in every tooth, we can take multiple samples. Also, it is a non-invasive or slightly invasive procedure. As periodontal disease involves few teeth, we can take the sample from those teeth (Bibi *et al.*, 2021; Lamster, 1997; Subbarao *et al.*, 2019). GCF can be collected using intracrevicular washing technique, microcapillary technique, and absorption technique (Nazar Majeed *et al.*, 2016).

1.5.2(a) Intracrevicular washing technique

The method is mentioned briefly as following, A gel loading capillary tip is carefully inserted into the crevice at a level of approximately 1 mm below the gingival margin. 5 sequential washes with 10 µl of 0.9% sodium chloride are performed using a micropipette in each case. The washes is transferred into a microcentrifuge tube (Guentsch *et al.*, 2011; Güntsch *et al.*, 2006; Sigusch *et al.*, 1992). The washing technique could be used when other techniques fail. One study reported that they could only detect cathelicidin LL-37 in GCF by western blot technique only if GCF were sampled using the washing technique (Guentsch *et al.*, 2011; Puklo *et al.*, 2008). This method is not very commonly used because it is technique sensitive, and there is also the possibility of blood contamination (Nazar Majeed *et al.*, 2016)

1.5.2(b) Microcapillary technique

The method is as follows; the sites are air-dried and isolated with gauze pieces. Supragingival plaque is removed to avoid contamination. For GCF collection, 1–5 µl calibrated volumetric microcapillary pipette is placed extracrevicularly for 5–20 minutes without touching the marginal gingiva. The collected GCF is transferred to a centrifuge tube (Yadav *et al.*, 2014). This method's advantage is that there is less possibility of volumetric error that can happen in GCF collection by paper strip as there are some chances of binding of product to periodontal strips (Yadav *et al.*, 2014).

However, this method also has some disadvantages. It is a very time-consuming method which also increases the possibility of saliva and blood contamination (Nazar Majeed *et al.*, 2016).

1.5.2(c) Absorption technique

These are of two types. 1) Extra crevicular, the strips are overlaid on the gingival crevice region to minimize the gingival trauma. 2) Intra crevicular, the strips are inserted into the gingival crevice. The most common method is intra-crevicular absorption. The method is as follows. The site is isolated by inserting the cotton rolls and air-dried, and the paper strip is inserted in the gingival crevice until resistance is felt. The paper strip is left in the gingival crevice for 30 seconds to 1 minute. The strip is removed and stored in a microcentrifuge tube (Bathla, 2011). The procedure of collecting GCF by paper strips is used in many studies, and it is significantly less time-consuming as it only takes 30 seconds (Barros *et al.*, 2016; Bibi *et al.*, 2021). However, this collection method can have volumetric errors (Nazar Majeed *et al.*, 2016). Some researchers have suggested placing the periopaper in gingival crevice until it is "visibly wet," or until 30 seconds. Putting the periopaper in 1-2 mm into the periodontal pocket is sufficient to collect the GCF (Wassall and Preshaw, 2016).

1.6 Cytokines

1.6.1 Introduction

Cytokines are a group of small proteins responsible for cell signalling and communication and initiate several cellular changes (Andrukhov *et al.*, 2011). They control cell proliferation, immune and inflammatory response. Cytokines play a significant role in periodontitis. They activate and play a vital role in the differentiation of osteoblasts, activation, fibroblasts' proliferation, collagen production and

neovascularisation (Araujo-Pires *et al.*, 2014; Ferreira *et al.*, 2019). Cytokines include the subfamilies such as chemokines, interleukins, interferons, lymphokines, and tumor necrosis factor (Ramadan *et al.*, 2020). Chemokines attract lymphocytes, neutrophils, and macrophages to the inflammation site. They are responsible for the pathogenesis of various human immune diseases as they coordinate leukocyte recruitment and activation (Bonecchi, 2009; Ferreira *et al.*, 2019).

1.6.2 Cytokines in periodontitis

During inflammation, different immune cells release cytokines and chemokines. The T helper cells regulate the release of cytokines. Th1 cells induce the cellular immune response by releasing interleukins as TNF-α, IL-12, IL-1, IL-2, and IFN-γ. Th2 cells induce the humoral immune response by releasing IL-10, IL-5, IL-6, IL-4, and IL-13 (Ferreira *et al.*, 2019; Kany *et al.*, 2019; Mosmann and Sad, 1996). During inflammation, cytokine IL-17 stimulates the response of the Receptor activator of nuclear factor-κB ligand (RANKL), which is also called osteoprotegerin ligand (OPGL) or osteoclast differentiation factor (ODF) or TNF related activation-induced cytokine (TRANCE). It is essential for bone remodelling and repair as it regulates osteoclast formation, activation, and survival. The osteoclast is also responsible for bone resorption. During bone resorption, osteoclast attaches to the bone surface due to podosome and forms a tight seal with the bone. The release of proteolytic enzymes such as cathepsin K and hydrochloric acid (HCl) degrade and dissolve the bone minerals. The communication of RANK and RANKL starts the osteoclastogenic activity. RANKL is expressed on osteoblast and T cells, while its receptor RANK is on osteoclasts. Osteoprotegerin (OPG), a decoy receptor of RANKL, is produced by osteoblasts, and its function is to block the interactions of RANKL with RANK. Thus, it can also be termed as a bone protector. In the continuous phase of inflammation,

upregulated IL-17 promotes the inflammatory response, increases the production of RANKL, and promotes inflammatory bone loss (Teitelbaum and Ross, 2003; Wright *et al.*, 2008; Xiong *et al.*, 2019; S. Zhang *et al.*, 2017).

1.6.3 Pro-inflammatory cytokine

An inflammatory cytokine is an induced cytokine in the inflammatory process linked with the initiation and progression of inflammation. The activated macrophages release inflammatory cytokines. The pro-inflammatory cytokines include IL-1α, IL-1β, IL-6, IL-8, and TNF-α (Kany *et al.*, 2019; Okada and Murakami, 1998), IL-8 activates the neutrophil to degranulate, causing tissue damage. IL-1 and TNF are inducers of endothelial adhesion molecules necessary for leukocyte adhesion to the endothelial surface. IL-1 and TNF act together and initiates the cascade of inflammatory mediators. They also cause the synthesis of IL-8 by mesothelial cells (Bonaventura *et al.*, 2018; Dinarello, 2000; Elias and Lentz, 1990).

1.6.4 Anti-inflammatory cytokine

The increased production of pro-inflammatory cytokine increases the immunity of the host organism; however, it also causes damaging effects (C. L. Reid *et al.*, 2002). Anti-inflammatory cytokine includes IL-1 receptor antagonist, IL-4, IL-10, IL-11, and IL-13, whereas Leukemia inhibitory factor, interferon-alpha, IL-6, and transforming growth factor TGF-β are categorized as either anti-inflammatory or pro-inflammatory cytokines, depending on conditions. The anti-inflammatory cytokine control pro-inflammatory cytokine response (Pawluk *et al.*, 2020; J.-M. Zhang and An, 2007). They either block or suppress the intensity of the inflammation caused by pro-inflammatory cytokine. Anti-inflammatory cytokine suppresses genes for pro-inflammatory cytokines, e.g., IL-1, TNF, and chemokines (Dinarello, 2000; Tawfig, 2016). Such as, TGF-β reduces cytokine production by inhibiting macrophage and Th1 cell activity. It

also reduces IL-1, IL-2, IL-6, and TNF and stimulates the production of IL-1ra 6. TGF-β controls the development of innate cells, such as natural killer (NK) cells, macrophages, dendritic cells, and granulocyte (Dinarello, 2000; Roberts and Sporn, 1993; Sanjabi *et al.*, 2017).

IL-4, IL-10 and IL-13 activate B lymphocytes (Dinarello, 2000). They increase the bactericidal capacity of phagocytes. Also, these cytokines attract more innate immune cells to the infection sites and direct the response towards the invading pathogens (Hornef *et al.*, 2002; Tawfig, 2016; Xiao *et al.*, 2020). IL-10 activates the macrophages and thus reduces the expression of inflammatory cytokines such as TNF-α, IL-6, and IL-1. Moreover, IL-10 can irregulate the production and function of pro-inflammatory cytokine at different levels (Bakiri and Mingomata, 2019; Dinarello, 2000; Wieseler-Frank *et al.*, 2004). The balance between pro-inflammatory and anti-inflammatory cytokines may disturb and shift towards pro-inflammatory cytokine results in periodontal disease (Dinarello, 2000; Duruel *et al.*, 2020; Tawfig, 2016). In this thesis, we focus on the role of IL-6, IL-1, and IL-8 in periodontal disease. Hence, the detailed discussion about these cytokines is discussed in the following paragraph.

1.6.5 IL-6

IL-6 is a pro-inflammatory cytokine, and its family consists of IL-6, IL-11, ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin 1 (CT-1), cardiotrophin-like cytokine (CLC), and IL-27 (Rose-John, 2018). IL-6 family cytokines use the common signalling receptor subunit glycoprotein 130 kDa (gp130). Gp130 protein is expressed in all body cells (Rose-John, 2018; Rose-John *et al.*, 2015). IL-6 signalling requires the IL-6R and gp130. When IL-6 binds with IL-6R, it binds to gp130 and induces intracellular signalling (Ogata *et al.*, 2019; Reeh *et al.*, 2019; Taga *et al.*, 1989). IL-6 family induces B-cell

stimulation. Blockage of IL-6 was efficient in the blockage of tumor necrosis factor α in patients with rheumatoid arthritis (Gabay *et al.*, 2013; Pandolfi *et al.*, 2020). IL-6 is also associated with multiple sclerosis, Neuromyelitis optica spectrum disorder, metabolic syndrome, cardiovascular disease, autoimmune diseases, and cancer (Balkwill and Mantovani, 2001; Hirano, 1998; Ishihara and Hirano, 2002; Kong *et al.*, 2017). IL-6 are secreted by many immune cells such as dendritic cells, macrophages, B cells, and T cells, and non-immune cells, including fibroblasts, keratinocytes, and endothelial cells (Velazquez-Salinas *et al.*, 2019; J. Wolf *et al.*, 2014). IL-6 is also linked with plasma cell development and immunoglobulin production (Choy and Rose-John, 2017; Jones *et al.*, 2010; Lin *et al.*, 2015).

IL-6 also plays a vital role in bone homeostasis. It also up-regulates the expression of receptor activator of RANKL in osteoblast, leading to differentiation of osteoclasts and bone resorption (De Benedetti *et al.*, 2006; Q. Wu *et al.*, 2017). IL-6 has an essential role in enhancing T-cell proliferation and also increases osteoclast formation that causes bone resorption (Harmer *et al.*, 2019; Tamura *et al.*, 1993). IL-6 with TNF stimulates the production of matrix metalloproteinases which are involved in tissue destruction. MMP-8 is associated with gingival and periodontal ligament collagen degradation (Checchi *et al.*, 2020; Hill *et al.*, 1994). An experiment conducted on monkeys indicated that IL-6 in the initiation phase of periodontitis was high, but it remained low during the progression and resolution phase (Ebersole *et al.*, 2014; Pan *et al.*, 2019). This shows that IL-6 may play a significant role in the periodontitis initiation and acute phase (Pan *et al.*, 2019).

IL-6 is produced by bacterial lipopolysaccharide or IL-1β or TNF-α in inflamed tissues in response to cellular activation (Irwin and Myrillas, 1998; Ishihara and Hirano, 2002; Tawfig, 2016). Moreover, inflamed periodontal tissue locally produces IL-6

(Takahashi *et al.*, 1994; Tawfig, 2016). IL-6 levels in GCF have also been higher in periodontitis subjects than healthy subjects (Pan *et al.*, 2019; Stadler *et al.*, 2016). Moreover, a significant correlation between periodontal pocket depth and IL-6 in the gingival crevicular fluid has been reported (McGee *et al.*, 1998; Tawfig, 2016).

1.6.6 IL-1

IL-1 is a cytokine and includes IL-1α, IL-1β, IL-18, IL-33, IL-36, IL-37, and IL-38. IL-1 family consists of 11 molecules and 10 receptors. IL-1 family belongs to innate and adaptive immunity and is also related to inflammation, autoimmunity, cardiovascular disorder, and cancer (Mantovani *et al.*, 2019). The binding with the receptor recruits the adaptor protein myeloid differentiation factor-88 (Myd88) by the shared intracellular signalling domain named the Toll-IL-1 resistance (TIR) domain. The binding activates the proteins such as IL-1R-associated kinases (IRAKs) and tumor necrosis factor receptor-associated factor 6 (TRAF6), which leads to the activation of inflammatory-related transcriptional factors including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and c-Jun N-terminal kinase (JNK) (Dinarello, 2009; Saikh, 2021). IL-1α, with the help of IL-23 and IL-6, drives the type 3 immunity and also the expression of IL-17 (Langrish *et al.*, 2005; Pan *et al.*, 2019; Tsukazaki and Kaito, 2020).

The release of IL-1α and IL-1β from oral epithelial cells is triggered by LPS and leukotoxin from the bacterial pathogen contributing to periodontal destruction (Chompunud Na Ayudhya *et al.*, 2020). Many studied have reported a higher level of IL-1 in periodontitis in comparison with healthy. The level of IL-1 in gingival tissue reduced after treatment which indicates the active role of IL-1 in periodontal disease (Kurtiş *et al.*, 2005; Romano *et al.*, 2018; Thunell *et al.*, 2010; Yucel-Lindberg and Båge, 2013). However, another study showed the concentration of IL-1in moderate pocket depth didn't reduce, even after periodontal treatment, which could indicate the

residual pro-inflammatory periodontal pocket state (Romano *et al.*, 2020). IL-1 can induce fibroblast to secrete prostaglandin E_2. It induces many cell types to secrete cytokines and MMP's thus causes bone resorption. IL-1 also activates B and T lymphocyte for Immune response (Arasa *et al.*, 2019; Dinarello, 2000; Yucel-Lindberg and Båge, 2013). Thus, IL-1 plays an essential role in the homeostasis of periodontal disease, and its unrestrained production can cause tissue damage (Okada and Murakami, 1998; Papathanasiou *et al.*, 2020; Tawfig, 2016). IL-1β and IL-6 have been linked with vasodilation, inflammatory cell migration, and osteoclastogenesis (Cekici *et al.*, 2014; Fonseca *et al.*, 2009; Kany *et al.*, 2019). IL-1β has been considered an important biomarker to detect the periodontitis severity, progression, tooth loss, and success of the periodontal therapy (Graves and Cochran, 2003; Papathanasiou *et al.*, 2020).

1.6.7 IL-8

IL-8 is the pro-inflammatory chemokine that acts on CXCR1 and CXCR2 receptors. Other chemokine members such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL7 also bind to CXCR1 and CXCR2 receptors. IL-8 is found to be associated with atherosclerosis, cancer, inflammatory bowel disease, ischemia & reperfusion injury, acute lung injury, asthma, Chronic obstructive pulmonary disease, Pulmonary fibrosis, multiple sclerosis, and urological & reproductive diseases (Ha *et al.*, 2017; Q. Liu *et al.*, 2016; Russo *et al.*, 2014). IL-8 is released by different cells such as epithelial and endothelial cells, monocytes, lymphocytes, and fibroblasts (Ha *et al.*, 2017; George T.-J. Huang *et al.*, 1998).

IL-8 is found to be present in the damaged periodontal tissues, and their production is involved in chronic leukocyte recruitment, tissue, and bone destruction (Bascones *et al.*, 2005; George T.-J. Huang *et al.*, 1998; Madianos *et al.*, 2005). The expression of IL-8 has been found to increase in gingival tissue of aggressive

periodontitis (Kozak *et al.*, 2020; R. K. Liu *et al.*, 2001). The level of IL-8 in the periodontitis sites was increased compared to healthy sites (Ertugrul *et al.*, 2013; Finoti *et al.*, 2017; Jorge Gamonal *et al.*, 2001). Moreover, IL-8 concentration decreased after the periodontal treatment (Finoti *et al.*, 2017; Jorge Gamonal *et al.*, 2001).

IL-8 recruits the neutrophils at the site of bacterial biofilm, which in response secrete cytokines and metalloproteinases 8 and 9, which degrades the extracellular matrix and signalling other effector cells to produce matrix metalloproteinases. The matrix metalloproteinases 8 is found in GCF and saliva in diseased periodontal tissue and causes degradation of interstitial collagens (Cekici *et al.*, 2014; Checchi *et al.*, 2020; Sorsa *et al.*, 2006). IL-8 is a polymorphonuclear leukocyte chemoattractant. It is detected in both healthy and diseased periodontal tissues and also found in inflammation (Baggiolini *et al.*, 1995; Cekici *et al.*, 2014; Mathur *et al.*, 1996; Payne *et al.*, 1993).

1.7 Interleukin Correlation with Bacteria

1.7.1 Introduction

Bacteria contain lipopolysaccharide in their outer membrane, triggering the innate immune response (Hans and Hans, 2011). The in vitro studies reported that *Porphyromonas ginigvalis* activates the immune response, which challenges the human gingival fibroblast and immune cells like monocytes and macrophages, and results in an increase of inflammatory cytokine and chemokines in 6 hours (Ehrnhöfer-Ressler *et al.*, 2013; Josino Soares *et al.*, 2014; Schueller *et al.*, 2015, 2017). Microorganisms, specially *Porphromonas gingivalis* lead to the production of inflammatory cytokines such as IL-8, IL-6, and IL-1β (P. L. Wang and Ohura, 2002). In normal healthy tissues, the innate immune response is in homeostasis to maintain a healthy environment and low inflammatory cytokine level (Richard P Darveau, 2010; Schueller *et al.*, 2017).

However, it is disrupted in an inflammatory state like gingivitis and periodontitis. Bacterial communities shift, and the host homeostasis pathway is changed that causes migration of macrophages and increases levels of inflammatory markers. It also activates fibroblasts and endothelial cells (Bartold and Narayanan, 2006).

1.7.2 Dysbiosis in periodontium

The shift in the cytokines can be associated with a switch in the composition of the microbial community. They also affect the inflammatory reaction in the host (Zhou *et al.*, 2017). Several studies have demonstrated that many bacteria affect cytokine production during health and disease (Andrukhov *et al.*, 2011; Shaker and Ghallab, 2012), while every element of the biological system affects the other element of the system. The single microbiome is not responsible for the disease, but the whole ecology is responsible for the shift (Abusleme *et al.*, 2013; Schwarzberg *et al.*, 2014; Zhou *et al.*, 2017). The microorganism present in the gingival crevice can also affect the cytokine level, such as *Parvimonas micra, Corynebacterium matruchotii, Actinomyces sp., Fusobacterium nucleatum*, and *Pseudomonas fluorescens.* The shift in the cytokines can be associated to the shift in the composition of the microbiota, which effects the host inflammatory reaction (Zhou *et al.*, 2017). *Fusobacterium nucleatum* produces metabolites such as hydrogen sulfide (H2S). The H2S can induce the secretion of pro-inflammatory cytokines IL-1β and IL-18 in monocytes in vitro by the formation of NLRP3 inflammasome (Basic *et al.*, 2017; Dahlen *et al.*, 2019). *Pseudomonas aeruginosa* causes diseases by secretion of various compounds such as exotoxin A, elastase, exoenzyme S. Exotoxin A causes disease by inhibiting protein synthesis and interfering with host immune functions (Weber *et al.*, 2016; Wick *et al.*, 1990; P. Wolf and Elsässer-Beile, 2009).Therefore, microbial community and the relationship between cytokine and microbiomes can be a dominant etiological pattern.

1.7.3 Interaction of bacteria and interleukin

Bacterial pathogens repeatedly interact with gingival epithelial cells, fibroblast, and inflammatory cells and activate the toll-like receptor essential for the innate host immune system. Bacterial lipopolysaccharide binds to TLR-4 with the help of CD-14, adapter proteins including MyD88, IL-1-receptor-associated kinase (IRAK), IRA2, Tollip, TNF receptor-associated factor-6 (TRAF-6), and TAK-I are recruited. Several kinases are activated, which results in the activation of transcription factors. Thus, leading to the production of IL-1β, TNF-α, and IL-6 (O'Neill, 2002). Gingival epithelial cells, fibroblast, and inflammatory cells act in response to lipopolysaccharides by increasing the expression of cytokines and growth factors. This is facilitated by Toll-like receptor and CD-14 (Putnins *et al.*, 2002; P. L. Wang and Ohura, 2002).

1.8 Dental plaque analysis

There are many methods to analyse the bacteria in plaque. In the following section, cell culture, nucleic acid hybridisation, polymerase Chain reaction (PCR), Sanger sequencing method, pyrosequencing, shotgun sequencing, and metagenomics are discussed.

1.8.1 Bacterial culture

In 1673, dutch microscopist Antonie Van Leeuwenhoek observed the bacteria for the first time and termed them as "animalcules" (Summers, 2009). Bacterium were first grown in artificial liquid culture medium in a reproducible way by Louis Pasteur in 1860. Before that bacterial growth was only observed on some food (Bonnet *et al.*, 2020). Robert Koch proposed that a pure culture is the foundation of all research in infectious disease. The bacterial culture is important for the study of bacteria virulence,

antibiotic susceptibility, and genome sequence. Which makes it easier to understand and treat the disease caused by that bacterium (Lagier et al., 2015).

The method of bacterial culture is as follows: the bacterial samples are transferred to an appropriate medium, transported, and stored, followed by dispersion. Bacteria are then plated onto various culture media in the laboratory. The bacteria are then isolated and characterised by colony morphologies and biochemical testing (Benn *et al.*, 2018). However, there are limitations in this method, such as that it is not feasible to culture all the bacteria. Especially, strictly anaerobic bacteria, which can die in oxygen, and viable bacteria that are non-culturable, are difficult to culture in the lab. Also, it is a time-consuming and labour-intensive procedure (Hudu, 2016; Lagier *et al.*, 2015). A specific biochemical test is required to identify certain microbes, for which facility may not be present in a few labs in low or middle-income countries (Nkengasong *et al.*, 2018).

1.8.2 Nucleic acid hybridization

Nucleic acid hybridization is another common method for the identification of bacteria. In nucleic acid hybridization, DNA, RNA, or protein sequence needs to be identified, and probe molecule which identifies the target by hybridization. Hybridization that takes place on the solid carrier is called blotting. The blotting is divided into 4 types, 1) Southern blots, whereby DNA molecules are identified using small DNA probes, 2) Northern blotting whereby specific RNA molecules are identified using RNA or DNA probes, 3) Western blotting whereby protein sequences are identified using specific antibodies (Bhagavan and Ha, 2015), 4) Eastern blotting whereby proteins, carbohydrates and lipids are identified (S. Thomas *et al.*, 2009). The method involving hybridisation is DNA–DNA hybridisation. The checkerboard DNA–DNA hybridization technique was applied extensively by Socransky (S S Socransky *et*

al., 1994) and involved the hybridisation of 45 DNA samples against 30 DNA probes on a single membrane. The bacterial DNA is deposited in vertical lines on a nylon membrane. The DNA of a plaque sample is extracted and labelled with digoxigenin to be used as a probe. Digoxigenin-labelled whole genomic DNA probes are run horizontal to the samples. Then, washing is performed, and the bound probe is detected and quantified by an anti-digoxigenin antibody with a chemifluorescent substrate. The main advantage of this technique is that it is a very high throughput technique, and thousands of samples can be analysed. However, the major disadvantage is that unknown cross reactivity may occur with unknown taxa present in the sample. Also, this technique can only provide information about culturable taxa and the information about unculturable taxa is lost (Bass *et al.*, 2017; Spratt, 2004).

1.8.3 Polymerase chain reaction (PCR)

PCR is a commonly used detection method for the oral microbiome. The method is as following, DNA molecule is denatured at 95°C, which causes double-stranded DNA molecule to separate. The temperature is then reduced to 72°C, which allows oligonucleotide primers to anneal. The enzyme *Taq* polymerase is added, which can sustain high temperature and only copy those DNA molecules that have primers. The primers then direct *Taq* polymerase to synthesise a complementary strand of DNA from the free nucleotide. The process is repeated multiple times with DNA with the target sequence. Two partially double strands of DNA double molecules are formed. The temperature is again raised to 95°C, which initiates the reoccurrence of the reaction. Many amplicons are formed, which are the copies of the short fragments of the target DNA. The significant advantage of PCR is its high sensitivity compared to culture and staining, and it can be quickly performed (4-8 hours). However, it still has some