"MICROBIAL ANALYSIS OF ORANGE COMPLEX ORGANISMS OF THE WHOLE SALIVA IN PATIENTS WITH GINGIVITIS AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING"

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfilment of the degree

MASTER OF DENTAL SURGERY

BRANCH II

PERIODONTOLOGY

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **MICROBIAL ANALYSIS OF ORANGE COMPLEX ORGANISMS OF THE WHOLE SALIVA IN PATIENTS WITH GINGIVITIS AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. K.V.ARUN, M.D.S.,** Professor and Head of the department, Department of Periodontology, Ragas Dental college & Hospital, Chennai.

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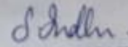

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CERTIFICATE

This is to certify that this dissertation titled **MICROBIAL ANALYSIS OF ORANGE COMPLEX ORGANISMS OF THE WHOLE SALIVA IN PATIENTS WITH GINGIVITIS AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING** is a bonafide record of work done by **Dr INDHUMATHI S** under my guidance during my study period of 2017-2020.

The dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY** in partial fulfilment of the degree **MASTER OF DENTAL SURGERY, BRANCH II – PERIODONTOLOGY.** It has not been submitted (Partial or full) for the award of any other degree or diploma.

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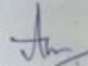

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LIST OF ABBREVATIONS

ABBREVATION	EXPANSION
PSD	Polymicrobial Synergy and Dysbiosis
DNA	Deoxyribonucleic acid
rRNA	Ribosomal Ribonucleic acid
HOMIM	Human Oral Microbiome Identification Microarray
NGS	Next Generation Sequencing
MSR	MiSeq Reporter software
HOMD	Human Oral Microbiome Database
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
SoLiD	Supported Oligonucleotide Ligation and Detection
BLAST	Basic Local Alignment Search Tool
HOT	Human Oral Taxon number

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ABSTRACT

ABSTRACT

Background:

Gingivitis is a reversible non-specific inflammatory reaction of the marginal gingiva but always precedes periodontitis. Gingival recession may occur as loss of attachment and inflammatory exacerbation due to accumulation of local factors, which results in the apical migration of gingival margin. It may be a phenotypic form of periodontitis. Individual susceptibility may be important for transition from gingivitis to periodontitis and has been examined using various risk markers such as genetic, microbial and immunological. The orange complex bacteria were thought to be the bridging species that represent a change between gingivitis and periodontitis, may be used as putative risk markers. Therefore, this study aims to analyse and compare the prevalence of salivary orange complex bacterial species in periodontal health, gingivitis and gingival recession.

Materials and methods:

In this study, Subjects were Periodontally evaluated and allocated into three groups as healthy controls (ten subjects), Gingivitis patients (ten subjects) and Gingival recession (ten patients). Orange complex microbiome was evaluated from Gingival health, Gingivitis and Gingival recession individuals using NGS technology with Illumina sequencing. Amplicons from V3-V4 hypervariable regions of 16S rRNA gene were sequenced. The frequency of distribution of Orange complex bacteria in Health, Gingivitis and Gingival recession were measured with Chi-square test.

Results:

There was a statistically significant increase in the distribution of 5 organisms (Prevotella nigrescens P=0.008, S. constellatus P= 0.001, C. rectus P=0.014, P. intermedia P=0.015, C. gracilis P=0.001) in gingivitis and gingival recession group when compared to health. There was no statistically

significant difference in distribution of Orange complex organisms between the gingivitis & gingival recession group.

Conclusion:

Members of the orange complex seem to be suitable candidates for use as microbial risk factors in gingivitis and gingival recession.

Keywords:

Salivary microbiome, Illumina Sequencing, Next Generation Sequencing, 16S rRNA, Periodontitis, Dysbiosis.

INTRODUCTION

INTRODUCTION

Periodontal diseases are polymicrobial, multifactorial diseases with many host related factors that are involved in determining the individual susceptibility to the disease[1]. Over the past 50 years, the role of dental plaque has undergone significant shift from Nonspecific plaque hypothesis and Specific plaque hypothesis to Ecological plaque hypothesis. Currently, pathogenesis of periodontal disease is explained by "Polymicrobial Synergy and Dysbiosis Model (PSD)" proposed by Hajishengalis et al[2]. This model states that the dysbiotic environment and polymicrobial synergy are the key events that lead to development of periodontitis rather than individual bacterial species.

The microbiome consists of an ecological community of commensal, symbiotic and pathogenic organisms found in all multicellular organisms studied till date ranging from plants to the highest order of the animal kingdom. They have been found to be crucial for the immunologic, hormonal and metabolic homeostasis of the host[3].

The salient clinical features of periodontal disease are gingival inflammation, formation of periodontal pockets and gingival recession. Gingivitis is a reversible non-specific inflammatory reaction of the marginal gingiva to plaque accumulation, whereas Periodontitis is a non-resolving and irreversible condition resulting in loss of attachment apparatus potentially leading to tooth loss.

Existing evidence indicates that gingivitis precedes the onset of periodontitis; However, not all gingivitis cases develop into periodontitis[4]. The reason for this is that accumulation of plaque bacteria is a necessary but not a sufficient prerequisite for the development of periodontitis[5,6]. Individual disease susceptibility plays a major role in the progression from gingivitis to periodontitis[7]. This individual susceptibility has been examined extensively and a host of genetic, inflammatory, host tissue and microbial biomarkers have been developed to assess periodontal disease progression. Microbial profiling from tissue fluids like GCF and saliva may be one approach to identify susceptibility for Chronic periodontitis.

Saliva is a body fluid essential for the maintenance of health of the oral cavity including the periodontium. Whole saliva is a complex mixture of oral fluids including secretions of the major and minor salivary glands; constituents of non-salivary origin derived from GCF, desquamated epithelial cells and food debris[8]. It plays an important role in maintaining the homeostasis of the periodontal tissues through the anti-bacterial effects exerted by the Immunoglobulins and other antimicrobial peptides present in it.

Although saliva does not have a resident microflora, it has been postulated that salivary microorganisms may play an important role in the aetiology and propagation of periodontal diseases[9]. Bacterial translocation through saliva has been proposed to play a role in transfer of subgingival

bacteria from uninfected to infected sites and recolonization of treated sites in the periodontium[10].

Subgingival microbiota has been divided into complexes, based on their association with health and various disease severities. The yellow, green and purple complexes are thought to be early colonizers that favour colonization of orange and red complexes that have been associated with periodontal disease activity[11,12].

The orange complex is constituted by Fusobacterium nucleatum, Fusobacterium periodonticum, Prevotella intermedia, Prevotella nigrescens, Peptostreptococcus micros, Streptococcus constellatus, Eubacterium nodatum, Campylobacter showae, Campylobacter gracilis, Campylobacter rectus and Parvimonas micra. There is voluminous literature on the relative abundance and virulence of these organisms in sub-gingival plaque world over[13–15], but it has not been as extensively studied in Indian populations.

We have previously reported on the sub-gingival microflora in Health and Periodontal pockets using Next generation Sequencing technology.[16] This study is to ascertain the variation of microbial profile in Gingivitis and Gingival recession level compared to the Healthy state.

Recent trends in sequencing of microbiome are based on next generation sequencing. Next-generation sequencing (NGS) is a type of DNA sequencing technology that uses parallel sequencing of multiple small

fragments of DNA to determine genetic sequences[17]. In contrast to Sanger sequencing, the speed of sequencing and amounts of DNA sequence data generated with NGS is exponentially greater and are produced at significantly reduced costs[18].

We have included only Gingivitis and Gingival recession as the disease group. Gingival recession in this study population is a phenotypic representation of progressive periodontal lesion that is characterized by the presence of attachment loss and inflammatory exacerbation and not that caused by the tooth brush trauma.

This study proposes to evaluate the orange complex species in the saliva of individuals in Gingival health, Gingivitis and Gingival recession using next generation sequencing so as to ascertain the possibility of using them as microbial risk markers of disease activity.

AIM & OBJECTIVE

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AIM:

➢ To evaluate the Orange complex species in the saliva in Gingival health, Gingivitis and Gingival Recession patients using Next Generation Sequencing technique with Illumina sequencing method.

OBJECTIVE:

➢ To evaluate the Orange complex species in the saliva in Gingival health, Gingivitis and Gingival Recession patients using Next Generation Sequencing technique with Illumina sequencing method.

➢ To compare the frequency distribution of salivary Orange complex bacteria in healthy, Gingivitis and Gingival Recession individuals.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The human mouth harbors one of the most diverse microbiomes in the human body, including viruses, fungi, protozoa, archaea and bacteria. The microorganisms in the human oral cavity is also referred to as the oral microflora, oral microbiota, or more recently as the oral microbiome. The term **Microbiome** was coined by **Joshua Lederberg**[19] "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that share our body space and have been all but ignored as determinants of health and disease". The human microbiome can be classified into two distinct types: a core microbiome and a variable microbiome[20,21]. The core microbiome is shared among all individuals and is comprised of the predominant species that exist under healthy conditions at different sites of the body[20–22]. The variable microbiome is exclusive to the individual and has evolved in response to unique lifestyle, and phenotypic and genotypic determinants. Although individuals share microbiota at similar sites of the body, there are varying differences at the species and strain level of the microbiome that can be as inimitable to the individual as is the fingerprint[23].

An ecosystem is the complex of organisms in a specified environment and the nonmicrobial surroundings with which the organisms are associated. The ecosystem includes the assemblage of species and the organic and inorganic constituents characterizing that particular site. The organisms inhabiting a given site constitute a community. The habitat is the site at which a population or

community grows, reproduces or survives. The role of an organism in a habitat is its niche. Niche does not connote location but rather function. A species can have one niche in one habitat and a different niche in another habitat.

Dental plaque:

Dental plaque has been defined as the microbial community that develops on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin. Dental plaque forms via an ordered sequence of events, resulting in a structurally and functionally organized species-rich microbial biofilm. Marsh et al stated that oral diseases initiate with the growth of the dental plaque, a biofilm formed by the accumulation of bacteria in a timely manner together with the human salivary glycoproteins and polysaccharides secreted by the microbes[24]. The subgingival plaque, located within the neutral or alkaline subgingival sulcus, is typically inhabited by anaerobic Gram negatives and is responsible for the development of gingivitis and periodontitis. The supragingival dental plaque is formed on the teeth surfaces by acidogenic and acidophilic bacteria, which are responsible for dental caries.

The Plaque formation occurs in 6 distinct stages- 1) formation of acquired pellicle 2) Reversible adhesion 3) Irreversible adhesion 4) Co-adhesion 5) Multiplication of attached cells 6) Detachment from the biofilms. The acquired pellicle is formed by the molecules that are adsorbed to the tooth surface within

seconds immediately after cleaning or following initial exposure to the oral environment, and remain functional. These molecules are derived mainly from saliva, but, in the subgingival region, molecules originate from gingival crevicular fluid.

Theories of Plaque Hypothesis:

According to the criteria proposed by Socransky and Haffajee[11], a periodontal microorganism has to meet certain conditions to be considered a potential pathogen: to be associated with the disease by means of increased number in diseased patients and sites; to be reduced or eliminated after treatment and, with the healing, to be capable of provoking the destructive host response; to possess the capacity to cause the disease in experimental animal models; to demonstrate production of virulence factors known to cause periodontal destruction. The literature evidence has shown the changes in dental plaque relate to a shift from oral health to disease have changed over time. The understanding and characterization of dental plaque have undergone significant evolution over past 50 years.

Non-specific Hypothesis:

The Non-specific plaque hypothesis was based on research work of Black[25] and Miller[19]. This hypothesis stated that the cause of periodontal disease is due to bacterial accumulation, rather than its composition. No one specific bacterial

species is any more significant than another in its ability to cause periodontal disease[26]. Any accumulation of microorganisms at or below the gingival margin would produce

irritants, resulting in inflammation which in turn, was responsible for the periodontal tissue destruction[11]. It was proposed that the entire microbial community of plaque that accumulated on tooth surfaces and in the gingival crevice contributed to the development of periodontal disease through the production of virulence factors and noxious products that initiated inflammation, challenged the host defense system, and resulted in the destruction of periodontal tissues. Thus, the quantity of micro-organisms in plaque, as opposed to the quality of micro-organisms found in the plaque, were viewed as being primarily responsible for inducing Periodontal disease and its progression[27]. It concludes that the different combinations of indigenous bacteria, rather than just a single species, can produce the pathogenic potential necessary to cause progression from gingivitis to destructive periodontitis[28].

Theilade and Attstorm (1985)[28] observed inflammation and loss of hemidesmosomes between the junctional epithelium and the teeth in dogs are seen ahead of the bacteria. Although the amount of plaque present may correlate well with disease severity in cross-sectional studies, it correlates poorly in longitudinal studies. This hypothesis is valid for the development of gingivitis but

not for the development of periodontitis, which is multifactorial in nature[6]. It also failed to explain why all gingivitis not progress to periodontitis and why some individuals with increased plaque showed little overt periodontitis and some individuals with very little plaque manifested with aggressive and advanced forms of periodontitis[8,9]. It does not consider variations in the dental biofilm which may affect its pathogenicity or, most importantly, host determinants.

Specific plaque hypothesis:

This hypothesis was proposed by Walter J. Loesche. According to specific plaque hypothesis, periodontal disease is the result of an infection with a single specific pathogen. The microbial etiology of various forms of periodontitis like Aggressive Periodontitis supports the specific plaque hypothesis, which proposes that only certain microorganisms within the dental plaque complex are pathogenic. For example, Aggregatibactor actinomycetamcomitans was identified as a specific pathogen in localized aggressive periodontitis[29]. Despite the presence of numerous species of microorganisms in periodontal pockets, fewer micro-organisms are routinely found in increased proportions at periodontally diseased sites. The virulence factors produced by specific bacterial species activates the host's immune and inflammatory responses that then cause bone and soft tissue destruction.

Socransky and colleagues, in 1998[15] recognized that early plaque consists predominantly of gram-positive organisms and that if it is left undisturbed for days, it undergoes a process of maturation resulting in a more complex and predominantly gram-negative flora. The organisms of the subgingival microbiota are classified into groups, or complexes, based on their association with health and various disease severities[27]. The yellow, green and purple complexes were the early colonizers that favor the colonization of orange and red complexes. The red complex bacteria included Tannerella forsythia, Porphyromonas gingivalis and Treponema denticola and they were significantly associated with periodontitis. This hypothesis failed to explain why the putative periodontal pathogens of red complexes are frequently found in healthy periodontal sites. **(Figure 1)**

Ecological plaque hypothesis:

This hypothesis was proposed by Philip D. Marsh in 1994[17,24]. It was proposed to describe and explain the dynamic relationship between the resident microflora and the host in health and disease in ecological terms. According to this theory, the etiology of periodontal disease is that changes in the environment, increase in the competitiveness of the putative pathogens (which, if present in health, are generally only at low and clinically insignificant levels) at the expense of species associated with oral health and upregulate the expression of virulence factors. There is a clear link between local environmental conditions and

the activity and composition of the biofilm community. Any change to the environment induces a response in the microflora, and vice versa. **(Figure 2)**

Keystone Pathogen Hypothesis:

The Keystone Pathogen Hypothesis indicates that certain low abundance microbial pathogens can cause inflammatory disease by increasing the quantity of the normal microbiota and by changing its composition. When disease develops and advanced stages are reached, the keystone pathogens are detected in higher numbers[11,12,30].

Polymicrobial Synergy and Dysbiosis Model:

PSD model of pathogenesis states that periodontitis is initiated by a broadly based dysbiotic, synergistic microbiota as against the traditional view that it is caused by a single or several periopathogens like red complex bacteria. This dysbiotic, synergistic microbiota environment retards the host-microbe homeostasis and offers its transition to a chronic inflammatory state. It is the interaction between the subgingival community of microorganisms and local immune responses that ultimately leads to bone and connective tissue attachment loss[2,10] .(**Figure 3)**

In the periodontal ecosystem wide variety of bacteria (or specific combinations of genes within the community) are present, which might be

able to fulfil distinct roles that converge to form and stabilize a disease provoking microbiota. Hence, there will be a number of core requirements for a potentially pathogenic community to arise. (i) Bacterial constituents will express the relevant adhesins and receptors to allow assembly of a heterotypic community. (ii) Individual members of the community will be physiologically compatible or at least non-antagonistic. (iii) The combined activities of the community will resist the host innate and acquired immune responses and contribute to tissue inflammation through, that is by proteolytic activity and induction.

PERIODONTAL DISEASE- ETIOLOGY AND PATHOGENESIS

Periodontal diseases are a heterogeneous group of chronic conditions that reflect a cellular inflammatory response of supporting periodontal tissues of the teeth against bacterial challenges. It is a dysbiotic disease characterized as being polymicrobial and multifactorial in nature exhibiting a shift from predominantly gram-positive bacteria found in healthy sites to mostly gram-negative bacteria found in clinically diseased sites. The initiation and progression of the inflammatory and destructive periodontal lesion is related to the lack or minimal proportions of beneficial microorganisms in a susceptible host. The end outcome of untreated periodontal disease is loss of attachment apparatus and subsequent loss of teeth often leaving patients unable to eat and function properly.

Löe et al (1978)[5] demonstrated the natural progression of periodontal disease through a series of studies over several years following a population of Sri Lankan tea workers which represented a relatively uniform population that had little to no dental care and also had extremely poor oral hygiene. Several lines of evidence indicate that bacteria are necessary for the development of inflammation in the periodontal tissues. In a study by Mitchell and Johnson[31], bacteria were implicated in periodontal disease with the observation that administration of penicillin inhibited periodontitis in laboratory animals, and Keyes and Jordan[32] demonstrated the infectious nature of periodontitis by its transmissibility in animal models.

The current concept concerning the etiology of periodontal disease considers three groups of factors which determine whether active periodontal disease will occur: a susceptible host, presence of pathogenic species, and absence of so-called "beneficial bacteria"[33]. The American Academy of Periodontology defined gingivitis as a non-destructive disease that occurs around the teeth. Bacterial biofilms that are attached to the tooth contribute to the most common form of gingivitis known as plaque-induced gingivitis, which acts to initiate the body's host response thereby leading to the gingival tissues destruction resulting in the destruction of the periodontal attachment apparatus. Gingivitis is a reversible inflammatory reaction of marginal gingiva to plaque accumulation, whereas periodontitis is a destructive, nonreversible condition resulting in loss of tooth

connective-tissue attachment to bone, which ultimately leads to loss of the involved teeth. Existing evidence indicates that gingivitis precedes onset of periodontitis; however, not all gingivitis cases develop into periodontitis. The reason for this is that accumulation of plaque bacteria is necessary but not sufficient by itself for development of periodontitis: a susceptible host is necessary[34].

Gingivitis and periodontitis were associated with higher microbial community richness and Shannon indexes, and this association remained after adjustment for demographic factors, including age, body mass index (BMI), and socioeconomic status. This finding is consistent with previous research by various authors who proposed, with higher diversity meaning that, in periodontal disease, the oral microbiota is added rather than existing taxa undergoing replacement. This may correspond to primary ecological succession in a new environmental niche, as suggested by Abusleme et al[35].

Studies conducted by Liam shaw et al[36] stated that many taxa were associated with gingivitis and periodontitis. The abundance of the majority of these taxa increased with the severity of Gingivitis, and this pattern was not influenced by the presence of periodontitis. It would appear that relative bacterial abundances alone are insufficient to explain the presence of disease, which is consistent with a requirement for other factors, such as the host inflammatory response, to cause disease. It reveals that distinct signals associated with gingivitis and periodontitis

in subgingival plaque, with a dominant contribution from gingivitis. Network analysis of observed co-occurrence patterns was consistent with the role of bridging bacteria like F. nucleatum and F. alocis in the coaggregation of periodontal biofilms prior to entrance into subgingival regions. Although some periodontitis-associated bacteria were also associated with gingivitis, the major change with periodontitis is in the network of co-occurrences.

The initial stages of plaque were characterised by gram-positive cocci and rods while the latter by an increase in gram-negative rods, fusiforms, filaments, spirilla and spirochetes as the plaque matures which leads to periodontal disease. The association of plaque to gingivitis was confirmed by the study done by Loe on the Srilankan tea labourers which popularly came to know as the "Experimental Gingivitis" model[5].

Experimental Gingivitis:

Loe et al reported the development of gingivitis exclusively in a system of model known as experimental gingivitis. Gram-positive rods, gram-positive cocci and gram-negative cocci were the initial microbiota of experimental gingivitis. Gram-negative rods and filaments, spirochaetal and motile microorganisms increase in number resulting in inflammatory changes that leads to gingivitis. He founded that 56% gram-positive bacteria, 44%gram negative bacteria are present in plaque induced gingivitis; which also includes 59%

facultative organism and 41% obligate anaerobic organisms. Most predominant gram positive organisms includes S.sanguis, S.mitis, S.intermedius, S.oralis, A.viscosus, A.naeslundii, and P.micros. The most predominant gram-negative organisms includes F.nucleatum, P.intermedia, and V.parvula, Haemophilus , Capnocytophaga and Campylobacter species.

Experimental gingivitis in man:

Theilade et al[28] in an experiment carried out in 11 subjects who had excellent oral hygiene and healthy gingiva wherein they developed accumulations of plaque and generalized gingivitis after 9 to 21 days of refraining from oral hygiene aids. It was found that there is a correlation between the rate of plaque accumulation and the development of Gingivitis. Gram-positive cocci and rods were present initially in theclean and healthy gingiva. During the first two days without oral hygiene, there developed the first phase of plaque. Proliferation of gram-positive cocci and rods were seen along with an addition of about 20 to 30% gram-negative cocci and rods. Fusobacteria and filaments began to appear and increased about 7% of the total flora during the second phase that is about 1-4 days. Spirilla and spirochetes contributed for about 2% of the total flora during the third phase; that is after 4-9 days. The composition of the plaque correlated with the condition of the gingiva in certain areas which helped in the clinical diagnosis of the gingivitis which in turn correlates with the same time as this complex flora

begin to colonize but the sub-clinical inflammation started at the first phases of plaque development. After this experiment the subjects were advised to start the oral hygiene measures and it was noted that within 1 to 2 days, plaque began to disappear indicating that the gingivitis began to reduce one day after the reinforcement of oral hygiene practices.

Health to Gingivitis:

The classic experiments of Loe et al. (1965) demonstrated that without doubt the accumulation of microbial plaque results in the development of ginvititis and that its removal and control results in resolution of the lesions in humans, thereby proving the microbial etiology of the disease. More recent studies have confirmed this conclusion in humans and in experimental animal model[32–34].

Temporal shifts in the microbiome:

Conceptual model of microbial shift from health to periodontitis based on microbial clusters propose by **Hong et al**[37]**.** Communities from healthy subjects tended to occur in 2 clusters-Cluster L(large) and Cluster S(small). Cluster L consists of health associated species such as streptococcus, Actinomyces, Rothia, Kingella and cluster S consists of Gingivitis associated species and core species such as bacteroides, capnocytophaga, fusobacteria, prevotella and others.

Communities in periodontitis also occurs in 2 clusters- Cluster A and Cluster B. Cluster A consists of gingivitis associated species and core species and cluster B consists of red complex species, filifactor alocis, freitobacterium and others. Greater number of species enriched in the health cluster S and in the periodontitis cluster A are gingivitis associated species with some core species. Hence whether gingivitis represents a transitional stage between health and periodontium is in question**. (Figure 5)**

A shift in microbial species in the gingival sulcus from gram-positive, facultative, fermentative microorganisms to predominantly gram-negative, anaerobic, chemoorganotrophic, and proteolytic organisms have been strongly associated with periodontal tissue breakdown. However, the level of periodontal breakdown has long been associated with the degree of host predisposition. There are no actual pathogenic oral bacteria, only opportunistic commensal bacteria.

Gingival Recession:

Wensstrom J et al[38] stated that gingival recession refers to exposure of root surface caused by apical displacement of gingival margin beyond cementoenamel junction. Gingival recession, either localized or generalized, is one of the clinical features of periodontal disease and is frequently associated with clinical problems such as root surface hypersensitivity, root caries, cervical root abrasions, erosions, plaque retentionand aesthetic dissatisfaction[39]. According to

Page & Sturdivant[40], the distinction between two different phenotypes are described as 1)Periodontal atrophy, where the gingiva retains a very healthy aspect and are quite free of pain and inflammation, and yet will gradually recede; 2) Destructive periodontal disease with presence of deepened periodontal pockets and underlying bone loss.

Etiology of Gingival Recession:

The etiology is multifactorial in nature which includes excessive or inadequate teeth brushing, destructive periodontal disease, tooth malposition, alveolar bone dehiscence, high muscle attachment, aberrant frenal pull, occlusal trauma, iatrogenic factors (such as orthodontic, or prosthetic treatment) and smoking[41].

The mechanism of gingival recession due to localized inflammatory processes in connective tissues with the accumulation of mononuclear cells was reported by Baker and Seymour (1976)[42]. In the initial stage there is normal or subclinical inflammation, followed by inflammation clinically and histologically where there is proliferation of epithelial rete pegs. Stage three presents with increased epithelial proliferation resulting in loss of connective tissue core and finally there is merging of oral and sulcular epithelium resulting in separation and recession of the gingival tissues due to loss of nutritional supply.

Van der Velden et al[43] stated that there was an association between gingival recession and periodontitis severity in a Java population. The results were

concluded together with results from the study by Sarfati in 2010, may indicate a bi-directional association between gingival recession severity and periodontitis severity, rather than a causal relationship. It may be assumed that inflammatory reaction to dental biofilm is the predominant biologic feature shared by gingival recessions and periodontitis.

Keratinized tissue width as a parameter for gingival recession:

Lang and Loe (1972)[44] stated that although tooth surfaces may be kept free of clinically detectable plaque, areas with less than 2 mm of keratinized gingiva tend to remain inflamed. However, studies conducted by Dorfman HS[45], Freedman AL et al[46], Kisch et al[47] and Miyasato[48] have evaluated sites with less than 2 mm of keratinized tissue and concluded that these sites do not necessarily develop gingival recession solely as a result of a narrow width/band of keratinized tissue. Serino et al reported that teeth with a positive history of progressive gingival recession have increased susceptibility to additional apical displacement of gingival margin[49].

In 1999, Albandar and Kingman[50] reported that gingival recession occurs primarily as a consequence of periodontal diseases and aggressive use of mechanical oral hygiene measures. A positive history of low grade chronic inflammatory periodontal disease (e.g. plaque-induced gingivitis and localized chronic periodontitis) can be considered an important factor associated with

gingival recession, especially for teeth with thin gingival tissues and/or mucogingival conditions.

Microbial etiology in Gingivitis and Gingival recession:

The subgingival microbiome is the community of microorganisms inhabiting the subgingival environment. Haffajee and Socransky[9] and Zambon[51] have extensively studied the microbial composition of subgingival plaque at periodontally diseased sites. In a landmark study by Socransky and Haffajee, they attempted to define bacterial communities existing as different complexes in subgingival plaque by studying 13,261 plaque samples from 185 subjects using whole genomic DNA probes and checkerboard DNA DNA hybridization. They defined 5 major bacterial complexes identified by different clustering and ordination techniques. The complex most significantly associated with periodontitis and to clinical measures like probing depth and bleeding on probing was the red complex, comprising of Tanerella forsythia, Porphyromonas gingivalis and Treponema denticola.

The culture studies by Moore and Moore[52] involving analysis of subgingival plaque taken from subjects with different forms of periodontal disease and health reported a shift in the subgingival microbiota as the periodontium progressed from health through gingivitis to periodontitis. Liu et al and Chen et al investigated bacterial diversity between periodontal health and disease status using

16S rRNA amplicon sequencing and reported that there is a shift in the composition of the oral microbiota between healthy and diseased samples.

Kumar et al[14] studied the subgingival microbiome based on 16S rDNA cloning and sequencing and showed that 40% of bacterial species present were either novel species or phylotypes. The molecular technique developed by Paster BJ and Dewhirst (2006) was to detect oral biofilms using a 16S rRNA-based microarray technology called Human Oral Microbiome Identification Microarray (HOMIM). This system provides information on the 9 most commonly found oral bacterial flora namely Bacteroidetes, Firmicutes, Proteobacteria, Synergistetes, Fusobacteria, Spirochaetes, Actinobacteria, SR-1 and TM-7.

Clinical significance of the intra-oral translocation of bacteria;

Impact of a full-mouth tooth extraction on distribution of periodontopathogens: Danser et al[53] studied the prevalence of selected periodontopathogens on the oral mucous membranes and in the saliva, it seemed plausible that at least one of the periodontal pathogens such as A. actinomycetemcomitans, P. gingivalis or P. intermedia must have been present before tooth extraction, their detection frequencies were extremely low 0/26 for A. actinomycetemcomitans, 2/26 for P. gingivalis or 7/26 for P. intermedia, respectively.

Saliva as a Bio-marker:

Saliva is a complex biological fluid composed of water and organic and inorganic substances. Most salivary constituents are produced locally in the salivary glands, but some molecules pass into saliva from blood through biological processes such as diffusion, active transport and ultrafiltration. Water is the main component of saliva, representing 99% of saliva's total composition. The inorganic components of saliva are minerals and ions. The organic species consist of body secretion products, lipids and hundreds of proteins and peptides. It plays a major role in maintaining a 'healthy mouth'; Studies have shown that reduction in saliva flow markedly increases the risk of dental caries. Saliva lubricates oral surfaces and is fundamental to maintaining the structural integrity of teeth by reducing demineralization (through buffering the potentially damaging acids produced by dental plaque biofilms following the metabolism of dietary carbohydrates by bacteria), clearing food, promoting remineralization and providing components of the adaptive and innate arms of the host defenses.

Collection of saliva is easy, inexpensive, noninvasive and requires little training or experience. Saliva can be collected under unstimulated or stimulated (following chewing a piece of paraffin wax or applying citric acid onto the tongue) conditions. Whole saliva (which contains saliva, gingival cervicular fluid and epithelial transudate) can be easily collected by the drooling method, the spitting method, the swabbing method and the suction method. Saliva can also be

collected from individual glands by cannulation of glandular ducts or by using specialized collection devices. This later approach, however, is complex, invasive and expensive, and requires experienced personnel; therefore, it has limited use. Measurement of saliva flow rate (sialometry) is generally performed by collection of whole unstimulated saliva. **(Figure 6)**

Since the mid-1990s, the use of whole saliva as a clinical diagnostic fluid has gained increasing attention because of its rich content of biologically active molecules and the practical, simple, inexpensive and noninvasive collection, transport and analysis methods. In addition, advances in proteomics, genomics, metabolomics and nanotechnology have increased the sensitivity and reliability of saliva in diagnosis, monitoring and treatment responses. It also contains non-salivary elements, such as gingival crevicular fluid, desquamated cells, nasopharyngeal discharge, extraneous debris, and bacteria and bacterial by-products.

The term ‘biomarker’ refers to biologic substances that can be measured and evaluated to serve as indicators of biological health, pathogenic processes, environmental exposure and pharmacologic responses to a therapeutic intervention. The biologic parameters of health and disease can be better understood and allow better implementation of appropriate and personalized preventive and therapeutic strategies to maintain optimal health.

Zhang et al[54] recently identified, with high sensitivity and specificity, salivary messenger RNA biomarkers that are discriminatory for the detection of respectable pancreatic cancer without the complication of chronic pancreatitis.

Longitudinal analyses for periodontal pathogens and host-response biomarkers have been investigated for the ability to determine periodontal disease progression. The 'red-complex' pathogens (Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia) were able to predict periodontal disease progression and demonstrated an association with the inflammatory biomarkers interleukin-1β, interleukin-8 and MMP-8. Certain putative periodontal pathogens, when elevated, were able to predict periodontal disease progression; these include the aforementioned 'red-complex' pathogens, as well as Fusobacterium nucleatum, Campylobacter rectus and Prevotella intermedia. When clustered together, these microbial markers and inflammatory markers offer a strong relationship to current disease status. Alkaline aminotransferase and P. gingivalis demonstrated high levels of predictive ability for disease progression. The microbial stand-outs in that study were P. gingivalis and P. intermedia for their ability to predict periodontal disease progression.

Personalized medicine for periodontal diseases may soon involve utilization of saliva to develop subclinical profiles, identifying and measuring specific genotypes, phenotypes, putative pathogens, inflammatory

markers and collagen-degradation biomarkers to make informed clinical decisions about disease susceptibility, site-specific risk and treatment interventions. screening, disease detection, monitoring of treatment outcomes and identification of refractory or progressing cases[55,56]. During the screening phase, the use of saliva to identify patients at risk for future disease activity opens the door for heightened risk management strategies, preventive care and/or behavior change on the part of the patient to prevent the onset of disease. At the diagnostic stage, identifying the presence of disease at the earliest possible stage may allow for less-invasive, less-costly treatment. Saliva as a simple mechanism for monitoring treatment outcomes, as well as identification of refractory sites, additionally provides both the patient and the clinician with valuable information regarding the present state of the disease.

MICROFLORA IN SALIVA

TAUBMAN ET AL. 2007[57] have demonstrated that host susceptibility is of primary importance with an uncharacterized defect of the immune system, which causes defects in the regulation of osteoclast recruitment, differentiation and activation, causing affected individuals to mount an inappropriately aggressively inflammatory response to the normal microbiota.

ASIKAINEN et al (1991)[58] compared the recovery of *A. actinomycetemcomitans* from subgingival sites, the dorsum of the tongue and saliva. When *A. actinomycetemcomitans* was recovered from subgingival sites it was also found in

69.9% and 35.9% of the samples of stimulated and unstimulated saliva, respectively.

Salivary levels of *A. actinomycetemcomitans*, *P. gingivalis*, *P.intermedia*, *Campylobacter rectus*, and *Peptostreptococus micros* were determined by bacterial culture and related to clinical periodontal status in 40 subjects with varying degrees of periodontitis[59].

DETECTION AND ENNUMERATION OF BACTERIAL SPECIES FROM PERIODONTAL SAMPLES:

Microscopic studies

The earliest studies of subgingival biofilm composition were identified with the techniques of light microscopy. These techniques were reasonably rapid, but limited in the precision of identification of individual bacterial species. Thus, while about only nine morphotypes could be recognized, there were actually as many as 500 bacterial species in oral biofilm samples.

The development of the electron microscope permitted examination of biofilm samples with greater resolution. Electron microscopic techniques allowed a somewhat finer distinction of microbial groups based on cell wall ultrastructure and the presence and arrangement of various appendages to the microbial cell such as axial filaments or flagella. Electron microscopy by itself could not precisely identify a cell to the species level; however, in combination with

immunocytochemical techniques or in situ hybridization, the technique permitted precise localization of bacterial cells in relation to each other and the host.

The great strength of the microscopy techniques, including the promising confocal microscopy, is the delineation of spatial arrangements of the organisms. The great weakness of these techniques from an ecologic perspective is that they are slow and labor intensive and thus limit the number of samples that can be examined. In addition, precise speciation using immunologic or hybridization techniques can only be performed for a very limited number of species in any given sample[30].

Culture based methods

For many years, the major technique available to researchers to identify plaque bacteria was to cultivate the organisms and identify the species by their phenotypic traits; a rather time-consuming, labor-intensive, and expensive undertaking. As a result, relatively few plaque samples in small numbers of subjects could be examined. Culturing can be done on selective and non-selective media. Blood agar is a common non-selective medium as it allows growth of a broad spectrum of organisms. More specific media include Gram negative anaerobic medium supplemented with vancomycin to selectively allow growth of Gram-negative anaerobic rods while inhibiting Gram positive bacteria[60]. Another example of a selective medium is Staphylococcus sp. isolation on mannitol salt

medium, as fermentation of this salt by Staphylococcus aureus will turn the medium from pink to yellow.

The classic studies of Moore & Moore[52], in which they examined the composition of subgingival plaque samples in periodontal health and different states of periodontal disease, employed cultural techniques to examine over 17,000 isolates from over 600 periodontal sites. This represented a huge body of work on, by current standards, a limited number of samples. The major strength of culture is that, in theory, the majority of the bacterial species sampled will grow and be identified. However, difficult to grow species and uncultivable species, such as many of the spirochetes, will not be detected by this technique. Other species require special conditions for their growth. If these conditions are not met, their numbers will be severely underestimated[30]. It is well recognized that the main drawback of this method is its narrow spectrum. It has been estimated that 50% to 60% of distinct bacterial phyla in oral cavity still have no cultivable representatives[3,61,62].However, cell culture is still essential to assess bacterial sensitivity to antibiotics and, also for verifying the presence of known species.

Immunologic and enzymatic assays

Antibody-based methods were among the first to be used to enumerate specific species of microorganisms without their cultivation. Immunofluorescence techniques and enzyme-linked immunosorbent assay (ELISA) techniques have been successfully employed to examine a limited range of bacterial species in

larger numbers of plaque samples than had been examined using cultural techniques. These techniques are dependent on the specificity of the developed antibodies to specific taxa. Properly prepared and evaluated monoclonal or polyclonal antisera provide a sensitive and specific method of detecting specific bacterial species in dental plaque samples[30].

These techniques have the advantage that samples do not have to be cultured for enumeration, and they are rapid and less expensive than culture. However, they are limited to species for which reagents have been developed. In addition, it is difficult to use these techniques, particularly immunofluorescence techniques, to evaluate large numbers of species in very large numbers of plaque samples. Another disadvantage of antibody-based techniques is the time required to develop and validate specific antisera to new species (Socransky SS and Haffajee AD, 2005).

DNA – DNA hybridization or checkerboard

DNA-DNA hybridization is a molecular approach that has been used in a large number of studies. This method detects bacteria based on hybridization of target species to labeled genomic DNA that has been attached to nylon membranes previously. The levels of a limited number of species have been studied with this method in adult periodontitis, periodontal health, refractory periodontitis and response to therapy parts[9,63,64]. DNA-DNA hybridization has the advantage of detecting multiple species from each sample simultaneously, and the capability of

studying large numbers of samples for large numbers of species does provide a major benefit for studies of oral microbial ecology. The checkerboard technique is rapid, sensitive, and relatively inexpensive but this method is also dependent on culture technique to cultivate the target species for creating genomic probes. Like antibody-based assays, cross reactivity can be verified only with cultivated species and so specificity of the probe is an unknown variable. Selected species may not be representative of the entire microbiome and during result interpretation this fact should be considered.

Polymerase chain reaction

Kary Mullis[63] in 1993first developed the polymerase chain reaction (PCR) and this technique amplifies specific genes or parts of genes which are then to be used to identify the bacterial species they originated from. In the last decade, polymerase chain reaction (PCR) has been used to detect the presence of selected bacterial species in subgingival plaque samples. Species specific PCR primers were designed and used in individual PCR reactions to detect the prevalence of target species in plaque samples of healthy subjects and diseased subjects[14]. These studies confirmed that several more species, including uncultivated, were associated with oral health or periodontitis.

Given the appropriate primers, this method is rapid and simple and is able to detect very small numbers of cells of a given species. It has the disadvantage of not providing quantitative data, but usually indicates the presence or absence of a

species in the sample. For applications where the relative levels of species are important, PCR may not be ideal. In addition, examining large numbers of species in large numbers of samples is difficult and may not be cost effective[30].

Real-time PCR is also referred to as qPCR, qRT-PCR, RT-qPCR and kinetic PCR. The procedure relies on the same basic principles of PCR; the additional feature is that the amplified DNA is detected and quantified simultaneously as the reaction progresses in real-time. Real time PCR has been added to the potential methods for examining the composition of biofilm samples. Real-time PCR has been used to detect and quantify several periodontal pathogens including A. actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, the tetQ gene and total bacteria, in clinical samples.

DNA Probes

DNA probes provide another approach to identification and enumeration of bacterial species in complex communities such as dental plaque. Oligonucleotide probes are short probes designed to identify unique regions of DNA within cells of a given bacterial species. It has been suggested that these probes are highly specific and the likelihood of cross-reactions with other species is very low. A number of studies have utilized these probes to identify periodontal bacteria[30]. Because they target a limited segment of the DNA of an organism, oligonucleotide probes tend to be less sensitive for the detection of low numbers of bacteria than whole genomic probes.

Whole genomic DNA probes have been used extensively in studies evaluating the composition of subgingival plaque. Whole genomic probes are constructed using the entire genome of a bacterial species as the target and thus can be quite sensitive. One of the criticisms of these probes is that the use of the entire genome may increase the probability of cross-reactions between species because of common regions of DNA among closely related species. The whole genomic DNA probes might not detect all strains of a given species and the probes may have a low sensitivity in terms of the numbers of cells that they detect.

The technique can detect only species for which DNA probes have been prepared. Thus, this method would not detect novel pathogens or environmentally important species that might be detected in culture or by other molecular techniques. The probes must be used to detect organisms in samples of the appropriate size. Probes optimized to detect species in the 10^4 to 10^7 range often will provide cross-reactions if much larger samples are employed.

OPEN ENDED APPROACHES- 16S rRNA sequencing analysis:

In 1977, 2 landmark articles describing methods for DNA sequencing were published. Allan Maxam and Walter Gilbert reported an approach in which terminally labeled DNA fragments were subjected to base-specific chemical cleavage and the reaction products were separated by gel electrophoresis. In an alternative approach, Frederick Sanger and colleagues described the use of chain-

terminating dideoxynucleotide analogs that caused base-specific termination of primed DNA synthesis.

Open ended approaches allow identification of even uncultivated and previously unknown species. These approaches are based on 16 S rRNA sequencing. This method is based on amplification and analysis of the 16S rRNA genes in a microbiome sample[64]. 16S rRNA has proven to be the most useful phylogenetic marker to identify bacteria and to determine their evolutionary relationships. Ribosomal RNA gene is essential for life and present in all prokaryotes. It contains nucleic acid sequences with highly conserved and variable regions. The conserved regions are used to design universal PCR primers capable of recognizing segments of the 16S rRNA gene sequence of all bacterial species. The hypervariable regions can be used as signatures to discriminate one species from another. 16S rRNA gene is large enough (about 1500 bases) to provide sufficient sequence variability among bacteria, thereby making comparisons possible at different taxonomic levels.

Recently, next generation sequencing technologies have emerged, which are high throughput and able to generate three to four orders of magnitude more sequences and are also relatively less expensive[65].

NEXT GENERATION SEQUENCING TECHNOLOGY:

Next generation sequencing methods employ a wide spectrum of technologies such as sequencing by synthesis, sequencing by ligation, single molecule DNA sequencing and polony sequencing. The next-generation sequencing is done by repeated cycles of polymerase-mediated nucleotide extensions or by machinery automated cyclical ligation of oligonucleotides[66,67].

Types of NGS are

1. Roche/454 FLX (Life Sciences, Branford, CT, Margulies et al[68].)
2. Illumina/ Solexa Genome Analyzer (Illumina, San Diego, CA, Bentley DR[69], Korbel et al[65])
3. SOLiD (Life Technologies, Carlsbad, CA, Mardis[66], Voelkerding et al[67])
4. HiSeq and the Ion Torrent Personal Genome Machine (PGM) (Rothberg et al[70]).

Fundamentals of NGS platforms:

NGS platforms share a common technological feature—massively parallel sequencing of clonally amplified or single DNA molecules that are spatially separated in a flow cell. This design is a paradigm shift from that of Sanger sequencing, which is based on the electrophoretic separation of chain-termination products produced in individual sequencing reactions. In NGS, sequencing is

performed by repeated cycles of polymerase-mediated nucleotide extensions or, in one format, by iterative cycles of oligonucleotide ligation[67]. As a massively parallel process, NGS generates hundreds of megabases to gigabases of nucleotide sequence output in a single instrument run, depending on the platform. The three commonly used platforms for massively parallel DNA sequencing at present are the Roche/454 FLX (Life Sciences, Branford, CT, Margulies et al)[68], the Illumina/ Solexa Genome Analyzer (Illumina, San Diego, CA, Bentley DR, 2006)[69] and the Applied Biosystems / SOLiD (Life Technologies, Carlsbad, CA, Mardis, Voelkerding et al.,)[66,67]. The most recent powerful NGS platforms have significant reductions in the run time and remarkable data output, they include HiSeq and the Ion Torrent Personal Genome Machine (PGM) [70].

The 2 basic procedures in the first generation NGS platforms are the ligation of DNA fragments with oligonucleotide adaptors and the fragments immobilization to a solid surface, such as a bead.

ROCHE 454 LIFE SCIENCES SYSTEM

The 454 technology is derived from the technological convergence of pyrosequencing and emulsion PCR. In 2000, Jonathan Rothberg founded 454 Life Sciences, which developed the first commercially available NGS platform, the GS 20, launched in 2005. Combining single-molecule emulsion PCR with pyrosequencing, Margulies and colleagues performed shotgun sequencing of the

entire 580 069 bp of the Mycoplasma genitalia genome at 96% coverage and 99.96% accuracy in a single GS 20 run[67].

A single GS FLX run generates approximately 1x 10^6 sequence reads, with read lengths of 400 bases yielding up to 500 million base pairs (Mb) of sequence. A recognized strength of the 454 technology is the longer read length, which facilitates de novo assembly of genomes.

The protocol includes (i) clonal amplification of templates on beads; (ii) deposition of the beads onto pico titerplate wells; (iii) controlled delivery of deoxyribonucleotide triphosphates by laminar fluidics, and (iv)a high-resolution charge-coupled device camera that detects the luminescent burst upon deoxyribonucleotide triphosphate incorporation. One of the major drawbacks of this system is that sometimes more than one nucleotide is incorporated in the DNA template during a cycle, making it difficult to resolve homopolymeric stretches of sequence (e.g. CCCCC or AAAAA).

APPLIED BIOSYSTEMS SOLiD

The SOLiD (Supported Oligonucleotide Ligation and Detection) System 2.0 platform is a short-read sequencing technology based on ligation. This approach was developed in the laboratory of George Church and reported in 2005 along with the resequencing of the Escherichia coli genome[67].

Sample preparation shares similarities with the 454 technology in that DNA fragments are ligated to oligonucleotide adapters, attached to beads, and clonally

amplified by emulsion PCR. Beads with clonally amplified template are immobilized onto a derivitized-glass flow-cell surface, and sequencing is begun by annealing a primer oligonucleotide complementary to the adapter at the adapter–template junction. Instead of providing a 3' hydroxyl group for polymerase-mediated extension, the primer is oriented to provide a 5' phosphate group for ligation to interrogation probes during the first "ligation sequencing" step. A 6-day instrument run generates sequence read lengths of 35 bases. Thus system can generate 4 GB of sequence but the reads are only 35 nucleotides[67].

ILLUMINA/SOLEXA GENOME ANALYZER

In 1997, British chemists Shankar Balasubramanian and David Klenerman conceptualized an approach for sequencing single DNA molecules attached to microspheres. They founded Solexa in 1998, and their goal during early development of sequencing single DNA molecules was not achieved, requiring a shift toward sequencing clonally amplified templates. By 2006, the Solexa Genome Analyzer, the first "short read" sequencing platform, was commercially launched. Acquired by Illumina in 2006, the Genome Analyzer uses a flow cell consisting of an optically transparent slide with 8 individual lanes on the surfaces of which are bound oligonucleotide anchors. Template DNA is fragmented into lengths of several hundred base pairs and end-repaired to generate 5' phosphorylated blunt ends[67]. The analyzer uses a specific number of cycles, where fluorescently labeled reversible-terminator nucleotides are detected on clonally amplified DNA

templates that are immobilized to an acrylamide coating on the surface of a glass flow cell[69].

DNA is fragmented and addition of adaptor sequences to each end of the fragments is done. The fragments are then sent to a lawn of immobilized oligonucleotides which are then grafted to the surface of a microfluidic chamber. The DNA templates are hybridized to the immobilized oligonucleotides by the adaptors. Once attached, the DNA templates are copied using bridge amplification[71]. Bridge amplification involves the tethered DNA template arching over and hybridizing to an adjacent anchored oligonucleotide, forming a bridge. Amplification of a single DNA molecule results in a cluster of molecules composed of the same sequence. Following amplification, the reverse strands of the DNA are denatured and washed away, resulting in clusters of unique immobilized ssDNA. DNA sequencing begins with the addition of polymerase, fluorescently labeled deoxyribonucleotide triphosphates and a primer that hybridizes to one of the adaptors. The incorporation of a complementary base results in a burst of light that is recorded by a charge-coupled device camera. Unlike the 454 Sequencing system, the fluorophore is removed from the incorporated base, washed away and the cycle is repeated. This prevents the addition of more than one base per cycle. **(Figure 7)**

The newest platform, the Genome Analyzer II, has optical modifications enabling analysis of higher cluster densities. Coupled with ongoing improvements in sequencing chemistry and projected read lengths of 50-plus bases, further

increases in output should be realized. Illumina and other NGS technologies have devised strategies to sequence both ends of template molecules. Such “paired-end” sequencing provides positional information that facilitates alignment and assembly, especially for short reads[67].

The advantage of the Solexa system is that it can generate 1.5 GB of sequence per run with read lengths that range from 35 to 100 bases. Each run requires 3–5 days to complete (Rothberg JM, 2008)[70]. To deal with short read length, the confidence of the sequence reads is improved by using pair-end sequencing, which means that both ends are sequenced.

A technical concern of Illumina sequencing is that base-call accuracy decreases with increasing read length. This phenomenon is primarily due to “dephasing noise”[67]. Dephasing noise occurs when a complementary nucleotide is not incorporated or when the fluorophore is not properly cleaved at the end of the cycle – blocking the incorporation of the next nucleotide base. As a consequence, the sequence is out-of-phase for the remainder of the template. Another shortcoming is that short read lengths tend to produce biased sequence coverage that occurs in AT-rich repetitive sequences[72].

Modifications in sequencing chemistry and algorithms for data-image analysis and interpretation are being pursued to mitigate dephasing. Investigators at the Wellcome Trust Sanger Institute published a series of technical improvements for library preparation, including methods for increasing the

reproducibility of fragmentation by adaptive focused acoustic wave sonication, enhanced efficiency of adapter ligation by use of an alternate ligase, and reducing the G-C bias that has been observed in Illumina reads via a modified gel-extraction protocol[67].

THE HUMAN ORAL MICROBIOME DATABASE

Research over the past 20 years has focused on defining breadth and diversity of oral microbiome by obtaining 16S rRNA gene sequence information for both cultivable and as yet uncultivated oral bacteria. The majority of bacterial species isolated from the oral cavity are included in 4 of the 10 bacterial phyla; Phylum 1 (Proteobacteria), Phylum 2 (the gram-positives), Phylum 5 (the spirochetes) and Phylum 6 (the flavobacter-bacteroides group). There are no known human oral representatives from the other 6 phyla. Though human oral microbiome is the most studied human microflora, 53% of species have not been named yet and 35% of species are uncultivated. The uncultivated taxa are identified mainly by 16S rRNA sequence information.

The ***Human Oral Microbiome Database (HOMD)*** is a specifically designed database to provide a provisional naming scheme where each oral taxon is given a human oral taxon (HOT) number linked to comprehensive information and tools for examining and analyzing each taxon in the human oral microbiome at both taxonomic and genomic level. This dynamic database provides a curated taxonomy of oral prokaryotes, a curated set of full-length 16S rRNA reference sequences, and

BLAST tools that allow identification of unknown isolates or clones based on their 16S rRNA sequence; along with this phenotypic, bibliographic, clinical and genomic information are linked for each taxa. Organisms of the human oral cavity are organized in a taxonomy hierarchy, which leads to individual pages for every oral taxon with comprehensive information and links. The genomic component of HOMD contains both static and dynamically updated annotations as well as bioinformatics analysis tools for all the genomic sequences, and curated 16S rRNA gene reference sequences for all human oral microbes. HOMD may serve as an example of a body site-specific tool for other communities.

More recently, a similar database was set up by **Griffen A et al**[13] known as CORE, a phylogenetically curated 16S rDNA database of the core oral microbiome, which offers improved and more robust identification of human oral bacterial 16S rRNA gene sequences compared with other methods. Its main goal is to provide a comprehensive and minimally redundant collection of oral bacteria at the genus and species level, as well as providing support for inferring community divergence and analysis of large datasets.

The basic list of oral bacteria came from the literature works of **Dzink JL**[15]**, Sockransky**[30]**, Tanner**[73] and **Moore WE**[52]. In 2010, **Dewhirst** [74] identified 1,179 taxa of which 24% were named, 8% were cultivated but unnamed, and 68% were uncultivated phylotypes. Upon validation, 434 novel non-singleton taxa were added to the HOMD. **Liu B et al**[74] reported using a metagenomic

approach by applying next-generation sequencing to sequence entire microbial DNA within a sample directly, and reconstructing genomes of microbiota via de novo assembly or mapping against a reference genome database.

According to **Blainey P**[75]**,** the emerging field of single-cell genomics is also currently being implemented for bacteria and Archaea. The issue with these commercially available tests is the question of their true value in terms of reliability for detecting causative agents of disease, given our limited knowledge of the complex ecosystem involved. The other major concern lies in the ability of clinician or diagnostic company to interpret results correctly and in such a way as to provide benefit for patients.

Studies on Plaque microbial analysis:

The first complete sequence of a microbial genome was published in 1995 by Fleischmann RD et al. In the past 50 years, numerous studies by Paster et al[76], Baker et al[42], Kumar et al[14] and Aas et al[77] have characterized the community composition of oral microbiota. Using culture-dependent and independent methods, estimates of oral biodiversity have implicated more than 700 different microbial species. Culture analysis of subgingival plaque samples of early periodontitis by Tanner et al detected Tannerella forsythia, Campylobacter rectus, and Selenomonas noxia associated with progressing disease pattern compared with non-progressing disease subjects, whereas Porphyromonas gingivalis was associated, by whole genomic DNA probes, with progressing periodontitis.

Abusleme et al[35] conducted a study in 2013 to and found a higher biodiversity and biomass in periodontitis compared to health, with periodontitis having higher proportions of Spirochetes, Synergistetes, Firmicutes and Chloroflexi; while the proportions of Actinobacteria like Actinomyces were increased in health. They also showed an association between biomass and community structure in periodontitis with proportions of specific taxa correlating with bacterial load.

Tanner AC et al[73] conducted a cross-sectional evaluation of 141 healthy and periodontitis individuals to compare microbiota of subgingival and tongue samples between early periodontitis and health using oligonucleotide probes and PCR. Porphyromonas gingivalis and Tannerella forsythia were associated with early periodontitis by direct PCR, and they found that microbiota of tongue samples was less sensitive than that of subgingival samples in detecting periodontal species.

G Xie et al[78] studies on metagenomic analysis of a healthy human plaque sample using a combination of second-generation sequencing platforms, and revealed the presence of 12 well-characterized phyla, members of the TM-7 and BRC 1 clade, and unclassified sequences. 73% of the total assembled counting sequences were predicted to code for proteins, 2.8% of the predicted genes coded for proteins involved in resistance to antibiotics and toxic compounds.

Griffen et al[13] conducted a study using 454 pyrosequencing of 16S rRNA genes and identified and reported 16 phyla, 106 genera and 596 species. Community diversity was higher in disease, 123 species were significantly abundant in disease and 53 species in health. Spirochaetes, Synergistetes and Bacteroidetes were more abundant in disease whereas Proteobacteria were found in higher levels in healthy controls. Within the phylum Firmicutes, the class Bacilli was health associated whereas Clostridia, Negativicutes and Erysipelotrichia were associated with disease.

Zheng et al[79] performed a study to analyze the microbial characteristics of oral plaque around implants using pyrosequencing of 16S rRNA gene, and reported an increase in microbial diversity in subgingival sites of ailing implants compared with healthy implants. Periodontal pathogens like P. gingivalis, T. forsythia and P. intermedia were clustered into modules in the peri-implant mucositis network.

Hong BY[37] et al conducted a study to explore the existence of different community types in periodontitis and their relationship with host demographic, medical and disease-related clinical characteristics. Their results suggested 2 types of communities (A and B) existed in periodontitis. Type B communities harbored greater proportions of certain periodontitis associated taxa like P. gingivalis, T. forsythia and T. denticola and other recently linked periodontitis associated ones.

REVIEW OF LITERATURE FIGURES

REVIEW OF LITERATURE FIGURES

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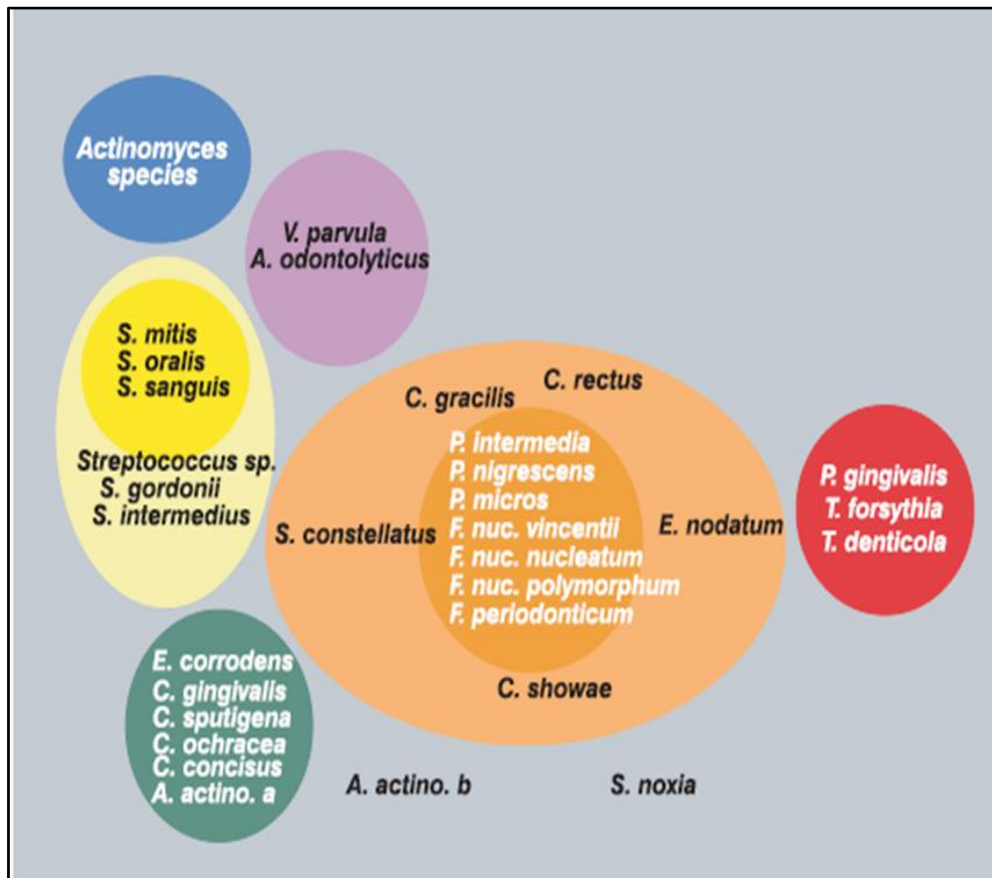


Figure 1- Bacterial complexes

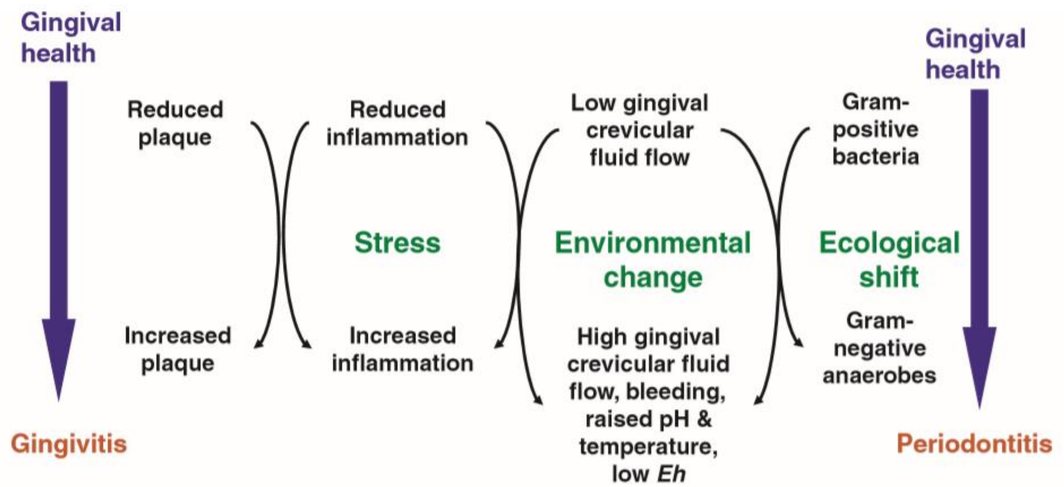


Figure 2- Marsh plaque hypothesis

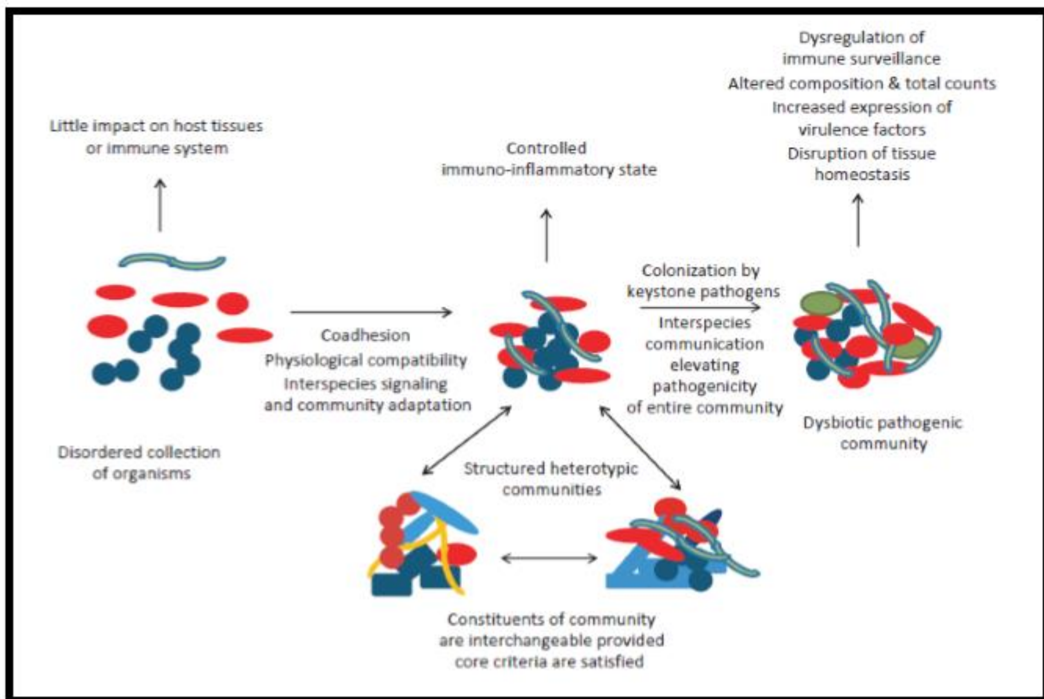


Figure 3- PSD MODEL

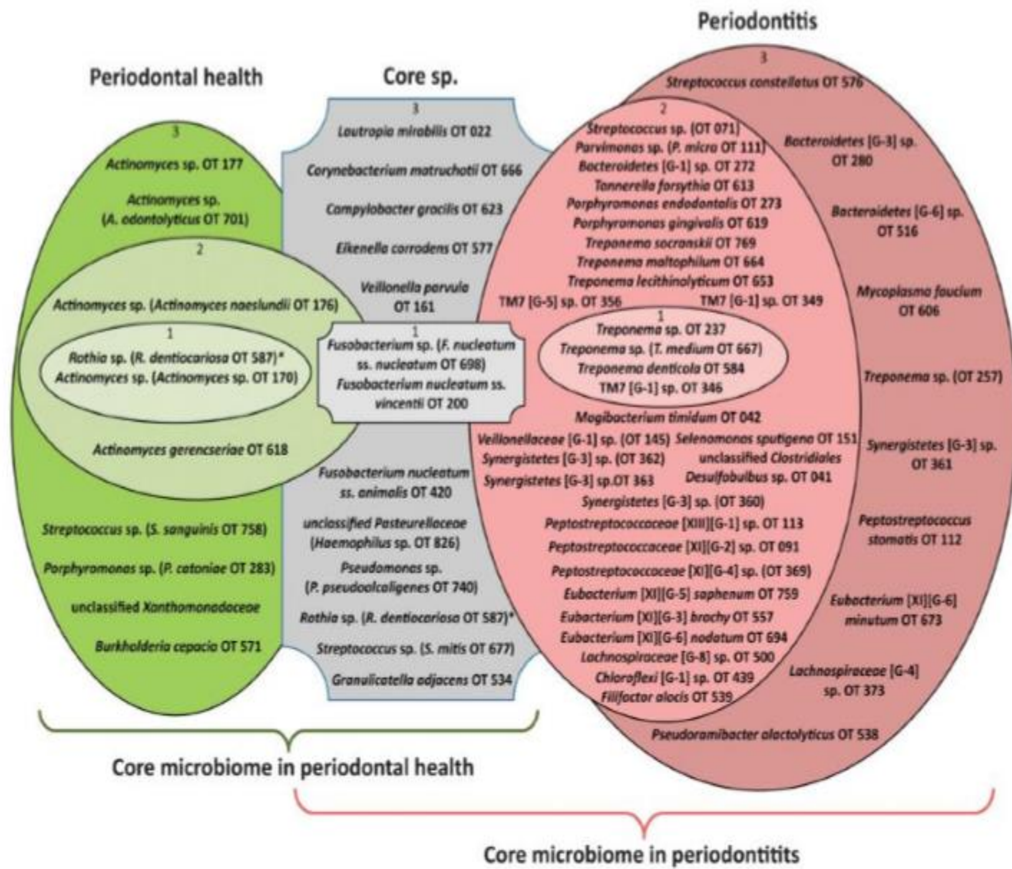


FIGURE 4 - CORE SUBGINGIVAL MICROBIOME IN HEALTH AND PERIODONTITIS

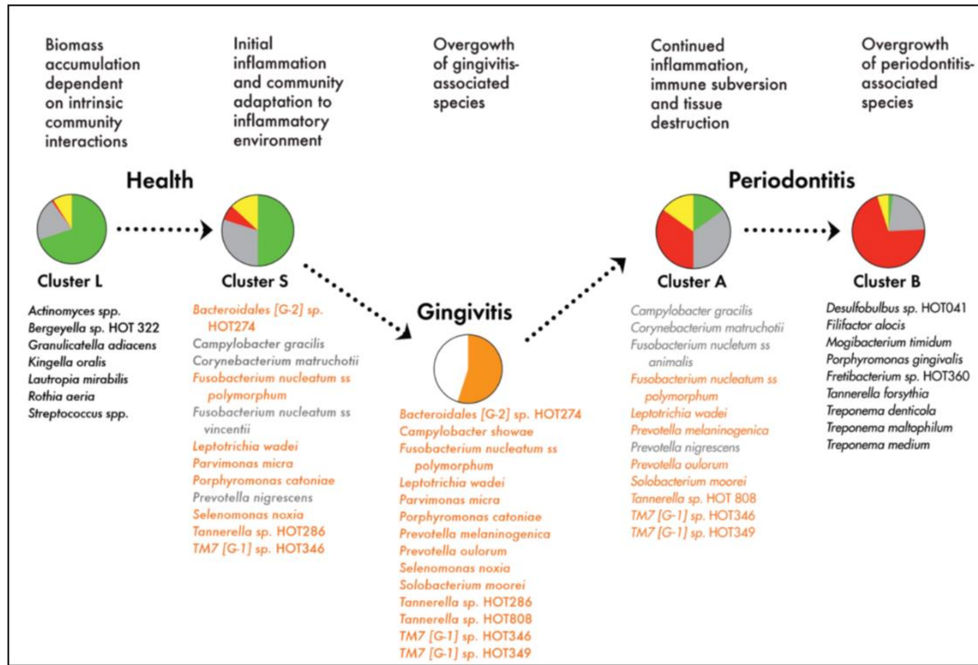


FIGURE 5- TEMPORAL SHIFTS IN THE MICROBIOME (HONG ET AL)

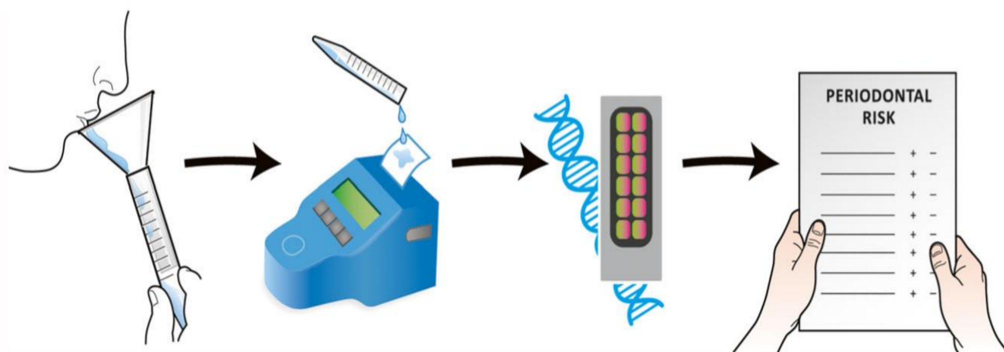


FIGURE 6 - SALIVA COLLECTION TUBES

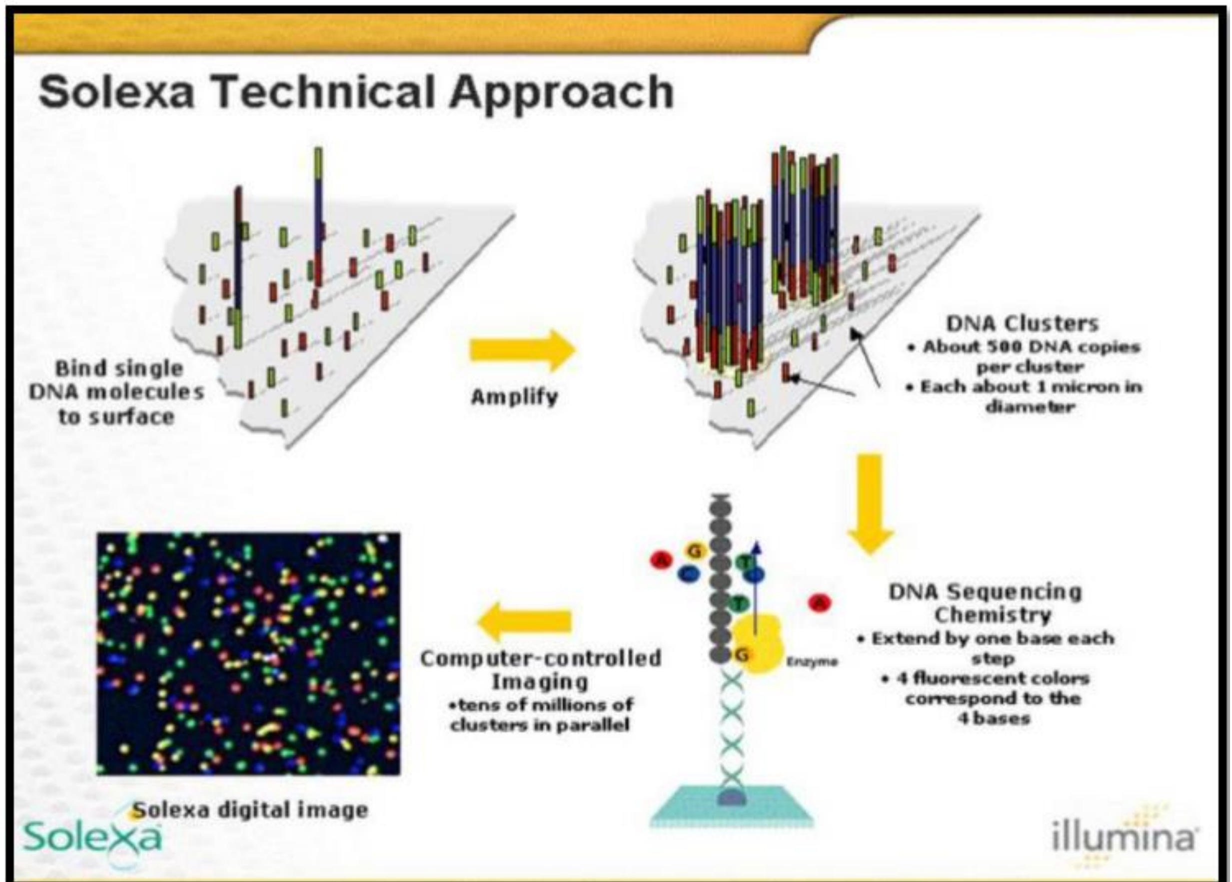


FIGURE 7 - ILLUMINA SOLEXA

MATERIALS AND METHODS

Materials and Methods

STUDY POPULATION

Considering the cost involved and sheer complexity of the technology used and data obtained microbiome studies are difficult to perform in large population. Our study utilized a sample of 10 patients in each group as per previous studies by Zheng et al[79], Dzink et al[15], who used a similar sample size in their study.

INCLUSION CRITERIA:

A total of 30 individuals seeking dental treatment in Ragas Dental College and Hospitals, Chennai, were involved in the present study, of which 10 were periodontally healthy individuals (control group) and 10 were Gingivitis patients and 10 were Gingival Recession patients (test group). A diagnosis of Gingivitis and Gingival recession was determined based on the American Academy of Periodontology parameters. Control Group consisted of 10 subjects with clinically non-inflamed, healthy gingiva (probing pocket depth {PPD} ≤ 3mm, no clinical attachment loss {CAL}, no bleeding on probing {BOP}). Test Group 1 consisted of 10 subjects with gingivitis with PPD ≤ 3mm, no CAL and BOP >30% & Test group 2 consisted of 10 subjects of gingival recession PPD ≤3mm and CAL ≥ 3mm with BOP >30% as per the new classification of Periodontal diseases and conditions.

The study protocol was explained, and written informed consent was received from each individual before clinical periodontal examinations and saliva sampling. Medical and dental histories were obtained.

EXCLUSION CRITERIA:

1) Patients with systemic disorders, such as diabetes mellitus or immunological disorders, HIV.
2) Patients on drugs that have potential to interfere with microbial characteristics such as immunosuppressant drugs or steroids.
3) Patients with history of tobacco usage.
4) Patients with history of periodontal treatment in the past 6 months.
5) Patients under antimicrobial therapy for the past 6 months.

SALIVA SAMPLING:

All examinations were performed by a single, calibrated examiner. The test and control group patients were selected and the sample was collected in a sterile salivary tub. Unstimulated whole saliva was collected in the morning and subjects had to refrain from eating, drinking, smoking or performing any oral hygiene for at least 2 hours prior to the collection. The samples obtained were frozen and stored at -80°C until the sample collection period was completed. All

the samples were collected within 2 days and then sent for processing so as to avoid any degradation.

DNA extraction, 16S rRNA amplification, library construction and sequencing:

Genomic DNA was extracted from 30 saliva samples of 10 from Gingivitis patients, 10 from gingival recession patients and 10 from healthy control with the Qiagen powersoil kit according to manufacturer's recommendations.

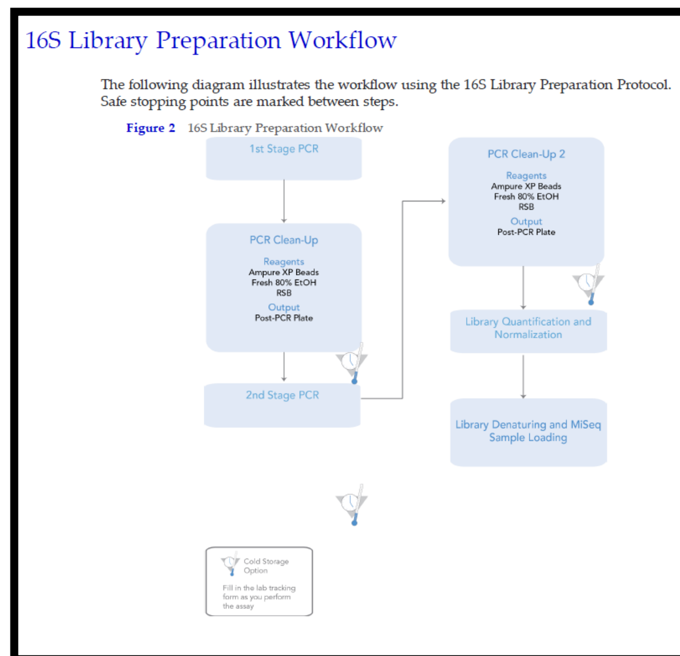
DNA QUALITY CONTROL:

DNA samples were quantitated using Nanodrop. All the samples passed QC and were taken for further library preparation.

16S Metagenomic Sequencing Library Preparation

Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System.

Metagenomic studies are commonly performed by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. Variable regions of 16S rRNA are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations.



This study used the variable V3 and V4 regions of the 16S rRNA gene. After sequencing the V3 and V4 regions a benchtop sequencing system, on board primary analysis, and secondary analysis using MiSeq Reporter or Base Space, provides a comprehensive workflow for 16S rRNA amplicon sequencing.

Workflow Summary:

1) To Order amplicon primers, this protocol included the primer pair sequences for the V3 and V4 region that create a single amplicon of approximately ~460 bp. The protocol also included overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters.

2) In DNA library preparation, this protocol described the steps to amplify the V3 and V4 region and using a limited cycle PCR, add Illumina sequencing adapters and dual-index barcodes are added to the amplicon target. By using the full complement of Nextera XT indices, up to 96 libraries was pooled together for sequencing.

3) On MiSeq sequencing, paired 300-bp reads and MiSeq v3 reagents were used, the ends of each read are overlapped to generate high-quality, full-length reads of the V3 and V4 region in a single 65-hour run. The MiSeq run output is approximately > 20 million reads and assuming 96 indexed samples, can generate > 100,000 reads per sample, commonly recognized as sufficient for metagenomic surveys.

4) Analyzing on MSR or BaseSpace- The Metagenomics workflow was a secondary analysis option built into the MiSeq Reporter (on-system software) or available on BaseSpace (cloud-based software). The Metagenomics Workflow performed a taxonomic classification using the Green genes database showing genus or species level classification in a graphical format.

AMPLICON PCR:

Reactions were cleaned up with Agencourt AMPure XP beads (Beckman Coulter Genomics) according to the manufacturer’s protocol. Attachment of dual indices and Illumina sequencing adapters was performed using 5µl of amplicon

PCR product DNA, 5µl of Illumina Nextera XT Index 1 Primer (N7xx) from the Nextera XT Index kit, 5 µl of Nextera XT Index 2 Primer (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10µl of PCR-grade water (UltraClean DNA-free PCR water; MO BIO Laboratories, Inc., Carlsbad, CA, USA), with thermocycling at 95°C for 3 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes.

Library Quantification, Normalization, and Pooling:

This study utilized the Illumina's recommendation quantifying libraries with a fluorometric quantification method that used dsDNA binding dyes. The concentrated final library was diluted using Resuspension Buffer (RSB) or 10 mM Tris pH 8.5 to 4 nM. 5 µl of diluted DNA was aliquoted from each library and mixed for pooling libraries with unique indices. Depending on coverage needs, up to 96 libraries can be pooled for one MiSeq run. For metagenomics samples, >100,000 reads per sample is sufficient to fully survey the bacterial composition. This number of reads allows for sample pooling to the maximum level of 96 libraries, given the MiSeq output of > 20 million reads.

Library Denaturing and MiSeq Sample Loading:

In preparation for cluster generation and sequencing, pooled libraries are denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq sequencing. Each run must include a minimum of 5% PhiX to serve

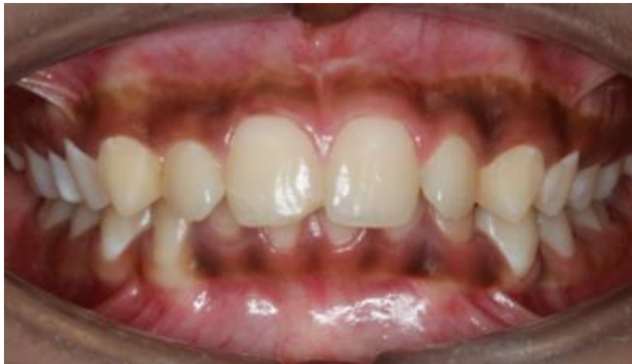
as an internal control for these low diversity libraries. Illumina recommends using MiSeq v3 reagent kits for improved run metrics.

MiSeq Reporter Metagenomics Workflow:

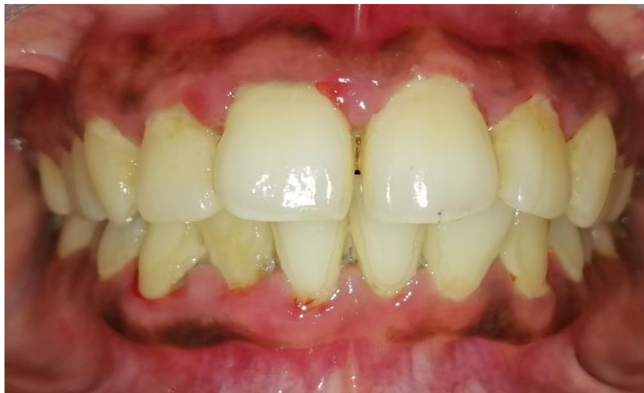
After samples were loaded, the MiSeq system provides on-instrument secondary analysis using the MiSeq Reporter software (MSR). MSR provides several options for analysing MiSeq sequencing data. For this demonstrated 16S protocol, select the Metagenomics workflow. By following this 16S Metagenomics protocol, the Metagenomics workflow classifies organisms from your V3 and V4 amplicon using a database of 16S rRNA data. The classification is based on the Greengenes database (http://greengenes.lbl.gov/). The output of this workflow is a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species. Data analysis was done by using 16s metagenomics tool from Base Space Onsite. Operational taxonomic units (OTUs) were assigned to each sequence using HOMD database. Statistical analysis was performed for individual bacteria using frequency distribution and intergroup comparison was done using Chi square test with statistical significance set as $P < 0.05$.

PHOTOGRAPHS

GINGIVAL HEALTH



GINGIVITIS

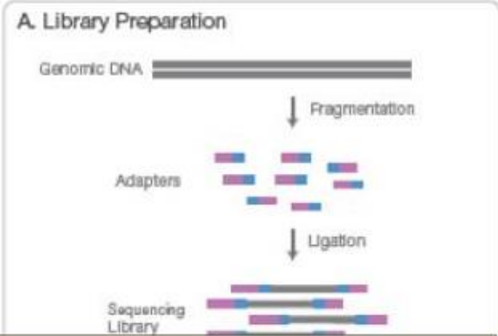


GINGIVAL RECESSION

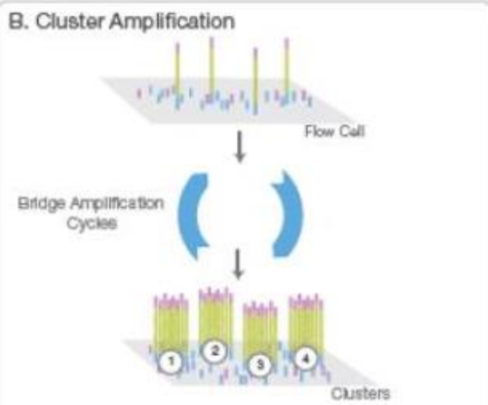
STERILE SALIVA TUBE

ILLUMINA SEQUENCING

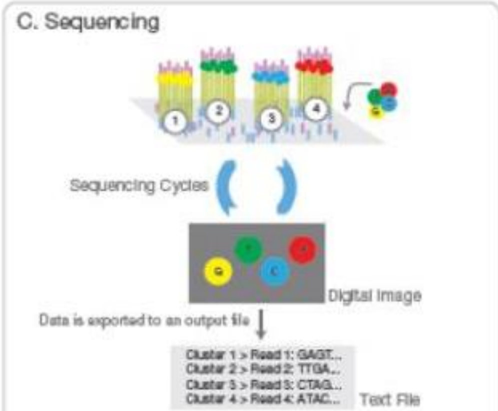
FOUR BASIC STEPS IN NGS ILLUMINA WORKFLOW



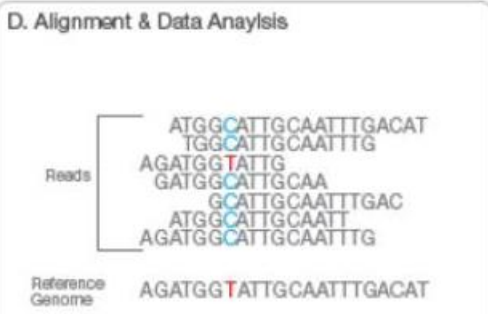
NGS library is prepared by fragmenting a gDNA sample and ligating specialized adapters to both fragment ends.



Library is loaded into a flow cell and the fragments hybridize to the flow cell surface. Each bound fragment is amplified into a clonal cluster through bridge amplification.

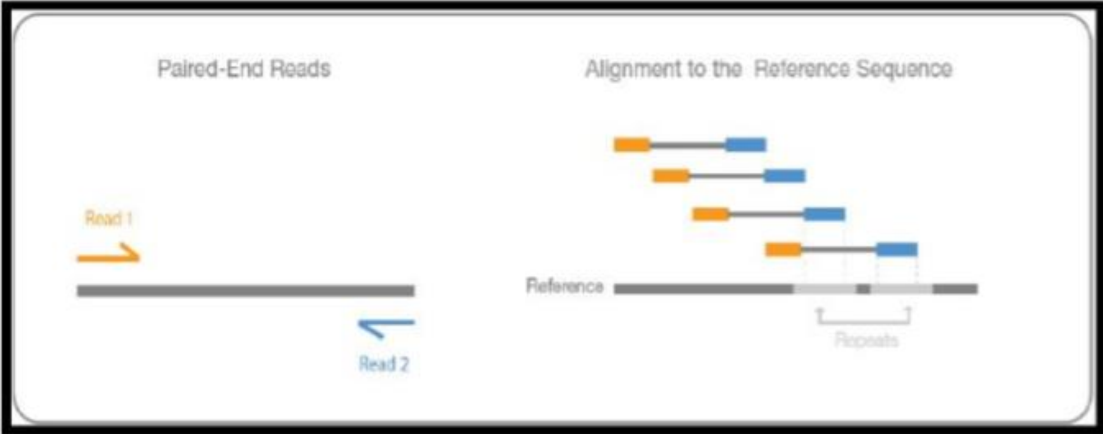


Sequencing reagents, including fluorescently labeled nucleotides, are added and the first base is incorporated. The flow cell is imaged and the emission from each cluster is recorded. The emission wavelength and intensity are used to identify the base. This cycle is repeated "n" times to create a read length of "n" bases.

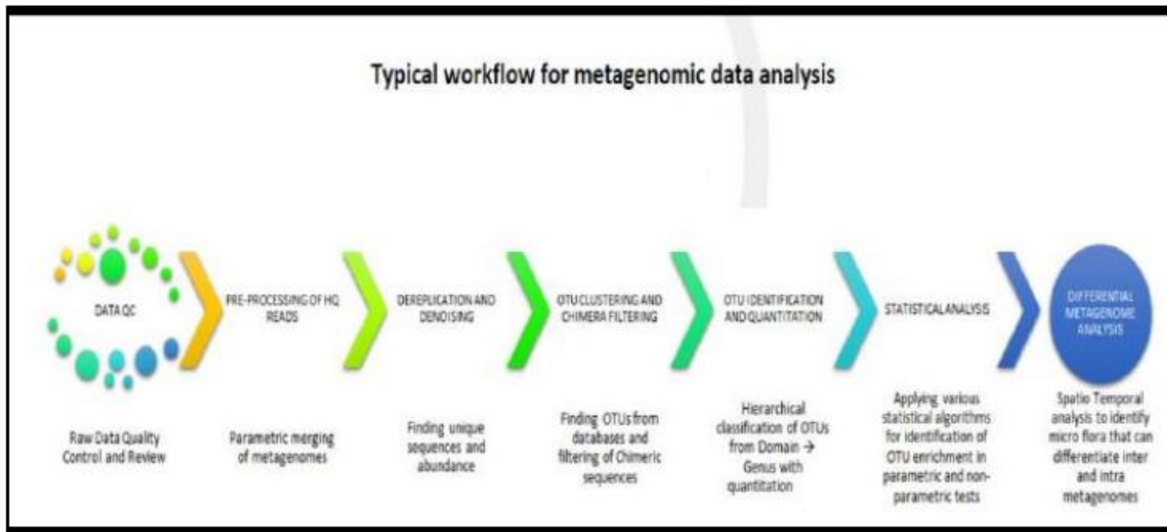


Reads are aligned to a reference sequence with bioinformatics software. After alignment, differences between the reference genome and the newly sequenced reads can be identified.

PAIRED END SEQUENCING AND ALINGMENT



WORK FLOW FOR METAGENOMICS DATA ANALYSIS



RESULTS

RESULTS

The present study was carried out among 10 individuals in each group, that is in health and diseased individuals seeking dental treatment in Ragas Dental College and Hospital, Chennai. The age distribution of the study participants ranged from 20-45 years.

Saliva samples were collected in a saliva tub from periodontally healthy individuals designated as control group (H1- H10); the two test groups were Gingivitis patients designated as (G1-G10) and Gingival recession as (P1-P10).

Orange complex bacteria includes Campylobacter rectus, Campylobacter gracilis, Campylobacter showae, Campylobacter concisus, Eubacterium nodatum, Fusobacterium nucleatum animalis, Fusobacterium nucleatum vincentii, Fusobacterium nucleatum polymorphum, Fusobacterium periodonticum, Prevotella intermedia, Prevotella nigrescens, Prevotella melaninogenica, Parvimonas micra, Peptostreptococcus micros and Streptococcus constellatus.

Health Group:

Our results in **Table 1** have shown that in Health group, Fusobacterium nucleatum vincentii and Fusobacterium periodonticum was present in all the 10 samples(H1-H10). Fusobacterium nucleatum polymorphum was present in 7/10 samples (H1, H2, H3, H4, H6, H7, H8 & H10); F. nucleatum animalis in 5/10 samples (H1, H2, H6, H7 & H10);

Campylobacter concisus was present in all the 10 samples; Campylobacter rectus in 4/10 samples (H1, H2, H6 & H9); Campylobater gracilias in 3/10 samples (H2, H2, H3 & H6).Prevotella melaninogenica was present in 8/10 samples(H1, H2, H3, H4, H5, H6, H9 & H10); P. nigrescens in 4/10 samples (H1, H2, H3 & H6); Parvimonas micra was present in 4/10 samples (H2, H5, H6, H7 &H10);); Peptostreptococcus micros was present in only one sample (H1).

Campylobacter showae, Eubacterium nodatum, Prevotella intermedia and Streptococcus constellatus were not detected in any of the health samples examined in this study. (Refer to **Table 1**)

Gingivitis Group:

In Gingivitis test group, Fusobacterium nucleatum vincentii, Fusobacterium nucleatum polymorphum, Fusobacterium periodonticum, Campylobacter concisus and Campylobacter gracilis were present in 9 samples (G1, G3, G4, G5, G6, G7, G8, G9& G10). Fusobacterium nucleatum animalis was present in 7/10 samples (G1, G3, G4, G5, G6, G7, G8&G9); Campylobacter rectus was present in 7/10 samples (G1, G3, G5, G6, G7, G8 & G9); Prevotella nigrescens was present in 8/10 samples (G1, G4, G5, G6, G7, G8, G9& G10); Prevotella intermedia was present in 4/10 samples (G1, G6, G7 & G10); Prevotella melaninogenica was present in 6/10 samples (G4, G5, G6, G7, G8 & G10); Streptococcus constellatus was present in 7/10 samples (G1,

G3, G4, G6, G7, G8 & G10); Parvimonas micra was present in 4/10 samples (G1, G4, G5 & G7).

Campylobacter showae, Eubacterium nodatum and Peptostreptococcus micros were not detected in any of the gingivitis samples examined in this study. Refer to **Table 2.**

Gingival Recession Group:

In Gingival recession test group, Fusobacterium nucleatum vincentii, Fusobacterium periodonticum, Prevotella nigrescens, Campylobacter gracilis, campylobacter rectus, Campylobacter concisus and Streptococcus constellatus were present in all samples. Fusobacterium nucleatum polymorphum was present in 9/10 samples (P1, P2, P3, P4, P6, P7, P8, P9 &P10); Fusobacterium nucleatum animalis was present in 3/10 samples (P4, P5 & P6); Prevotella intermedia is present in 6/10 samples (P2, P4, P6, P8, P9 & P10); Prevotella melaninogenica was present in 6/10 samples (P1, P5, P7, P8, P9 & P10) and Parvimonas micra was present in 7/10 samples (PP1, P2, P4, P6,P7, P9 & P10).

Campylobacter showae, Eubacterium nodatum and Peptostreptococcus micros were not detected in any of the Gingival recession samples examined in this study. (Refer to **Table 3**)

FREQUENCY OF DISTRIBUTION OF ORANGE COMPLEX IN THREE GROUPS:

The frequency of distribution of Orange complex bacteria in Health, Gingivitis and Gingival recession were measured with Chi-square test. There was a statistically significant difference in distribution of 5 organisms (Prevotella nigrescens P=0.08, S. constellatus P= 0.001, C. rectus P=0.014, P. intermedia P=0.015, C. gracilis P=0.001) among the three groups (Refer to **Table 4**).

INTER-GROUP COMPARISON BETWEEN HEALTH AND GINGIVITIS GROUPS:

The inter-group comparison between Health and Gingivitis group were measured with Chi-square test. There was a statistically significant increase in the following organisms, Prevotella nigrescens(P=0.04), Streptococcus constellatus(P=0.001), Prevotella intermedia(P=0.015) and Campylobacter gracilis(P=0.02) in Gingivitis group compared to the Health group (Refer to **Table 5**).

INTER-GROUP COMPARISON BETWEEN HEALTH AND GINGIVAL RECESSION GROUPS:

The inter-group comparison between Health and Gingival Recession were measured with Chi-square test. There was a statistically significant increase in the following organisms, Prevotella

nigrescens(P=0.014), Streptococcus constellatus(P=0.001), Prevotella intermedia(P=0.014), Campylobacter rectus(P=0.014) and Campylobacter gracilis(P=0.001) in Gingival recession group compared to the Health group (Refer to **Table 6**).

INTER-GROUP COMPARISON BETWEEN GINGIVITIS AND GINGIVAL RECESSION GROUPS:

The inter-group comparison between Gingivitis and Gingival recession groups were measured with Chi-square test. Hence the $P>0.05$ in **Table 7** shows that there was no statistically significant difference in distribution of Orange complex organisms among the Gingivitis & Gingival recession.

While comparing the shift from health group to gingivitis, there was a significant increase in P. intermedia, S. constellatus, P. nigrecens and C. gracilis in Gingivitis group. On comparing the microbial shift from Health to Gingival recession, there is a significant increase in P. intermedia, S. constellatus, P. nigrecens, C. rectus and C. gracilis in Gingival recession group. Interestingly, both the Gingivitis and Gingival recession group shows distinct microbial profile.

The salivary microbiome as a whole obtained in our study was represented at the genus level using the phylogenetic tree. (Refer to GRAPH 2)

TABLES AND GRAPHS

TABLE 1: DISTRIBUTION OF ORANGE COMPLEX IN HEALTH GROUP

S.no	Orange complex	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
1	Campylobacter gracilis	-	1	1	-	-	1	-	-	-	-
2	Campylobacter rectus	1	1	-	-	-	1	-	-	1	-
3	Campylobacter showae	-	-	-	-	-	-	-	-	-	-
4	Campylobacter concisus	1	1	1	1	1	1	1	1	1	1
5	F.nucleatum polymorphum	1	1	1	-	1	1	1	-	-	1
6	F. nucleatum vincentii	1	1	1	1	1	1	1	1	1	1
7	F. nucleatum animalis	1	1	-	-	-	1	1	-	-	1
8	F. periodonticum	1	1	1	1	1	1	1	1	1	1
9	Prevotella intermedia	-	-	-	-	-	-	-	-	-	-
10	Prevotella nigrescens	1	1	1	-	-	1	-	-	-	-
11	Prevotella melaninogenica	1	1	1	1	1	1	-	-	1	1
12	Eubacterium nodatum	-	-	-	-	-	-	-	-	-	-
13	Streptococcus constellatus	-	-	-	-	-	-	-	-	-	-
14	Peptostreptococcus micros	1	-	-	-	-	-	-	-	-	-
15	Parvimonas micra	-	1	-	-	-	1	1	-	-	1

TABLE 2: DISTRIBUTION OF ORANGE COMPLEX IN GINGIVITIS GROUP

S.N0	Orange complex	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
1	Campylobacter gracilis	1	-	1	1	1	1	1	1	1	1
2	Campylobacter rectus	1	-	1	-	1	1	1	1	1	-
3	Campylobacter showae	-	-	-	-	-	-	-	-	-	-
4	Campylobacter concisus	1	-	1	1	1	1	1	1	1	1
5	F.nucleatum polymorphum	1	-	1	1	1	1	1	1	1	1
6	F. nucleatum vincentii	1	-	1	1	1	1	1	1	1	1
7	F. nucleatum animalis	1	-	1	-	1	1	1	1	-	1
8	F. periodonticum	1	-	1	1	1	1	1	1	1	1
9	Prevotella intermedia	1	-	-	-	-	1	1	-	-	1
10	Prevotella nigrescens	1	-	-	1	1	1	1	1	1	1
11	Prevotella melaninogenica	-	-	-	1	1	1	1	1	-	1
12	Eubacterium nodatum	-	-	-	-	-	-	-	-	-	-
13	Streptococcus constellatus	1	-	1	1	-	1	1	1	-	1
14	Peptostreptococcus micros	-	-	-	-	-	-	-	-	-	-
15	Parvimonas micra	1	-	-	1	1	-	1	-	-	-

TABLE 3: DISTRIBUTION OF ORANGE COMPLEX IN GINGIVAL RECESSION

S.N0	ORANGE COMPLEX	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1	Campylobacter gracilis	1	1	1	1	1	1	1	1	1	1
2	Campylobacter rectus	1	1	1	1	1	1	1	1	1	1
3	Campylobacter showae	-	-	-	-	-	-	-	-	-	-
4	Campylobacter concisus	1	1	1	1	1	1	1	1	1	1
5	F. nucleatum polymorphum	1	1	1	1	-	1	1	1	1	1
6	F. nucleatum vincentii	1	1	1	1	1	1	1	1	1	1
7	F. nucleatum animalis	-	-	-	1	1	1	-	-	-	-
8	F. periodonticum	1	1	1	1	1	1	1	1	1	1
9	Prevotella intermedia	-	1	-	1	-	1	-	1	1	1
10	Prevotella nigrescens	1	1	1	1	1	1	1	1	1	1
11	Prevotella melaninogenica	1	-	-	-	1	-	1	1	1	1
12	Eubacterium nodatum	-	-	-	-	-	-	-	-	-	-
13	Streptococcus constellatus	1	1	1	1	1	1	1	1	1	1
14	Peptostreptococcus micros	-	-	-	-	-	-	-	-	-	-
15	Parvimonas micraa	1	1	-	1	-	1	1	-	1	1

TABLE 4: FREQUENCY OF DISTRIBUTION OF ORANGE COMPLEX IN THREE GROUPS

S.NO	ORANGE COMPLEX	Healthy	Gingivitis	Periodontitis	Chi square	P value
1	Fusobacterium nucleatum Vincentii	90%	90%	100%	1.071	0.58
2	Fusobacterium nucleatum polymorphum	70%	90%	80%	1.25	0.53
3	Fusobacterium nucleatum animalis	50%	70%	30%	3.20	0.20
4	Fusobacterium nucleatum periodonticum	90%	90%	100%	1.071	0.58
5	Prevotella nigrescens	40%	80%	100%	9.54	0.008
6	Streptococcus constellatus	0	70%	100%	21.48	0.001
7	Campylobacter rectus	40%	70%	100%	8.57	0.014
8	Campylobacter showae	0	0	0	*	*
9	Prevotella intermedia	0	40%	60%	8.40	0.015
10	Prevotella melaninogenica	80%	60%	60%	1.20	0.54
11	Campylobacter concisus	100%	90%	100%	2.06	0.35
12	Parvimonas micra	40%	40%	70%	2.40	0.301
13	Campylobacter gracilis	30%	90%	100%	14.65	0.001
14	Peptostreptococcus micos	10%	0	0	2.069	0.355
15	Eubacterium nodatum	0	0	0	*	*

TABLE 5: INTER-GROUP COMPARISON BETWEEN HEALTH AND GINGIVITIS GROUPS

S.NO	ORANGE COMPLEX	Healthy	Gingivitis	Chi square	P value
1	Fusobacterium nucleatum Vincentii	90%	90%	*	*
2	Fusobacterium nucleatum polymorphum	70%	90%	1.25	0.53
3	Fusobacterium nucleatum aimalis	50%	70%	3.20	0.20
4	Fusobacterium nucleatum periodonticum	90%	90%	*	*
5	Prevotella nigrescens	40%	80%	4.54	0.04
6	Streptococcus constellatus	0	70%	21.48	0.001
7	Campylobacter rectus	40%	70%	3.57	0.06
8	Campylobacter showae	0	0	*	*
9	Prevotella intermedia	0	40%	8.40	0.015
10	Prevotella melaninogenica	80%	60%	1.20	0.54
11	Campylobacter concisus	100%	90%	2.06	0.35
12	Parvimonas micra	40%	40%	*	*
13	Campylobacter gracilis	30%	90%	14.65	0.02
14	Peptostreptococcus micos	10%	0	2.069	0.355
15	Eubacterium nodatum	0	0	*	*

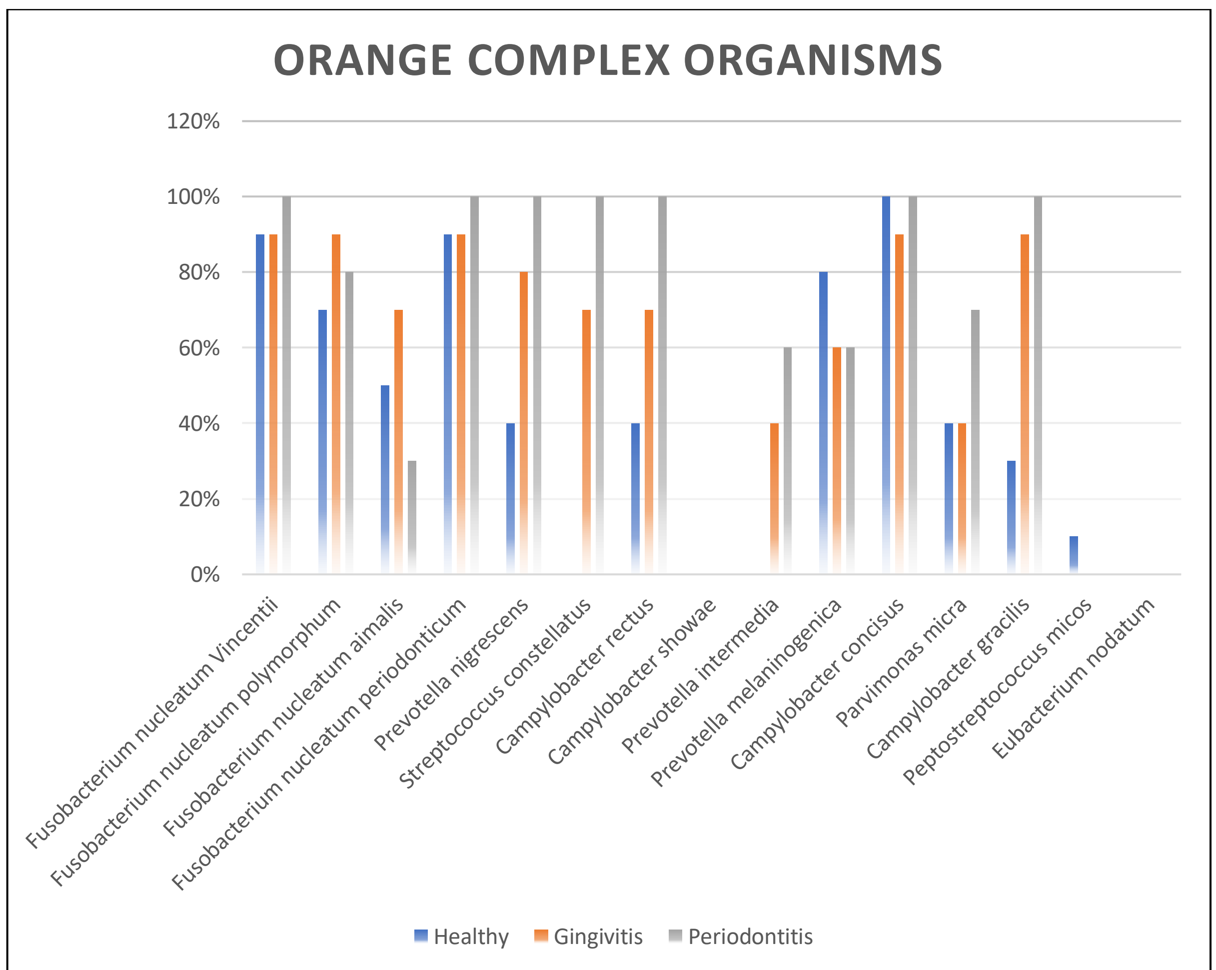
TABLE 6: INTER-GROUP COMPARISON BETWEEN HEALTH AND GINGIVAL RECESSION GROUPS

S.NO	ORANGE COMPLEX	Healthy	Periodontitis	Chi square	P value
1	Fusobacterium nucleatum Vincentii	90%	100%	1.071	0.88
2	Fusobacterium nucleatum polymorphum	70%	80%	1.25	0.53
3	Fusobacterium nucleatum aimalis	50%	30%	2.20	0.20
4	Fusobacterium nucleatum periodonticum	90%	100%	0.97	0.88
5	Prevotella nigrescens	40%	100%	8.57	0.014
6	Streptococcus constellatus	0	100%	21.48	0.001
7	Campylobacter rectus	40%	100%	8.57	0.014
8	Campylobacter showae	0	0	*	*
9	Prevotella intermedia	0	60%	8.40	0.015
10	Prevotella melaninogenica	80%	60%	1.20	0.54
11	Campylobacter concisus	100%	100%	*	*
12	Parvimonas micra	40%	70%	2.40	0.301
13	Campylobacter gracilis	30%	100%	14.65	0.001
14	Peptostreptococcus micos	10%	0	2.069	0.355
15	Eubacterium nodatum	0	0	*	*

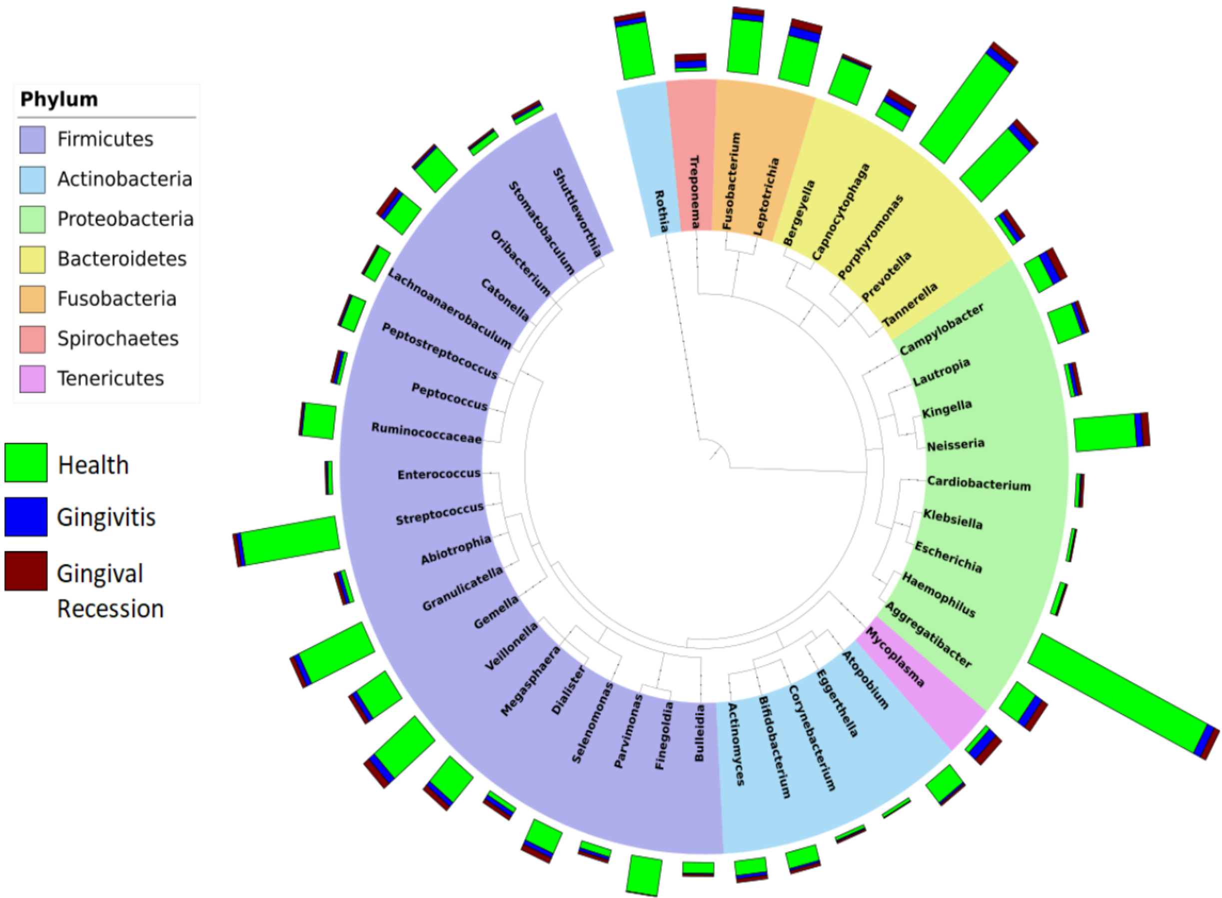
TABLE 7: INTER-GROUP COMPARISON BETWEEN GINGIVITIS AND GINGIVAL RECESSION GROUPS

S.NO	ORANGE COMPLEX	Gingivitis	Periodontitis	Chi square	P value
1	Fusobacterium nucleatum Vincentii	90%	100%	0.071	0.98
2	Fusobacterium nucleatum polymorphum	90%	80%	1.25	0.53
3	Fusobacterium nucleatum aimalis	70%	30%	3.20	0.20
4	Fusobacterium nucleatum periodonticum	90%	100%	1.071	0.58
5	Prevotella nigrescens	80%	100%	1.54	0.08
6	Streptococcus constellatus	70%	100%	1.04	0.07
7	Campylobacter rectus	70%	100%	1.04	0.07
8	Campylobacter showae	0	0	*	*
9	Prevotella intermedia	40%	60%	1.25	0.69
10	Prevotella melaninogenica	60%	60%	*	*
11	Campylobacter concisus	90%	100%	0.071	0.98
12	Parvimonas micra	40%	70%	2.40	0.301
13	Campylobacter gracilis	90%	100%	0.071	0.98
14	Peptostreptococcus micos	0	0	*	*
15	Eubacterium nodatum	0	0	*	*

GRAPH 1: DISTRIBUTION OF ORANGE COMPLEX ORGANISMS IN HEALTH, GINGIVITIS AND GINGIVAL RECESSION PATIENTS:



GRAPH 2: PHYLOGENETIC TREE



DISCUSSION

DISCUSSION

The orange complex includes bacteria which, as “bridge species”, form a link between the early colonizers and the highly pathogenic bacteria of the red complex[11]. The pathogenic potential of these marker bacteria is significantly increased as a result of bacterial interactions such as metabolic interactions or Co-aggregation[3]. The bacteria from the orange complex are thought to be responsible for progressive attachment loss and an increase in pocket depth[30]. Through their metabolism, these bacteria also create the living conditions for the strictly anaerobic bacteria of the red complex and their colonization of the sulcus.

In this study, orange complex has been evaluated in individuals in Gingival health, Gingivitis and Gingival recession for the following reasons.

1) The species in this group are closely associated with one another and coaggregate with the red complex bacteria. Coaggregation has been shown to be a highly specific mechanism by which dental plaque bacteria may interact physically with other bacteria. Most authors have described a potential role for coaggregation in formation of dental plaque biofilms and in particular secondary colonisation and development of a spatially organized community. Coaggregation may provide some metabolic advantages to bacteria through cross-feeding, enzyme complementation and physical proximity[3].

2) Hajishengalis et al have also proposed that these bacteria may act as pathobionts and favour progression of the inflammatory process that is probably initiated by the red complex bacteria[2].

Salivary diagnostic aids are used as the biomarkers for prediction of periodontal disease activity because of their non-invasive ease of collection and bio availability of most molecules present in the GCF and serum. As most studies have focused on host related biomarkers, salivary profiling has largely been restricted to genomic, transcriptomic and proteomic analysis. Salivary microbiome analysis and a potential profiling has received much less attention although it is widely accepted to be a medium for the translocation of oral bacteria[9].

Hitherto, culture based or Closed ended techniques like PCR have been largely relied upon to detect salivary microflora[80]. In the current study, we have used the NGS- Next Generation Sequencing technology for high-throughput genomic analysis.

NGS technology is known to be a high-throughput genomic analysis technique and is an open-ended diagnostic approach. This methodology is in accordance with previous studies of Griffen et al[13], Hong et al[37], Kumar et al[14], who have used NGS to characterize the subgingival microbiome.

The advantages of this method include that the entire bacterial species present in the subgingival environment are simultaneously identified and quantified.

1) Culture based methods cannot identify species whose culture characteristics are unknown. It has been estimated that there are nearly 300 and more uncultivable species are present in subgingival plaque.
2) Extremely sensitive closed ended techniques like DNA probes, RT-PCR can identify only targeted organisms against which specific primers have been designed.

Among the NGS technologies, Illumina sequencing has been used in this study for the following reasons,

1) It provides more sequence per run as a result of which there is a greater depth coverage than other technologies. This in turn helps to analyse a larger sample size, include more bar-coded time points and assess the total diversity in microbiome.
2) The low abundance taxa can be determined with generation and sequencing
of short 16S rRNA amplicons.

The salivary microbiome as a whole obtained in our study was represented at the genus level using the phylogenetic tree. (GRAPH 2)

The microbiome analysis includes segregation of various bacteria starting from Phyla, Order, Class, Family, Genus and Species. The overall difference in 3 groups at genus level has been represented under the phylogenetic tree as depicted in Graph 2. There are distinct differences in the microbial profile at genus level between health and disease groups examined, however this study has been focused on the orange complex.

According to Socransky's classification of bacterial complexes, the orange complex consists of four important phyla namely Fusobacteria, Proteobacteria, Bacteriodetes and Firmicutes; The genera includes Fusobacterium, Prevotella, Campylobacter, Streptococcus, Parvimonas, Eubacterium and Peptostreptococcus. The results of this study pertaining to the orange complex organisms depicted in the phylogenetic tree includes Fusobacteria, Campylobacter, Prevotella, Parvimonas and Streptococcus. (Graph 2)

The results of this study showed that Eubacterium nodatum, Peptostreptococcus micros and Campylobacter showae were not present in any of the 30 samples examined. While the reason for this was not immediately apparent, these results were in accordance with the previous studies that have reported wide variations in subgingival microflora among populations[35].

The results of this study showed that Fusobacterium nucleatum vincentii, Fusobacterium periodonticum and Campylobacter concisus were present in all the 30 samples examined. Our results were in agreement with the following studies by Savitt[29] and Sockransky[12], Moore et al[52] and Tanner et al[73],

These studies showed that F.nucleatum was ubiquitously present in oral cavity. These results are in line with microbiome data which suggested that most periodontal disease organisms tend to be oral commensal which acquire pathogenicity with specific environment/ susceptible groups[2].

Prevotella intermedia and Streptococcus constellatus are present only in Gingival disease group compared to Health. Hafstrom C et al[81], Haffajee et al[30], Magnusson et al[82] and Okayama et al[83] studies were in accordance to the study results. The inter-relationship between bleeding on probing and P. gingivalis and P. intermedia were demonstrated in many studies[81,84]. P. intermedia and P. gingivalis has several surface properties which may be regarded as potential virulence factors that includes LPS, capsule and surface appendages which may mediate attachment and causes periodontal tissue breakdown.

Prevotella melaninogenica was present in both the health and disease group but it was more significantly increased in Health group compared to the disease group. On contrary to this study results, Syed and Loesche et al[85] showed that Prevotella melaninogenica was present only in Gingival disease when compared to the healthy sites.

Prevotella nigrescens, Campylobacter gracilis and Campylobacter rectus were present in both the health and disease group but it was more significantly increased in Gingival disease group when compared to health group. These results were in agreement with the studies, conducted by P. J. Macuchi and Tanner et al. Campylobacter rectus with flagellum for motility has

been shown to reach the depth of periodontal pockets. It has been demonstrated to have a characteristic cell surface layer composed of hexagonally arrayed sub-units, with the capability to evoke antigenic responses. C. gracilis and C. concisus were increased in shallow sites than deeper periodontal pockets[86]. P. nigrescens is associated with either healthy or shallow active sites whereas P. intermedia is mostly isolated from periodontally diseased/ deeper active sites[87].

Parvimonas micra was present in all the three study groups but it was marginally increased in Gingival recession group (not statistically significant) when compared to Gingivitis and Health group. Our study results fall in line with Claudia Ota-Tsuzuki et al study results that P. micra isolates were detected from the sites with and without loss of attachment[88].

The results of this study may be interpreted as follows;
distinct microbial profiles with respect to orange complex organisms were observed in disease groups compared to health group. The health associated microbial profile showed a preponderance of F. nucleatum vincentii, F. nucleatum polymorphum, F. periodonticum and P. melaninogenica; the gingivitis associated microbial profile showed a preponderance of P. intermedia, P. nigrescens, C. gracilis and S. constellatus; the gingival recession associated microbial profile showed a preponderance of P. intermedia, P. nigrescens, C. rectus, C. gracilis and S. constellatus.

There was no statistically significant difference between gingivitis and gingival recession when the orange complex bacteria were examined. C.

rectus showed the highest difference between the two groups but this increase did not achieve statistically significance (P=0.07).

The lack of difference between gingivitis and gingival recession may be a result of the plaque environment being pretty much same in both groups. In Gingival recession, the plaque environment was provided with minimal pocket depth and with minimal anaerobic environment.

The microbial presence in saliva may/may not directly contribute to periodontal disease activity in individual sites. The microflora in saliva may originate from plaque, but helps in the translocation of bacteria from one site to another. The salivary microbial diagnostics can be used during the screening phase, the use of saliva to identify patients at risk for future disease activity opens the door for heightened risk management strategies, preventive care and/or behavioural change on the part of the patient to prevent the onset of disease.

Recent literature however suggested that there may be migrating microbiomes which may play a role in dissemination and subsequent pathology[89]. In the light of scanty information available to us, presently their role is yet to be fully understood. A relatively small sample size and the cross-sectional nature were some of the limitations associated with this study.

Within the limits of the study it appears that salivary Campylobacter gracilis, Prevotella intermedia, Prevotella nigrescens, Camylobacter rectus, Campylobacter concisus and Streptococcus constellatus

levels may be used as the risk markers of Gingivitis and Gingival recession. Further longitudinal studies with a greater sample size are required to substantiate these results.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

This study characterized the orange complex in the microbiome of saliva in Gingival health, Gingivitis and Gingival recession individuals. The whole unstimulated saliva was collected in each of 10 samples in Gingival health, Gingivitis and Gingival Recession individuals and salivary microbiome characterization was done with NGS technology using Illumina sequencing.

Among the orange complex bacteria, Fusobacterium nucleatum vincentii, Fusobacterium peridonticum and Campylobacter concisus were present in all the 30 samples examined including Gingival health, Gingivitis and Gingival Recession. Peptostreptococcus micros, Eubacterium nodatum and Campylobacter showae were not present in any of the 30 samples examined.

There was a statistically significant difference in distribution of 5 organisms (Prevotella nigrescens P=0.08, S constellatus P= 0.001, C rectus P=0.014, P intermedia P=0.015, C gracilis P=0.001) in gingivitis and gingival recession groups when compared to health group.

There was a statistically significant increase in the following organisms, Prevotella nigrescens(P=0.04), Streptococcus constellatus(P=0.001), Prevotella intermedia(P=0.015) and Campylobacter gracilis(P=0.02) in gingivitis group when compared to health group.

There was a statistically significant increase in the following organisms, Prevotella nigrescens(P=0.014), Streptococcus constellatus(P=0.001), Prevotella intermedia(P=0.014), Campylobacter rectus(P=0.014) and Campylobacter gracilis (P=0.001) in gingival recession group when compared to health group.

Further longitudinal studies with a higher sample size needed to be done to confirm these findings.

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ANNEXURE - I

RAGAS DENTAL COLLEGE & HOSPITAL

Department of periodontology

Patient record

DEMOGRAPHIC DATA

Name:	Date:
Age/Sex:	Occupation:
Address:	Sample no.:

Chief complaint:

History of presenting illness:

Past dental history:

Past medical history:

Family history:

Personal history & habits:

General examination:

CLINICAL EXAMINATION

Hard tissues:

Soft tissues:

GINGIVA

Colour:

Contour:

Consistency:

Position:

Surface texture:

Width of attached gingiva:

Pigmentation:

Vestibule:

Bleeding on probing:

Size & Shape:

Exudate:

Frenal attachment:

Tension test:

Fremitus:

PLAQUE SCORE:

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

BLEEDING SCORE:

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

CALCULUS SCORE:

B																
	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
P																

B																
	48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38
P																

Provisional diagnosis:

Prognosis:

Treatment plan:

ANNEXUTER - II

CONSENT FORM

I …………………………………… S/o / W/o / D/o …………………………… aged …… years, Hindu/Christian/Muslim ………………. residing at …………………………… …………………………………………… do solemnly and state as follows.

I am the deponent herein; as such I am aware of the facts stated here under

I state that I came to Ragas Dental College and Hospital, Chennai for my treatment for ………………………………………………………………………………………………..

I was examined by Dr………………………….. and I was requested to do the following

1. Full mouth Plaque Score
2. Full mouth bleeding score

3 Measurement of periodontal pocket depth and clinical attachment loss

I was also informed and explained about the collection of plaque during scaling in …………………………………(language) known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequence of the dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I also authorise the Doctor to proceed with further treatment or any other suitable alternative method for the study.

I have given voluntary consent to the collection of plaque for approved research.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

Signature of the patient/Attendant

The patient was explained the procedure by me and has understood the same and with full consent signed in (English/Tamil/Hindi/Telugu? ....) before me.

Signature of the Doctor

ANNEXURE- III

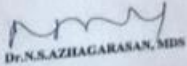
RAGAS DENTAL COLLEGE & HOSPITAL

(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr.M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi,Chennai- 600 119.INDIA
Tele:(044)24530002,24530003 - 06. Principal (Dir) 24530001 Fax:(044) 24530009

TO WHOM SO EVER IT MAY CONCERN

From | Date: 09-01-2020
The Institutional Review Board | Place: Chennai
Ragas Dental College and Hospital
Uthandi, Chennai-600119.

The Project topic titled "**MICROBIAL ANALYSIS OF ORANGE COMPLEX ORGANISMS OF THE WHOLE SALIVA IN PATIENTS WITH GINGIVITIS AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING**" submitted by **Dr. S INDHUMATHI** has been approved by the Institutional Review Board of Ragas Dental College and Hospital.



Dr.N.S.AZHAGARASAN, MDS

Member Secretary,
The Institutional Review Board,
Ragas Dental College and Hospital,
Uthandi, Chennai-600119.

ANNEXURE – IV

Urkund Analysis Result

Analysed Document: 4 INTRODUCTION-merged.pdf (D63077174)
Submitted: 1/28/2020 4:58:00 AM
Submitted By: indhu4192@gmail.com
Significance: 11 %

Sources included in the report:

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Instances where selected sources appear:

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ANNEXURE- V

The Tamil Nadu Dr.M.G.R. Medical University
69, Anna Salai, Guindy, Chennai - 600 032.

DEPARTMENT OF EPIDEMIOLOGY
CREDIT POINTS : 30

This certificate is awarded to Dr./Mr./Ms. INDHUMATHI S

for participating as a Delegate in the three days Workshop on 'Research Methodology and Biostatistics : How to do a Good Dissertation & Publish?' from 04-12-2019 to 06-12-2019.

Dr.G.SRINIVAS PROFESSOR & HEAD DEPARTMENT OF EPIDEMIOLOGY	Dr.PARAMESWARI SRIJAYANTH REGISTRAR	Dr.SUDHA SESHAYYAN VICE-CHANCELLOR

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