"ANALYSIS OF RED COMPLEX BACTERIA IN THE MICROBIOME OF WHOLE SALIVA IN PERIODONTAL HEALTH AND PERIODONTITIS INDIVIDUALS USING NEXT GENERATION SEQUENCING TECHNOLOGY"

Dissertation submitted to

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BRANCH II

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"ANALYSIS OF RED COMPLEX BACTERIA IN THE MICROBIOME OF WHOLE SALIVA IN PERIODONTAL HEALTH AND PERIODONTITIS INDIVIDUALS USING NEXT GENERATION SEQUENCING TECHNOLOGY"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. K.V. ARUN, M.D.S.**, Professor and HOD, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

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CERTIFICATE

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This dissertation is submitted to **THE TAMILNADU Dr. MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **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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Abstract

ABSTRACT

BACKGROUND

The red complex bacteria which includes *Porphyromonas gingivalis, and Tannerella forsythia (formerly Bacteroides forsythus), Treponema denticola* are described as climax colonizers and are thought to be play an important pathogen in progression of periodontal disease. These bacterial species are usually found together in periodontal pockets, suggesting that they may cause destruction of the periodontal tissue in a cooperative manner. Technological developments in sequencing and identifying DNA and powerful bioinformatics tools have helped in characterization of microbia of interest in relation to the total microbial load. The aim of our study was to evaluate the presence of the red complex bacteria in saliva using Next Generation Sequencing Technology in periodontal health and periodontitis.

MATERIALS AND METHODS

A total of 20 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 periodontitis patients (test group). The salivary red complex was investigated with NGS

technology using Illumina MiSeq sequencing method. Amplicons from V3-V4 hypervariable regions of 16S rRNA gene were sequenced.

RESULTS

The results of our study suggest that the Red complex bacteria were significantly higher in periodontitis [P. *gingivalis* (*P* = 0.020), *T. forsythia* (*P* = 0.005), and *T. denticola* (*P* = 0.033)] when compared to healthy subjects and may therefore be used as microbial risk markers. However, a longitudinal study with a greater sample size will be required to confirm the findings of this study.

KEYWORDS

Illumina MiSeq Sequencing, Next Generation Sequencing, 16S rRNA, Periodontitis, Dysbiosis, salivary microbiome

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LIST OF ABBREVATION

PSD	**POLYMICROBIAL SYNERGY AND DYSBIOSIS**
DNA	**DEOXYRIBONUCLEIC ACID**
rRNA	**RIBOSOMAL RIBONUCLEIC ACID**
HOMIM	**HUMAN ORAL MICROBIOME IDENTIFICATION OF 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**
MiSeq	**METAGENOMIC SEQUENCING**
RSB	**RESUSPENSION BUFFER**

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Introduction

INTRODUCTION

Every human being has a personalized set of microorganisms essential to maintain health, yet also capable of eliciting disease. The microbiome comprises, all these micro-organisms, their genomes and ecosystems[109]. In the human body, the total number of microbial cells is 10 times greater than the total number of human cells [148].

In the oral cavity, an estimated 300 to 400 different species contribute to the total number of microbial taxa [155]. Gingivitis and Periodontitis are common oral disease that are inflammatory in character. The chronic inflammation is evoked by the microbial antigens present in these diverse microflora. Gingivitis is a reversible inflammatory reaction of the marginal gingiva in response to plaque accumulation, whereas Periodontitis is an irreversible chronic inflammatory destruction of the attachment apparatus that may ultimately lead to loss of the involved teeth. Existing evidence indicates that periodontitis is always preceded by gingivitis; however, not all gingivitis develops into periodontitis. The reason being that the accumulation of plaque bacteria is necessary but not adequate by itself for the development of periodontitis .[139]

Over the past 5 decades, understanding and characterization of the dental plaque has undergone significant evolution from Nonspecific plaque hypothesis and Specific plaque hypothesis to Ecological plaque hypothesis. Currently,

"The Polymicrobial Synergy and Dysbiosis (PSD) Model" proposed by **Hajishengallis G et al** [49] explains the pathogenesis of periodontal diseases. This model proposed that, the key events that led to the development of periodontitis are dysbiosis and polymicrobial synergy; rather than an individual bacterial species. He proposed that keystone pathogens belonging to the red complex such as *Porphyromonas gingivalis, Tanneralla forsythia, Treponema denticola* may play a role in this dysbiotic change. The non-protective pro-inflammatory responses are thought to be enhanced by pathobionts such as gram-negative and gram-positive anaerobes that eventually damage the host tissues **.**[48]

Human saliva secreted by the major and the minor salivary glands, performs a wide variety of biological functions including lubrication, buffering, the maintenance of mucosal integrity and antimicrobial protection that are critical for the maintenance of the oral health**.**[34]

In addition to its protective effects, Saliva may play a significant role in the translocation of bacteria from one area of the oral cavity to the other. This intraoral translocation of the periodontopathogens has been shown to play a role in localized juvenile periodontitis patients and has been demonstrated well via periodontal probes [20]

The challenge for clinical investigators and practitioners alike lies in the ability to identify the transition from the gingivitis to the periodontitis and to

predict periodontitis sites that are undergoing progressive destruction. Biofluids such as saliva have been utilized to arrive at innovative diagnostic tests that focus on recognition and prediction of vulnerable sites and assess the response to periodontal interventions. [42,56]Microbial profiling of the saliva may be one such method of early diagnosis and prediction of progressive periodontitis in at risk population.

Next generation sequencing (NGS) is the current trend in sequencing of microbiome. 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.**[119]** The speed of sequencing and amounts of DNA sequence data generated with NGS are very high, enabling it to be considered as a "high-throughput technology" .[4]

There is a relative paucity in literature regarding the salivary microbiome in periodontal health and disease in our population.

Our aim was to evaluate the presence of the red complex bacteria (*Porphyromonas gingivalis, Tanneralla forsythia, Treponema denticola*) with the view that salivary microbial analysis may be a non-invasive method of detecting periodontal disease progression.

Aim and Objectives

AIM AND OBJECTIVES

Aim:

To evaluate the presence of the red complex bacteria (*Porphyromonas gingivalis, Tanneralla forsythia, Treponema denticola*) in saliva using Next Generation Sequencing Technology in health and periodontitis.

Objectives:

1. To evaluate the presence of the red complex bacteria in saliva using Next Generation Sequencing Technology in periodontal health and periodontitis.
2. To compare the presence of the salivary red complex in periodontitis with those of periodontally healthy controls.

Review of Literature

REVIEW OF LITERATURE

HUMAN ORAL MICROBIOME:

Every human being has a personalized set of microorganisms essential to maintain health, yet also capable of eliciting disease. The microbiome comprises all these micro-organisms, their genomes and ecosystems[109]**.** The number of microbial cells in the human body exceeds 10 times greater than the total number of human cells in the body [148]. The total number of microbial taxa that are able to colonize the oral cavity has been evaluated between 300 and 400 different species.

HUMAN ORAL MICROBIOME CONCEPT:

The human microbiome concept was first coined by Joshua Lederberg "to signify the ecological community of symbiotic, commensal, and pathogenic microorganisms that literally share our body space''[73]**.** The term oral Microbiome is defined as the totality of the micro-organisms and their collective genetic material present in the human body or oral cavity. [92]

Turnbaugh et al [148] was classified the human microbiome into two types such as a core microbiome and a variable microbiome**.**

Core microbiome:

Species that appear in most subjects and do not change in their proportions from health to disease. Metabolically versatile as they are capable of thriving under the environmental and nutritional conditions present in both health and periodontitis. Capable of synergistic interactions with health and disease associated species as they successfully grow with both groups. Act as metabolic cornerstones for the whole community and that their presence is important in the microbial shifts from health to periodontitis. These abundant phylotypes would maintain the homeostasis and functional stability essential for a healthy ecosystem [148]; [161].The most abundant core species is found to be Fusobacterium nucleatum.

Variable microbiome

These microbial species are exclusive to the individual and have evolved in response to unique lifestyle, genotypic and phenotypic requirements.

Fusobacterium nucleatum is the bridging organism and recent studies have indicated that the presence of *F. nucleatum* may be essential for the establishment and survival of other periodontopathic (anaerobic) bacteria in sub-gingival plaque, possibly by protecting them from oxidative damage. Its ability to interact physically through co-aggregation with a diverse range of oral species. Metabolically support the growth of taxa associated with periodontitis. By metabolizing oxygen through enzymatic activities such as NADPH oxidase,

it can easily be adapted to aerated conditions. Reducing environment to anaerobic levels in which *Porphyromonas gingival*is can thrive[14]**.** An individual's Oral microbiome is similar to that of their fingerprint even though individuals share microbiota at similar sites of the body, varying differences are observed at species and strain level of the microbiomes. [160]

It was suggested that the ability to maintain homeostasis within a microbial community increases with the diversity of its species. The diversity of the microflora in dental plaque is enhanced by the development of food chains between microbial species, and their use of complementary metabolic strategies for the catabolism of endogenous nutrients, such as glycoproteins and proteins[96].

The healthy microbiome:

Host-related commensal microbial communities have been well established to interfere with the colonization and establishment of pathogenic bacteria, a phenomenon often referred to as bacterial interference or colonization resistance. More recent studies have shown that commensal bacteria not only protect the host simply by niche[74]. More recently, in germ-free mice, **Stappenbeck** has been revealed that commensal microbiota induces angiogenesis, contributing to the development of the complex vascular beds found just underneath the mucosal surface. It has been found that intercellular adhesion marker-1 expression in these vessels is also regulated by the presence of the commensal microbiota[136]**.**

Dysbiosis can be defined as a symbiotic relationship that has disappeared due to a reduction in the number of beneficial symbionts and/or an increase in the number of pathobionts. Biological properties that help to maintain microbiome stability are important for sustaining symbiotic environment and for prevention of dysbiotic condition[112].

Role of plaque in periodontal disease:

Periodontal disease is a chronic inflammatory disease caused by biofilm that affects the tissue that supports the tooth or periodontium[113]and also increases the risk of developing diabetes, atherosclerosis and possibly rheumatoid arthritis[41,88]. The tooth-associated biofilm plays an important role in the initiation and progression of periodontal disease. The irreversible injury on the periodontium is due to the host inflammatory response, resulting in tooth loss in some case [113].

The relationship between the host and periodontal microbiota is generally recognized as benign however, once the specific microbial species overgrows within the sub gingiva this may lead to periodontal inflammation and destruction with attachment loss and bone loss. [114]

An experimental gingivitis study was done by **Loe[85] et al** from 1970 to 1986 among the tea labourers in Srilanka which included 480 males of age group between 14– 46 years. Etiologic role of plaque in periodontitis and its involvement in causation and progression of periodontal diseases was well established. It has been generally accepted that periodontal diseases seem to be

initiated by a complex dental film which comprises limited number of periodontal pathogen, and that they represent a small part of approximately 600 microbial species that are found to colonize dental surfaces over and below the gingival margin and oral mucous membranes.

Criteria for identification of bacterial species as periodontopathogens was proposed by **Socransky and Haffajee -1992**[134] which states that, in proximity to the periodontal lesion the organism must be found in relatively high numbers; in periodontally healthy subjects or in subjects with other forms of periodontal disease the organism must either be absent, or present in much smaller numbers; in periodontally diseased subjects the organism must have high levels of serum, salivary and gingival crevicular fluid antibody developed against it**;** the microbes must be found to produce virulence factors in vitro which can be correlated with clinical histopathology; similar pathogenic properties should be mimicked by the organism in an appropriate animal model ;following treatment must eliminate the putative pathogen from the periodontal lesion leading to clinical improvement.

Microbial aetiology of periodontitis:

Nonspecific plaque Hypothesis – Theilade

Specific plaque hypothesis – Loesche 1976

Ecological Hypothesis- Marsh 2003

Polymicrobial Synergy and Dysbiosis – Hajishengalis 2012

Nonspecific plaque hypothesis: [146]

It was stated that the pathogenicity was determined by the quantity of plaque than without discriminating between the levels of virulence of bacteria. The above said statement states that, the host would have a threshold capacity to detoxify bacterial products (e.g., saliva neutralizing acid) and disease would only develop if this threshold was exceeded and the virulence factors could no longer be neutralized.

Specific plaque hypothesis:

In 1976, Walter J. Loesche[87] introduced the "Specific Plaque Hypothesis" (SPH). The emergence of different species related to periodontal disease led to the idea that oral disease could be initiated by various specific pathogens [146]**.** This idea put forth the famous Socransky-complexes which comprises bacterial clusters based on their association with periodontal disease [131]**.**

Ecological hypothesis:

In 1994 Philip D. Marsh[96] proposed a hypothesis that included key concepts of the earlier hypotheses. In his "Ecological Plaque Hypothesis" (EPH), an imbalance in the total microflora due to ecological stress, resulting in an enrichment of some "oral pathogens" or disease-related micro-organisms

cause disease. He corelated the changes in microbial composition to changes in ecological factors such as the presence of nutrients and essential cofactors, pH and redox potential [95,96]

Polymicrobial Synergy and Dysbiosis:

Polymicrobial Synergy and Dysbiosis (the 'PSD model') is present in pathogenesis of periodontitis. According to **Hajishengallis[49] et al** pathogenesis by PSD model is described as a pathogenesis caused by a broad dysbiotic, synergistic microbiota against the traditional view that it is caused by a single or several peri pathogens like red complex bacteria. This PSD model alters host-microbe homeostasis and facilitate its transition to a chronic inflammatory state. **Lamont and Hajishengalis** revealed that the entire microbial community drives disease progression, representing the intercommunication between subgingival community of microorganisms and local immune responses which ultimately leads to bone and connective tissue attachment loss.

Keystone pathogen hypothesis:

Hajishengallis G and colleagues applied this concept to(oral) microbiology by introducing "TheKeystone-PathogenHypothesis" (KPH) [48]**.** The KPH states that certain low-abundance microbial pathogens increase the quantity of the normal microbiota and by changing its composition thus causing the inflammatory disease. *Porphyromonas gingivalis* can manipulate the native immune system of the host **(reviewed by Darveau,2009[25])**. Keystone

pathogens can trigger inflammation when they are present in low numbers, in contrast to dominant species that can influence inflammation by their abundant presence [48]. The keystone pathogen is detected in higher numbers, when disease develops and advanced stages are reached. [131]

Relative to its abundance, a pathogen with a disproportionately large effect on its environment, for example low-abundance *P. gingivalis* remodels a commensal microbial community into a dysbiotic and disease-provoking microbiota.

BIOFILM:

Biofilm is an aggregate of micro-organisms in which cells are adhere to each other or to the surface. These cells produced matrix known as extracellular polymeric substances (EPS) in which they are usually embedded. DNA, proteins and polysaccharides are the components of the extracellular polymeric substance. It is characterized by structural heterogenicity, structural diversity, ECM polymeric substances and complex community interactions [132].

It is well established that periodontitis is associated several different groups of bacteria but not a single micro-organism, participating in initiation and progression of disease process through the formation of biofilm &transfer of materials to the putative pathogens that are thought to cause disease [123].The formation of biofilms on tooth surfaces initiates the disease. After initial

colonization by the pathogens the onset of the periodontal diseases is usually delayed for prolonged periods of time[132].

The process of biofilm formation:

Development of supragingival plaque involves the following step [12]

- Step-1: the evolution of plaque biofilm begins with adsorption onto the tooth surface of a conditioning film derived from bacterial and host molecules that forms immediately following tooth eruption or tooth cleaning [2]. This adsorption is followed by passive transport of bacteria mediated by weak, long-range forces of attraction. Covalent and hydrogen bonds create strong, short-range forces that result in irreversible attachment with the formation of the pellicle, an acellular material on the tooth surface that is mostly composed of glycoproteins. (formation of acquired pellicle)

- Step-2: Micro-organism settle in the pellicle and form colonies. (initial adherence and colonization).
- Step-3: Maturation of plaque occurs (secondary colonization)

The predominant early colonizers of the subgingival plaque biofilms are *Actinomyces species* and *streptococci*. Within few days a complex microbial community then develops within the space and the secondary colonizers tend to be the more pathogenic species such as *Porphyromonas*

gingivalis, Tannerella forsythia, Treponema denticola, Fusobacterium nucleatum and *Aggregatibacter* adhesion that enable attachment to the earlier bacterial inhabitants of the region, often choosing partner that are metabolically compatible [145].

Mechanism of interaction or inter-species binding :

Co-aggregation

Quorum sensing and Autoinducer-2

Genetic exchange within communities

COAGGREGATION

According to **Koglenbrander**[65] a specific cell-to-cell recognition that occurs between genetically distinct cell types is termed as coaggregation. Adhesins / receptors are coaggregation mediators. **Gibbons & Nygaard** [43] termed it as "interbacterial aggregation".

Fusobacterium nucleatum which act as a co-aggregation bridges can bind to early and late colonizers *P. gingivalis, Aa. comittans and T. denticola*. *P. gingivalis* aids in co-adhesion and they are the constituent of both supra-

gingival and sub-gingival biofilm. Long fimbriae (Fim A), Short fimbriae (Mfa), Arg-gingipain (rgP)B of P. g's helps in the co-adhesion.

MORPHOLOGICAL CO-AGGREGATION PATTERNS:

ROSETTE FORMATION is where single coccus is surrounded by large number of cocci. It is usually found in early biofilm formation

According to **Listgarten [80]**- CORN COB PATTERN results from the growth of cocci on the surface of filamentous microorganism. It is present in teeth associated with gingivitis.

According to **Teughels W, Quirynen M,[145]** TEST TUBE BRUSH FORMATION composed of central axis of bacilli with perpendicularly associated with filamentous organism. It is present in subgingival plaque of teeth associated with periodontitis.

COMMUNICATION OF BIOFILM;

Communication with different bacteria & cell to cell signaling (e.g.: Quorum sensing) enhance biofilm formation.

QUORUM SENSING:

Bassler[7] stated that in response to soluble signals called auto-inducers, a cell density dependent regulation of gene expression, which is termed as quorum sensing. It helps in communication between inter-species and intra-species. Quorum sensing in biofilm was reported in oral biofilm by **Lijemark**[78]**.**

A response is triggered i.e., a change in expression of specific genes occurs due to the accumulation of the signalling molecule which is sent by the bacteria**.** Threshold concentration only at high cell density called a critical mass or Quorum. **Shao**[127] stated that the bacteria in a biofilm, have the ability to communicate with each other. One example of this is quorum sensing, in which a signalling molecule secreted by bacteria accumulates in the local environment and initiates a response such as a change in the expression of specific genes once they reach a critical threshold concentration. Only at a high-cell density the threshold concentration is reached, and therefore bacteria sense that the population has reached a critical mass, or quorum.

Kolenbrander[66] stated that peptides secreted by gram-positive organisms during growth and a "universal" signal molecule autoinducer 2 (AI-2) are the two types of signaling molecules have been detected from dental plaque bacteria. Oral streptococci produce peptide signals and are recognized by cells of the same strain that secrete them. Only when a threshold concentration of the peptide is attained responses are initiated, and thus the peptides act as cell

density, or quorum, sensors. AI-2 is produced and detected by various bacteria's, in contrast to the strain-specific competence-stimulating peptides. Wide-ranging changes in gene expression, in some cases affecting up to one-third of the entire genome in detection of AI-2. Important roles are played by Quorum sensing, for example encouraging the growth of beneficial species to the biofilm, modulates the expression of genes for antibiotic resistance, and suppressing the growth of competitors.

HORIZONTAL GENE TRANSFER:

Close cell-cell contact is required in horizontal gene transfer and also in presence of "mobile genetic elements". Genetic materials present in a donor cell and that can be released into the environment are referred as Mobile genetic element. A new genetic material is acquired when the recipient cell takes up the DNA. Cell death usually occurs in this method.

Mechanisms:

1. Transformation
2. Transduction
3. Conjugation

1)TRANSFORMATION:

A process in which genetic material is taken up and maintained in a cell. This does not rely only on the presence of live bacteria, even DNA secreted from

lysed bacteria can be taken up. One of the rate-limiting steps for transformation in the oral cavity is the longevity of DNA molecules in this environment**.** [37]

2)TRANSDUCTION:

Transduction is the process in which bacterial DNA or DNA segments gets incorporated into phage. Viruses that survive within the bacteria are called bacteriophage. Bacteriophages gain entry into the bacterial cells and replicates and its nucleic acid material undergoes replication. Bacterial DNA may be infected into phage DNA, during the process of DNA breakdown and assembly. This genetic material may be transferred into the new cell, when the phage infects another bacteria. Thus, the bacterial genetic material of primary cell can get transferred into that second cell. [120]**.** Presence of bacteriophage is seen in periodontal bacteria, such as *A. actinomycetemcomitans, Fusobacteria and T. denticola*[138]

3**)CONJUGATION:**

Conjugation is a process in which genetic material is transferred through direct cell-cell contact. Transfer of genetic element occurs through a conjugation tube when a donor bacterium (having sex pili) make physical contact with recipient bacterium. Conjugative transposon and conjugative plasmid mediate this process **(Roberts AP-2006**[2006]**).** A set of specialised

adhesives present on the surface of the cell membranes makes the bacterial interaction possible.

The tetracyclines resistant gene present in A.a is a result of process of conjugative transfer of a plasmid between different strains of A.a and also between different organisms. Conjugative transfer occurs between A. a and non-pathogenic organism *H. influenzae* is the most studied, a result of which tet-B gene is made accessible to the organism. A major component of antibiotic resistance exhibited by Aa to tetracycline is the Tet B gene[122]**.** Thus, horizontal gene transfer aids in Greater survival of pathogenic bacteria and increases virulence of pathogenic bacteria.

Bacterial succession[133]

Autogenic succession:

The sequence of microbial species is brought about because the resident populations alter their environment in such a manner that they are replaced by species better suited to the modified habitat**.**

Allogenic succession:

One variant of community is replaced by another variant because the habitat is altered by nonmicrobial factors such as, changes in the physical or chemical properties of the region or changes in the host.

Factors are contributing to succession are:

Alteration in inorganic nutrient concentration and making it available for one population of a constituent present in insufficient supply to allow for growth of a later population, provision by one community of a nutrient that confers an ecologic advantage to the species in the next stage of succession, changes of heterogeneous substrates such as animal tissue, an autointoxication effect, the appearance of barriers due to environmental feedback, elimination of an organism by physical means.

The microbial succession is well explained by the development of gingivitis.

Loe et al and Theilade et al[86] have been stated that gingivitis is caused by dental plaque. It was shown that 28 days withdrawal of toothbrushing in periodontally healthy volunteers resulted in the rapid accumulation of plaque on the teeth. Within 10–21 days gingivitis developed in all subjects. The gingivitis was reversed on re-establishment of oral hygiene procedures and on removal of the local factor.

The members of the yellow, green, and purple complexes along with Actinomyces species involves in initial colonization. This eventually leads to autogenic succession in which the predominant organisms are the members of the orange and then red complexes. A hypothesis was put forth that changes in the habitat occurs due to the presence of increased levels of the red and orange complexes which manifests clinically as gingivitis. Further proliferation by

members of not only the orange and red complexes, but also the members of the early colonizing species as well, are favoured by the gingivitis. In many ways this cycle could be broken. Elimination of all biofilm may be the primary step; this partially successful strategy is the one most commonly employed today. Members of the red and/or orange complexes elimination would be the second step, this probably limits gingivitis and its feedback effect of greater plaque development. The third step would be to decrease gingivitis by a nonantimicrobial approach, which leads to decreased plaque accumulation and possibly diminish red and orange complex development.

Climax community:

The communication between the nonmicrobial and microbial components of an ecosystem will eventually lead to a form of stabilization in which nonmicrobial and microbial forms exist in harmony and equilibrium with their environment.

This is a dynamic state in which cells are being replaced. Exogenous forces modifies the climax community from time to time. As the habitat returns to its original state the equilibrium tends to be restored.

The same general successional sequences will be initiated and fostered when the same initial physical and chemical site characteristics or identical hosts given, which leads to rise of remarkably similar climax communities.

Health to Gingivitis:

The classic experiments of **Loe et al [86]** demonstrated that without doubt that the development of gingivitis is a result of accumulation of microbial plaque and that its removal and control will lead to resolution of the lesions in humans, thereby proving the microbial aetiology of the disease. Recent studies by **Page & Schroeder [106], Moore et al. [99]**have also confirmed this conclusion in humans and in experimental animal models.

The oral commensal microbial community

Socransky et al[131] identified that many of the bacterial taxa appeared to cluster together including those associated with gingival health . *Capnocytophaga species, Campylobacter concisus, Eubacteria nodatum* and *Streptococcus constellatus* are the bacteria's of Green cluster. The yellow cluster was formed by a group of *streptococci, and Actinomyces odontolyticus* and *Veillonella parvula* comprise the purple cluster**.** These species tended to occur together in the periodontal crevice and did not associate with gingival bleeding or increasing pocket depth .

Periodontal health:

Early or primary colonizers such *as S mitis, S sanguis, Gemella spp, Atopobium spp, Capnocytophaga spp* and *Fusobacterium nucleatum* are the bacteria that are associated with periodontal health **[63], [112].** Species beneficial to

the host belongs to the genera *Capnocytophaga,Veillonella and Streptococcus* . Recent molecular studies has shown the presence of certain uncultivated species such as Bacteroides oral clone BU063 to be strongly associated with periodontal health [75].

Gingivitis:

Gingivitis have been associated with Gram-positive species (eg, *Streptococcus spp, Actinomyces viscosus, Peptostreptococcus micros*) and gram-negative species (eg, *Campylobacter gracilis, F nucleatum, Prevotella intermedia, Veillonella*) [91]. Microflora with a high proportion of *Prevotella intermedia* are seen in Pregnancy associated gingivitis[68].

Chronic periodontitis:

Longitudinal and the cross sectional studies has been revealed the bacterial profile of chronic periodontitis. The effect of various treatment methods in changing the microbial ecology has also been studied. Bacteria's commonly associated with chronic periodontitis are *Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia, Campylobacter rectus, Eikenella corrodens, F nucleatum, Actinobacillus actinomycetemcomitans, Peptostreptococcus micros, and Treponema spp* . Sites with active disease or with progressing disease reported high levels of *Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia, Campylobacter rectus, and F nucleatum*[31]

Gingivitis to Periodontitis:

Listgarten et al[80] conducted a longitudinal study of the periodontal status among 69 adult with gingivitis and found that, in 3 years, only 1 of 1000 individual tooth surfaces demonstrated an increase in pocket depth of 3 mm or more.

In 1965, Loe [86]**et al** performed a longitudinal study among tea laborers in Sri Lanka of age between 15-35 years and there was a marked increase in the amount of attachment loss and in the number of teeth affected.

Lindhe et al.[79] Observed an annual rate of 0.2 mm loss of attachment between baseline, 3 and 6 years , exhibited in a population in Sweden.

The authors concluded that these data did not support the hypothesis that periodontal disease in an individual was slowly progressive but rather that certain sites in a few individuals are affected by periods of exacerbation and remission, that is, a burst of activity, and that progressive disease may have occurred over a short time span.

Microbial load is low in the healthy sites. According to **Darveau et al.,**[26]mostly gram-positive streptococci (e.g., *Streptococcus gordonii*) and Actinomyces with about 15% gram-negative rod species, including Fusobacterium nucleatum are isolated from the culture of the healthy sulcus. In contrast, the microbial load is higher, and there is an increase in the number of

gram-negative organisms (15–50%), in periodontitis [26] when compared to clinically healthy sites.

VIRULANCE FACTORS:

The word virulence meaning full of poison is derived from Latin,' virulentus'. The properties to cause disease by microorganism or interfere with metabolic or physiologic functions of the host are referred to as ***virulence factors***. According to Curtis et al any description of microbial virulence is fundamentally reliant on an understanding of the relative susceptibility of the colonized host.

According to **Holt and Bramanti [54]:**

At least five integrated events are required for establishment of bacterial infection : Tissue surface is colonized initially ,Penetration of this surface either directly or indirectly, multiplication and emergence of the invading bacterium in this environment, the eventual destruction of the host's tissues; and the survival of the invading bacteria in the ecological niche by evasion of the host's defense mechanisms.

According to **Holt and Ebersole [55] :**

Multitude of functions can be performed by the virulence factors. The ability to induce microbe–host interactions (attachment), host tissue invasion,

the ability to grow in the confines of a host cell, the ability to evade / interfere with host defense are mediated by the virulence factor.

In the periodontal milieu virulence factors are responsible for the following characteristic 1. They grant the microorganism the ability to adhere to a substratum. 2. They aid the microbes to invade into the gingival tissue. 3.They help the organism to suppress the host responses that attempt to eliminate them. 4. They may be factors that can actually cause connective tissue breakdown or activate host enzymes/immune system to release substances that may do so.

ADHESINS:

Adhesins helps to adhere the microorganism to the host surfaces & in co-aggregation. Mostly they are surface molecules.

Factors involved in adhesion and persistence:

In oral cavity the bacterial colonization of the oral cavity takes place at birth and persist throughout the life of the host. The colonization is coordinated by the host and its surface receptors. The microbial adhesins that specifically fit to the host's receptors governs the selection of microorganisms and thereby constitute and build the commensal microbiota living in harmony and balance (microbial homeostasis) with the host[95]. In order to cause pathology the

periodontopathogens need to compete with the commensal primary colonizers and pioneer species. Periodontal bacteria are mostly less efficient in adhesion, some species like *Treponema* spp., *Campylobacter* spp., *Selenomonas* spp are motile, and the motile organism normally do not colonize smooth surfaces. *Treponema Denticola* lacks specific adherence structures such as fimbriae but the T.d adhere as a result of adherence factors on the surface of the microorganism[8].

Adhesins associated with biofilm formation:

***Porphyromonas gingivalis*:**

The cell surface of the P.g's shows the presence of the fimbriae arranged in a peritrichous manner. . Fimbriae attaches to host surface through their receptors salivary statherin and proline rich protein 1 . fimbrillin maturation involves Rgp and also makes a significant contribution to the processing of other virulence factors expressed on the bacterial surface[61].

Treponema denticola:

Adhesion to epithelial cells through Leucine rich repeat protein. Lectin like proteins mediates adhesion between major outer sheath protein to cells and ECM proteins. It act as porin and helps in transportation of molecules in and out of the host cells.. Adhesion to ECM proteins through outer membrane proteins

bind to plasminogen and fibronectin binds to fibronectin via bacterial pole. Binds to collagen I, IV, V via collagen binding protein.(Ishihara-2010).Co aggregation occurs through fimbriae binding protein binds to fimbriae of *Porphyromonas gingivalis*.(Onagawa-1994).Resistance to beta defensin at epithelial surface aid in initial colonisation and establishment in the oral biofilm[15].

***Tannerella forsythia*:**

Bsp A is a leucine rich repeat protein. This helps in interaction between the host and other bacteria. It co aggregates with Lrr A of Treponema denticola[3]

INVASINS:

Important step in the pathogenesis of bacteria, invasion into the gingival tissue. Tissue invasion makes it impossible to dislodge these bacteria by mechanical action**.** Periodontal disease is preceded by invasion into the tissue which causes greater host bacterial interaction[147]**.**

Invasion occurs during ulceration when the epithelial barrier (junctional epithelium) is disrupted. Spirochetes are shown to invade the underlying connective tissue by motility in acute necrotizing ulcerative gingivitis (ANUG)[80]**.** Bacterial invasion in periodontitis occurs in two routes. Motility

suggested to take place in intercellular route e.g., spirochetes[89].In health, the junctional epithelium is non-keratinized and thin (4–5 cells thick), and during inflammation, the cells are not tightly joined which facilitate gingival exudate and migration of PMN cells and macrophages through the gingival barrier. It is stated that this intercellular passage also allows motile bacteria such as *Treponema and Campylobacter* species to penetrate the barrier. Gingipains produced by the P.gs can degrade epithelial junctional proteins (E-cadherin and occludin) which impairs the junction-related structures [63]. Bacterial cells, mainly *streptococci* are usually seen in buccal epithelium. Interestingly, *P. gingivalis* and other periodontal bacteria have also been shown in such cells. Inside crevicular epithelial cells *T. forsythia, Prevotella intermedia, and C. rectus* have been identified[29].

EVASINS:

The bacteria to subvert host immune responses with the help of Evasin thereby preventing them from getting cleared from the gingival sulcus. The C3 molecule can be degraded by the Arg-X gingipains, which decreases bacterial opsonization [125]. This property concludes the increased resistance of *Porphyromonas gingivalis* to bactericidal activity. On the other hand, the C5a receptor on PMNs can be inactivated by Lys-X, an action that may actually impair their recruitment [59]. Lipid A of most Gram-negative species is a strong activator of TLR4 responses, *Porphyromonas gingivalis* lipid A is predominantly a TLR2 activator and may even act as antagonist to TLR4[25],

dampening the immune responses [50]. Gingipain converts prothrombin to thrombin which in turn converts C5 to C5a thus Gingipain act as C5 convertase, converting C5-C5a .Immunological paralysis and fail to carryout functions such as chemotaxis, phagocytosis and production of antimicrobial and inflammatory mediators caused by excessive C5a [159,98] stated that T.d dentilisin causes hydrolysis of human IL-1 beta,IL-6,8,TNF-α.cytokine down-regulation has been shown to be caused by the suppression of cytokine synthesis & cytokine degradation.

SUSTENINS:

Molecules that help these bacteria to meet their nutritional requirement & survive in adverse environmental stimuli are Sustenins. The red complex organisms are ready to express their pathogenicity after the initial steps of adhesion &invasion into the tissue. As enumerate numbers of organisms proliferate within the tissue , the rate limiting step is the availability of nutrition to the multiplying bacteria. Sustenins, a collection of enzymes &special molecular determinants in the organism which meets the nutritional demands of the organism. E.g.: *Treponema denticola* contains chymotrypsin like protease that shows a predilection for the proline, phenylalanine & leucine residues in protein structures[149].In addition dentilisin -serine peptidase cause degradation of host protein like laminin, fibronectin,type IV collagen, α1 antitrypsin[58].

Asaccharolytic bacteria such as *Porphyromonas gingivalis* secretes the proteases and peptidases whose primary function is to provide nutrients for

growth. Specific examples of attenuation of host defence mechanisms and tissue degradation include: the degradation of extracellular matrix proteins; activation of matrix metalloproteinases (MMPs). *Porphyromonas.gingivalis* thiol protease activate MMP-3 and MMP-1 from gingival fibroblasts. *Porphyromonas gingivalis* Rgp activates MMP-2. inactivation of plasma proteinase inhibitors; cleavage of cell-surface receptors; stimulation of apoptotic cell death; and disruption of PMN functions activation or inactivation of complement factors and cytokines; activation of the kallikrein-kinin cascade[55;61].

HOST-MICROBIAL INTERACTION:

Periodontal diseases are mostly inflammatory disease induced by bacteria characterized by a complex interplay between the pathogens and the host tissue. Innate or adaptive immune response of the host usually eliminates the pathogen. If the equilibrium between bacteria and host are not balanced, results in periodontal tissue destruction. A series of events occur in bacterial plaque, gingival sulcus, junctional epithelium, connective tissue, and bone, due to alteration in tissue homeostasis as periodontal disease progresses. Gram-positive aerobic and facultative anaerobic flora shift to gram-negative anaerobic flora gradually.

Microbial -Oral epithelium interactions:

A dynamic physical and chemical barrier against the pathologic properties of the microbial biofilm is present in the oral epithelium importantly the junctional and sulcular epithelia[13]**.** Epithelial tissue reacts by mobilizing their own antimicrobial mechanisms and by permitting cells of the innate and adaptive immune system when challenged by microbes [28]**.** Antibacterial peptides which includes calprotectin α and βdefensins, cathelicidin, and IL-37 contributes to host defense are expressed by cells of the junctional epithelium and resident leukocytes and up regulated in tissues of patients with chronic periodontitis[150]**.**

By increasing the expression of cytokines, growth factors, matrix components, matrix metalloproteinases (MMPs), Gingival epithelial cells, fibroblasts, and inflammatory cells respond to LPS and this is mediated through the TLRs and CD 14[116]. P38 and C-jun N-terminal kinase aid the LPSs to activate the expression of β-defensins by oral epithelial cells[21]**.** Nuclear factor kappa β (NF-Kβ) has been implicated in periodontitis and it is associated with the expression of IL-6 and other molecules that activate bone resorption[152]**.**

Immunologic response of host-microbial interaction:

T cells plays an important immuno-regulatory role rather than a defensive or destructive role in the pathogenesis of periodontal diseases. T cells are involved in the recruitment and activation of neutrophils at the site of infection.

In the stable lesion, activation of the neutrophils may be crucial in keeping the infection under control[40].

The production of IL-12 which in turn leads to a Th1 response are due to the innate immune response. The production of interferon-γ upregulates the phagocytic activity of both neutrophils and macrophages; hence, containment of the infection. The cross talk between innate and adaptive immunity controlled by dendritic cells via TLRs and the antigen-specific immune regulation is by particular subsets of dendritic cells and T cells[53].

Most studies on TLRs have focused on TLR-2 and TLR-4 because they identify gram- positive and gram-negative bacterial Pathogens-Associated Molecular Patterns (PAMPs), respectively. Particularly Porphyromonas gingivalis LPS, which preferentially utilizes TLR-2 and not TLR-4 within periodontal tissues, TLR-2 and TLR-4 expression appears to be increased in severe disease states[101]. The generation and modulation of the immune response to PAMPs are provided by the dendritic cells.

Initial lesion is most stable with a Th1 response due to intra cellular pathogens, in contrast advanced lesion is more consistent with Th2 response typically mounted to fight extra cellular pathogens. Th1 is characterized by IL-12 production which in turn stimulates interferon -γ production, leading to macrophage activation, increased phagocytic activity, and protective immunity. Th2response entails production of alternative cytokines such as IL-4, IL-10, and IL-13 leading to antibody protection. Chronic periodontitis is more related to Th2 response[158]. The levels of IgG and IgA against A. actinomycetemcomitans

and P. Gingivalis are increased during periodontal disease progression, and they correlate with the existing periodontitis and pathogen carriage in saliva.

T lymphocyte obtained from periodontitis lesion express Receptor Activator of Nuclear Factor-Ƙb ligand (RANKL). As T-cell specific for periodontopathic bacteria exists in the peripheral blood of periodontitis patients, they might potentially express RANKL when they encounter the specific bacterial antigen. Activated T-cells might regulate bone resorption through increased production of soluble RANKL[62]**.**The enrolment of new osteoclasts is dependent on the equilibrium between RANKL and its decoy receptor osteoprotegerin[128] in osteoblasts. Osteoblasts controls osteoclastic bone resorption which involves recruitment of new osteoclasts and activation of mature osteoclasts. Thus, the osteoclast differentiation and function are regulated by osteoblasts**. Choi *et al*.,**[19] reported that RANKL production and augmentation of osteoclastogenesis are mediated by B Lymphocytes. Lymphocytes, macrophages and neutrophils infiltrate the gingival connective tissue in periodontal lesion, and interact with osteoblasts, periodontal ligament fibroblasts, gingival fibroblasts, and inflammatory infiltrate (T & B lymphocytes and macrophages) macrophages and T-lymphocytes produce inflammatory mediators, including IL-1, IL-6, TNF-α, and prostaglandin E2 which can induce bone resorption indirectly by stimulating osteoblasts to produce RANKL. T lymphocytes can stimulate osteoclasts differentiation by

direct production of RANKL. Inflammatory infiltrate in periodontal lesions induces alveolar bone resorption directly or indirectly[144].

ROLE OF SALIVA IN PERIODONTAL DISEASE:

Various ecological niches in the oral cavity:

Bacteria that normally inhabit in the oral cavity (i.e. the indigenous microbiota) can select from different ecosystems for their habitat. The oral cavity can indeed be divided in several major ecosystems (also called niches),each with distinct ecological determinants: the supragingival tooth surface ,the buccal epithelium, the periodontal pocket (with its crevicular fluid, the root cementum and the pocket epithelium), the dorsum of the tongue the floor of the mouth, the vestibule, the palate and the tonsils on the basis of physical and morphological criteria. Most species are able to colonize all of them with the exception of spirochetes. Tonsillitis are also caused by some periodontopathogens (*Fusobacterium nucleatum and Provotella intermedia*) [17]. Even in the edentulous mouth of infants or of denture wearers the proportions of periodontopathogens [with the exception of *Aggregatobacer actinomycetemcomitans* and *Porphyromonas gingivalis*[67] can become very high. Most periodontopathogens also colonizes the maxillary sinus[153].

As saliva is an easily accessible and economical biological fluid, saliva has been thoroughly analyzed for biomarkers of health and disease over the past

decade[42]**.** Salivary proteins[64] and levels of proinflammatory cytokines in saliva[126] have been studied as possible biomarkers of periodontitis. The bacterial profile of saliva in relation to periodontitis has been described previously. In one report **Yamanaka et al[157].** Showed that periodontal therapy had only a small influence on the composition of the bacterial saliva profile[157]**,** and in another study that identification of multiple putative periodontal pathogens in saliva was related with periodontitis in a large Finnish population [107]**.**

The intra-oral translocation of periodontopathogens:

In the continuous intra-oral translocation of bacteria a crucial role is played by The microbial load in the saliva and its intra-oral spreading (during chewing, muscle activities and tongue movement).

Christersson et al. [20] demonstrated the translocation of A. actinomycetemcomitans through the periodontal probes in localised juvenile periodontitis patients. Previously non-colonized pockets with A. Actinomycetemcomitans successfully inoculated *by* a single course of probing with a probe previously inserted in a colonized pocket of the same patient.

Müller et al. [102], examined toothbrushes from juvenile periodontitis patients infected by A. actinomycetemcomitans and found that the brushes harboured this species about 69% and 23% immediately after brushing and after 24 hours, respectively. Immediately after a single period of brushing, with or without toothpaste, he also observed that a toothbrush contained 5.1×10^6 to 1.2×10^8 c.f.u. of anaerobic bacteria, and 6.8×10^5 to 4.6×10^7 of aerobic species, respectively. After 48 hours of drying, the brushes with toothpaste were nearly free of bacteria, whereas the brushes without toothpaste were still contaminated with more than 105 bacteria (primarily aerobic species, including *S. mutans* but without *Porphyromonas gingivalis* or *Prevotella intermedia*, which disappeared after 4 h), **[117].**

Glass&Lare [45]even suggest that, for immuno-suppressed patients such contaminated toothbrushes should be considered as a potential health risk.

Periodontal probes can also recruit high levels of potentially pathogenic species and might therefore lead to the intra-oral transmission of periodontopathogens. **Barnett et al[6]** examined periodontal probes after probing a single deep pocket (>6 mm) using light and electron microscopy and found that all specimens contained huge numbers of bacteria of which most had been morphologically connected with pathogenicity.

In another **study, Papaioannou et al.** [109] stated that the periodontopathogens adhered to the periodontal probes after probing a single site were cultured and quantified. The probes harboured up to10^7 bacteria with large extent of specific pathogens (e.g., *A. actinomycetemcomitans, Porphyromonas. gingivalis, Provotella intermedia and Camphylobacter. rectus*).

Preus et al.[115] stated that the syringe tips, used for repeated subgingival application of antibiotics, was found to become culture positive for antibiotic resistant bacteria. Cleaning these tips with paper tissue, soaked in 70% ethanol, before using next time may prevent transmission.

MOLECULAR MICROBIAL DIAGNOSTIC METHODS:

- Closed ended approaches:

  Directed DNA methods are closed -ended approaches that use short, synthetic nucleic acid sequences (oligonucleotides) specific to each target species.

  Which includes - polymerase chain reaction-based methods
  - DNA-DNA hybridization methods

- Open-ended approaches:

This technique is advantageous now since it can detect previously unknown microbes including those which are non -cultivable. These approaches are based on 16SrRNA sequencing.

SEQUENCING METHODS:

- ✓ Next-generation sequencing
- ✓ Real-time single molecule DNA sequencing
- ✓ Nanopore technologies

16Sr RNA SEQUENCING:

In recent decades, in bacterial taxonomy 16Sr RNA gene sequencing &phylogenetic analyses have been increasingly applied. Universal distribution is seen in 16S r RNA. It is almost resent in all bacteria. The 16Sr RNA function has not changed overtime. For informatic purpose it is large enough (contain 1500 nucleotide) .16s phylogeny is excellent for classification of bacteria. This technique is proven to be the most crucial in phylogenetic marker that amplifies & analyse 16Sr RNA genes in plaque sample & it is a culture independent technique as stated by **Spratt[135].**

NEXT GENERATION SEQUENCING:

PRINCIPLE:

The next generation sequencing works on the principle which involves oligonucleotide which undergoes cyclical ligation which is of machine automated, there will be repeated cycles of polymerase mediated nucleotide extension.[93,151]

In a single machine run there will be a enormous amount of nucleotide sequence as millions of reactions occur in a massively parallel process. Depending on the platform, NGS generates hundreds of megabases to gigabases of nucleotide sequence output in a single instrument run. The two basic procedures are ligation of DNA fragments with oligonucleotide adaptors and fragment immobilization to a solid surface, such as a bead.

The three main technologies for NGS:

Roche/454 FLX (Life Sciences, Branford, CT, [94]), the Illumina/ Solexa Genome Analyzer (Illumina, San Diego, CA, Bentley DR, 2006)

and the Applied Biosystems ⁄ SOLID (Life Technologies, Carlsbad, CA).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).

THE HUMAN ORAL MICROBIOME DATABASE

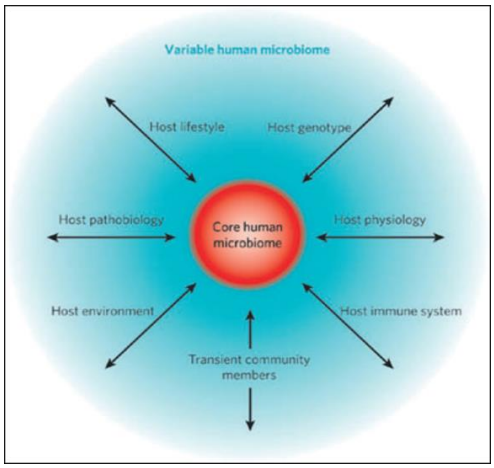
Research over the past 20 years has focused on defining breadth and diversity of oral microbiome by obtaining 16S rRNA gene sequence information for both cultivable and as yet uncultivated oral bacteria. The majority of bacterial species isolated from the oral cavity are included in 4 of the 10 bacterial 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 ***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; additionally 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.

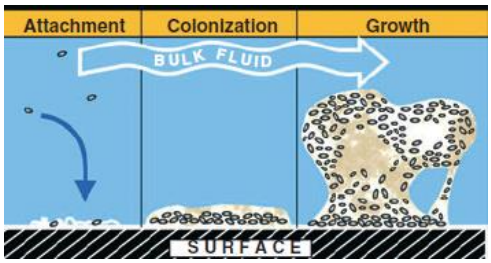
ROL FIGURES:

FIGURE NO:1



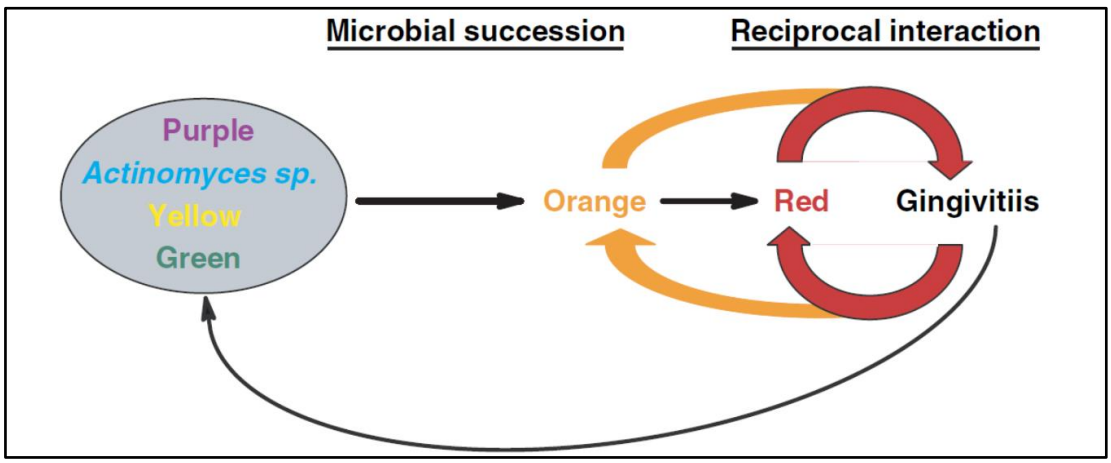
CORE AND VARIABLE HUMAN MICROBIOME

FIGURE NO:2



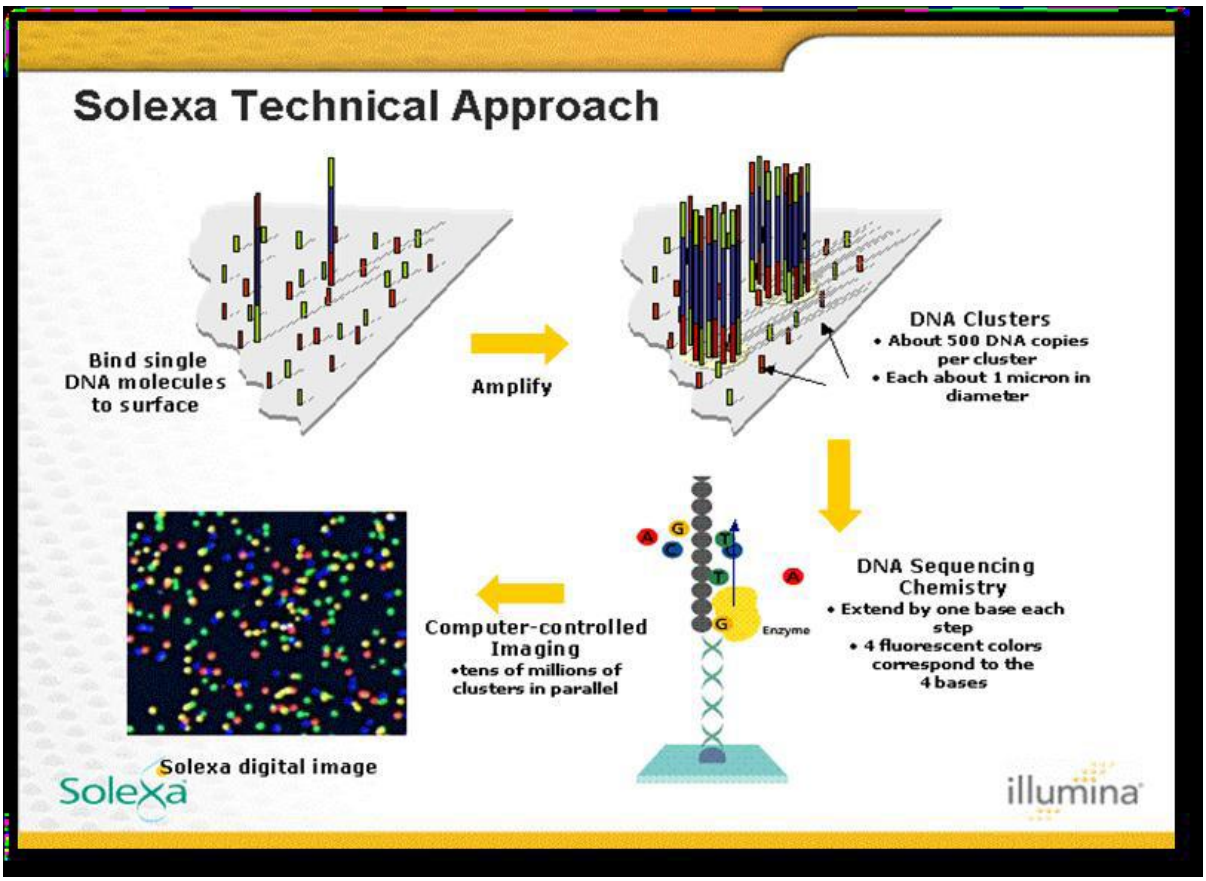
PROCESS OF BIOFLIM FORMATION

FIGURE NO:3



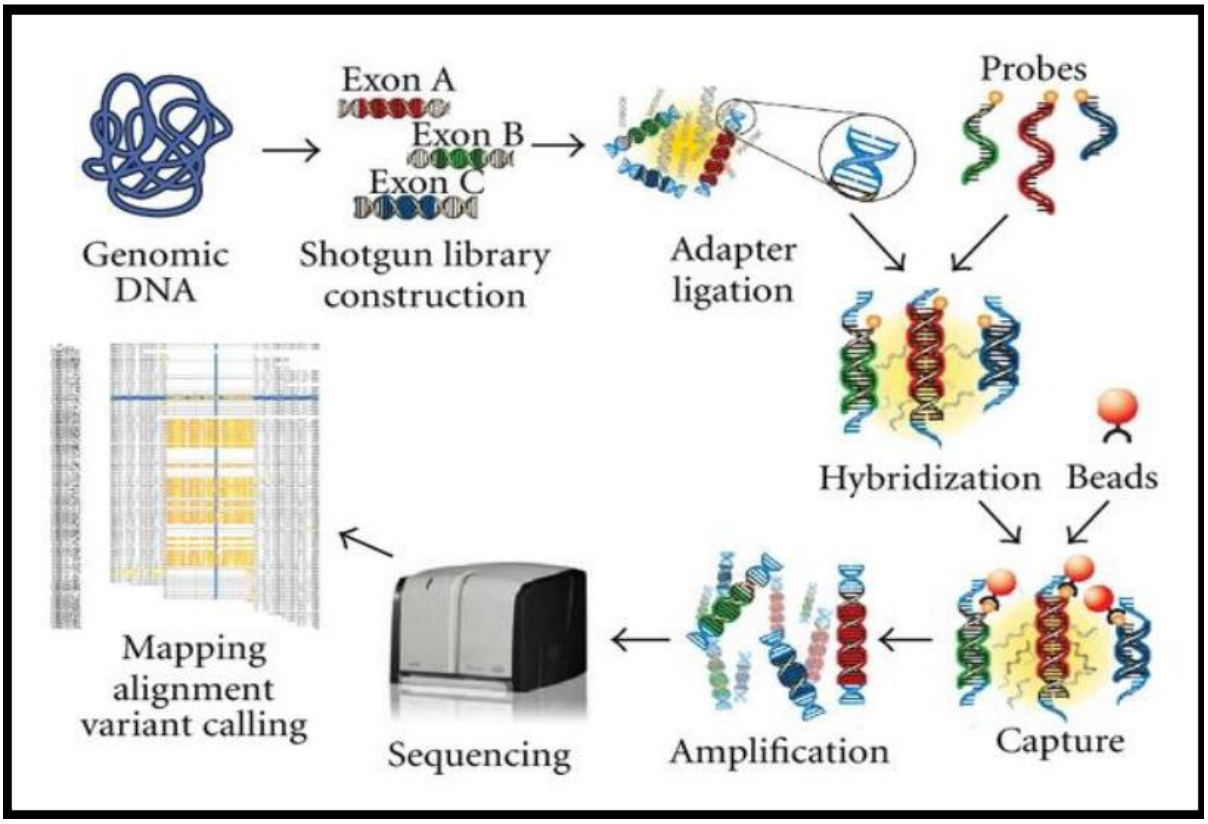
MICROBIAL SUCCESSSION FROM HEALTH TO GINGIVITIS

FIGURE NO:4



ILLUMINA SOLEXA GENOME ANALYZER

FIGURE NO:5



ROCHE 454 LIFE SCIENCES SYSTEM

Materials and Methods

MATERIALS AND METHODS

Study population

A total of 20 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 chronic periodontitis patients (test group). A diagnosis of chronic periodontitis 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 consisted of 10 subjects with chronic periodontitis with PPD ≥ 5mm and CAL ≥ 3mm in at least six sites.

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.

INCLUSION CRITERIA

- Subjects exhibiting good general health
- Subjects meeting the criteria of periodontal health and disease as described above were included in this study.

EXCLUSION CRITERIA

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

Saliva sampling

All examinations were performed by a single, calibrated examiner. The periodontitis patients were selected and the sample was collected in a sterile salivary tub. Unstimulated whole saliva will be collected in the morning and subjects refrained 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

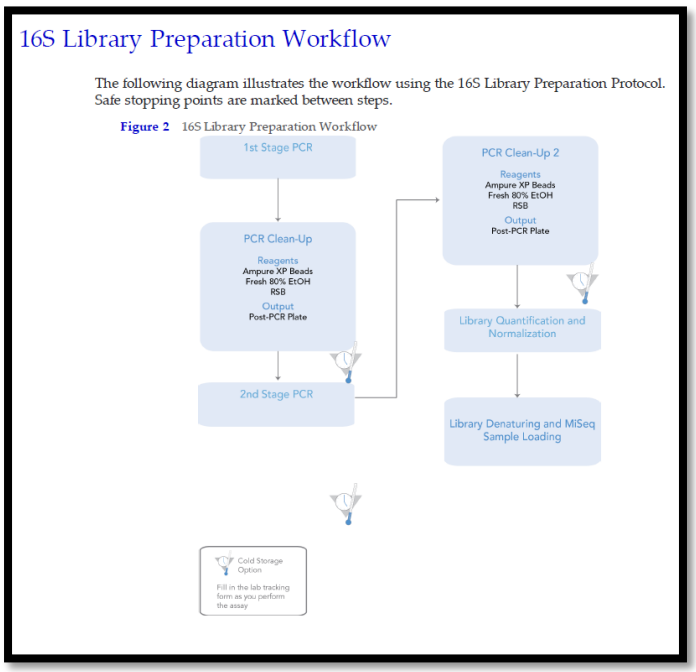
Genomic DNA was extracted from 20 saliva samples of periodontitis and health patients with the Qiagen powersoil kit according to manufacturer's recommendations.

DNA QUALITY CONTROL:

DNA samples were quantitated using Nanodrop. All the samples have passed QC and is 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 Order amplicon primers–The protocol includes the primer pair sequences for the V3 and V4 region that create a single amplicon of approximately ~460 bp. The protocol also includes overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters.

2 Prepare library–The protocol describes the steps to amplify the V3 and V4 region and using a limited cycle PCR, add Illumina sequencing adapters and dual-index barcodes to the amplicon target. Using the full complement of Nextera XT indices, up to 96 libraries can be pooled together for sequencing.

3 Sequence on MiSeq–Using paired 300-bp reads, and MiSeq v3 reagents, 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.

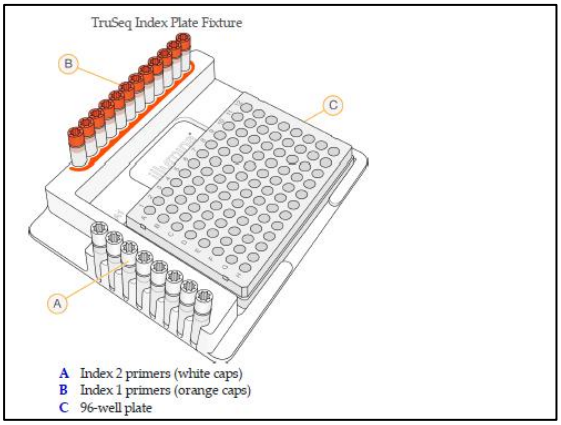
4 Analyze on MSR or BaseSpace–The Metagenomics workflow is a secondary analysis option built into the MiSeq Reporter (on-system software) or available on BaseSpace (cloud-based software). The Metagenomics Workflow performs a taxonomic classification using the Greengenes database showing genus or species level classification in a graphical format.

This protocol can be used to sequence alternative regions of the 16S rRNA gene and for other targeted amplicon sequences of interest. When using this protocol for amplicon sequencing other than 16S rRNA, use the Generate FASTQ Workflow (secondary analysis option).

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:

Following Illumina's recommendation quantifying libraries with a fluorometric quantification method that used dsDNA binding dyes was utilised in this study.

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 are 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

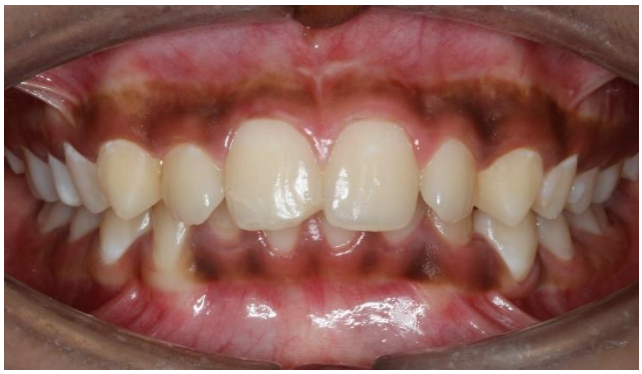
database(http://greengenes.lbl.gov/). The output of this workflow is a classification of reads at several taxonomic levels: kingdom, phylum,class, order, family, genus, and species.

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.

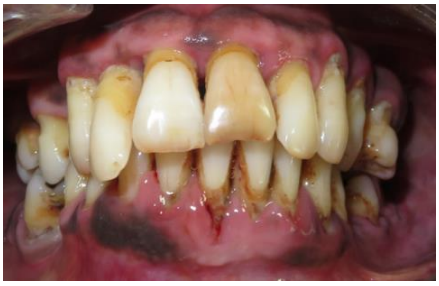
Statistical analysis was performed for individual bacteria using frequency distribution and intergroup comparison was done using Mann Whitney U test with statistical significant set as $P < 0.05$

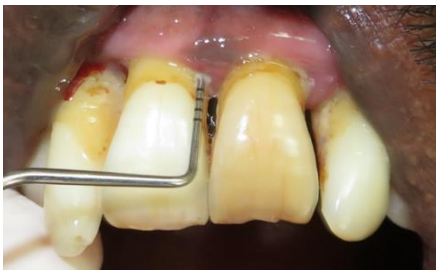
Photograph

HEALTH

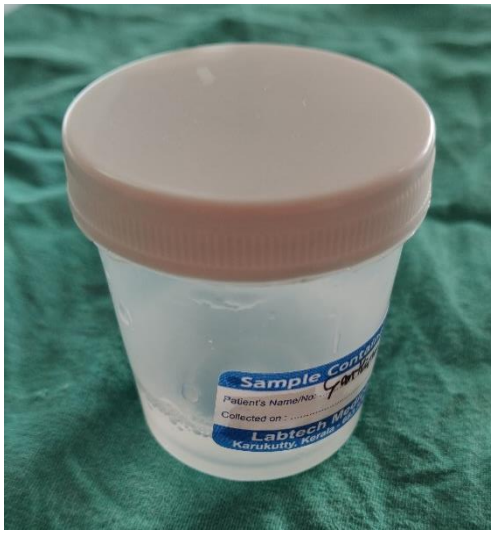


PERIODONTITIS





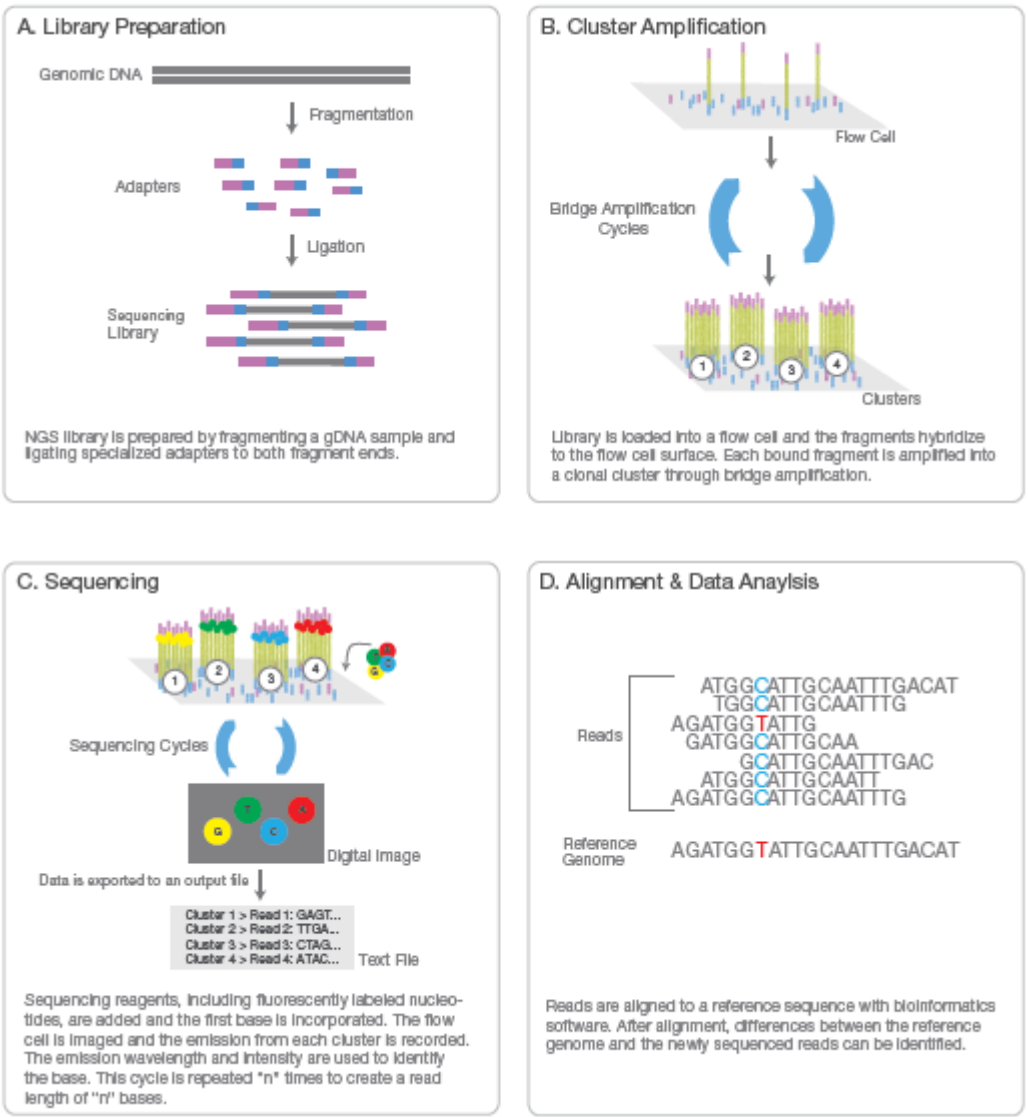
STERILE SALIVA TUB

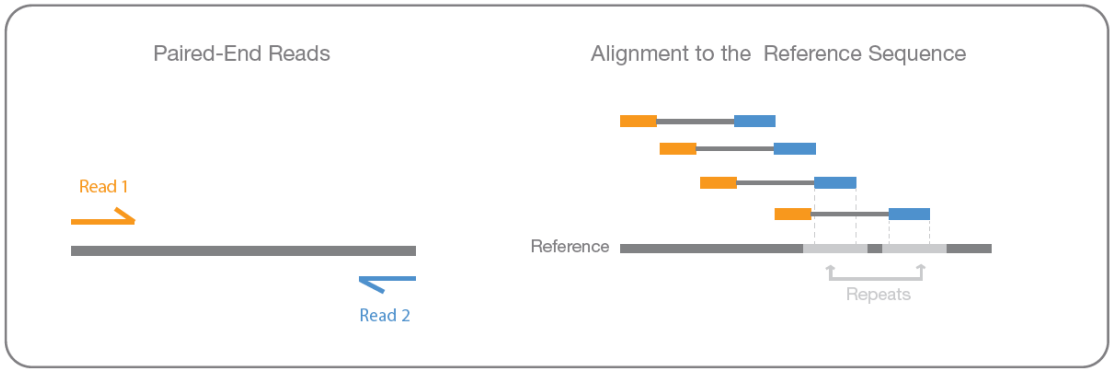


ILLUMINA SEQUENCING

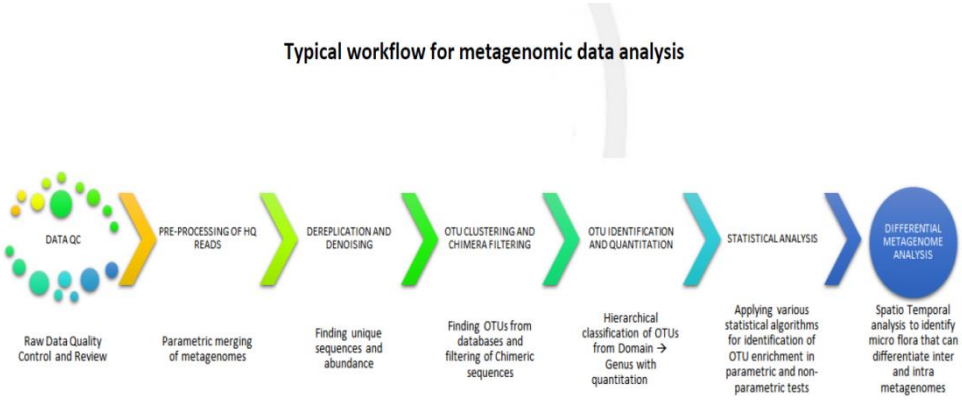
NextSeq Series

4 basic steps in Illumina NGS work flow




Paired-End Sequencing and Alignment
Paired-End Reads
Read 1
Read 2
Alignment to the Reference Sequence
Reference
Repeats


NGS Library Preparation Kits
Illumina Sequencing Instruments
Automated Data Analysis Tools
5–6 Hours
1–2 Days
1–2 Days


Typical workflow for metagenomic data analysis
DATA QC
PRE-PROCESSING OF HQ READS
DEREPLICATION AND DENOISING
OTU CLUSTERING AND CHIMERA FILTERING
OTU IDENTIFICATION AND QUANTITATION
STATISTICAL ANALYSIS
DIFFERENTIAL METAGENOME ANALYSIS
Raw Data Quality Control and Review
Parametric merging of metagenomes
Finding unique sequences and abundance
Finding OTUs from databases and filtering of Chimeric sequences
Hierarchical classification of OTUs from Domain → Genus with quantitation
Applying various statistical algorithms for identification of OTU enrichment in parametric and non-parametric tests
Spatio Temporal analysis to identify micro flora that can differentiate inter and intra metagenomes

Results

RESULTS

The present study was carried out in a population of 20 individuals, that is 10 in health and 10 diseased individuals seeking dental treatment in Ragas Dental College and Hospital, Chennai. The age distribution of the study participants ranged from 20-53 years with a mean age of 32.66 years.

Saliva samples were collected in a saliva tub from periodontally healthy individuals (designated as H1, H2, H3, H4, H5, H6, H7, H8, H9 and H10) and from the periodontitis patients (designated as P1,P2,P3,P4,P5,P6,P7,P8,P9 and P10), and subjected to 16SrRNA sequencing using NGS technology.

Table-1 **EXPRESSSION OF RED COMPLEXES IN HEALTH GROUP**

S.NO	RED COMPLEX	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
1	P. gingivalis	-	-	-	1	-	-	-	-	-	-
2	Tannerella forsythia	-	-	-	-	-	-	1	-	-	-
3	Treponema Denticola	-	-	-	-	-	-	-	-	-	-

In health group, the results have shown that *Porphyromonas gingivalis* was present only in one health sample (H4) and *Tanneralla forsythia* was present only in one sample(H7), whereas *Treponema denticola* was not identified in any of the samples.

Table-2 EXPRESSION OF RED COMPLEXES IN PERIODONTITIS GROUP

S.NO:	RED COMPLEX	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1	Porphyromonas gingivalis	**1**	**-**	**1**	**1**	**1**	**1**	**-**	**-**	**1**	**1**
2	Tannerella forsythia	**1**	**-**	**1**	**1**	**1**	**1**	**-**	**1**	**1**	**1**
3	Treponema denticola	**1**	**-**	**1**	**1**	**-**	**-**	**-**	**-**	**1**	**1**

In periodontitis group, the results have shown that *Porphyromonas gingivalis* was present in 7 of the 10 samples obtained (.i.e. P1,P3,P4,P5,P6,P9, P10) and *Tannerella forsythia* was present in 8 of the 10 samples taken (P1,P3,P4,P5,P6,P8.P9.P10) and *Treponema denticola* was present in 5 of 10 samples analysed (P2,P5,P6,P7,P8).

Comparison of frequency distribution of red complex bacteria in health &disease:

In the present study *T. forsythia* was the most prevalent microorganism, with a 80% detection frequency, followed by *P. gingivalis* with a 70% detection frequency, followed by *T. denticola* with a 50% detection frequency.

There was a statistically significant increase in the abundance of *P. gingivalis* (P = 0.020), *T. forsythia* (P = 0.005), and *T. denticola* (P = 0.033) in periodontitis group when compared to health group .

These results are represented in Table.no-3

They have also been depicted in the bar graphs 1,2,3.

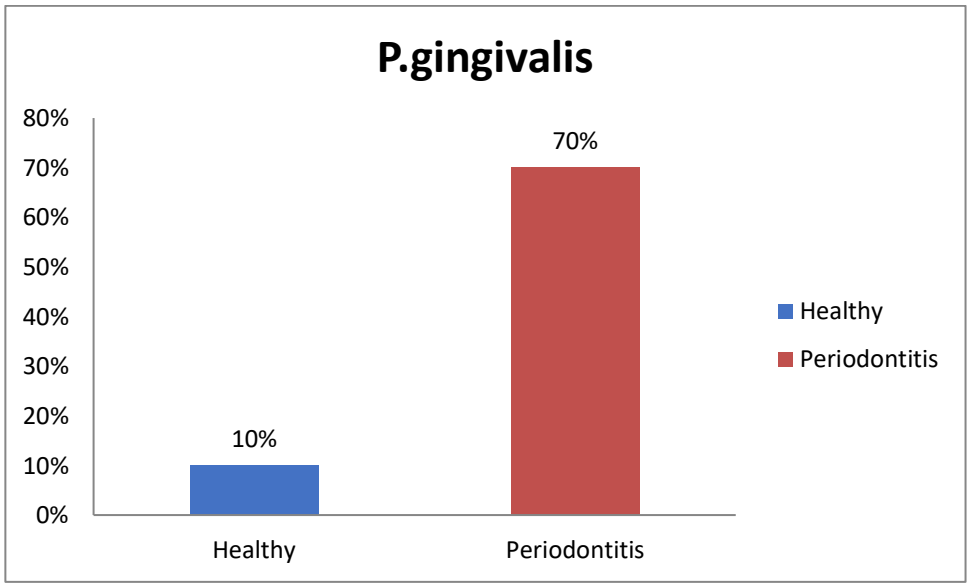
TABLES AND GRAPHS

Table-3 ABUNDANCE OF RED COMPLEX IN HEALTH AND PERIODONTITIS GROUP

Red complex	Expression	Healthy - n (%)	Periodontitis – n (%)	P value
P. gingivalis	0	9 (90%)	3 (30%)	0.020*
	1	1 (10%)	7 (70%)	
T. forsythia	0	9 (90%)	2 (20%)	0.005*
	1	1 (10%)	8 (80%)	
T.denticola	0	10 (100%)	5 (50%)	0.033*
	1	-	5 (50%)	

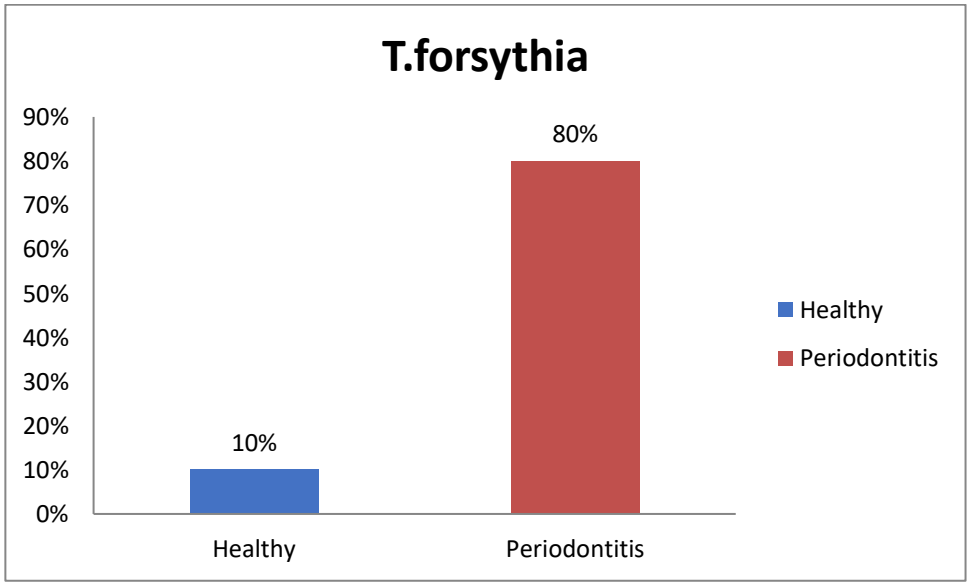
*Sig level at ≤0.05 (Mann whitney U test)

Graph no-1 FREQUENCY DISTRIBUTION OF *P. gingivalis* IN HEALTH &DISEASE



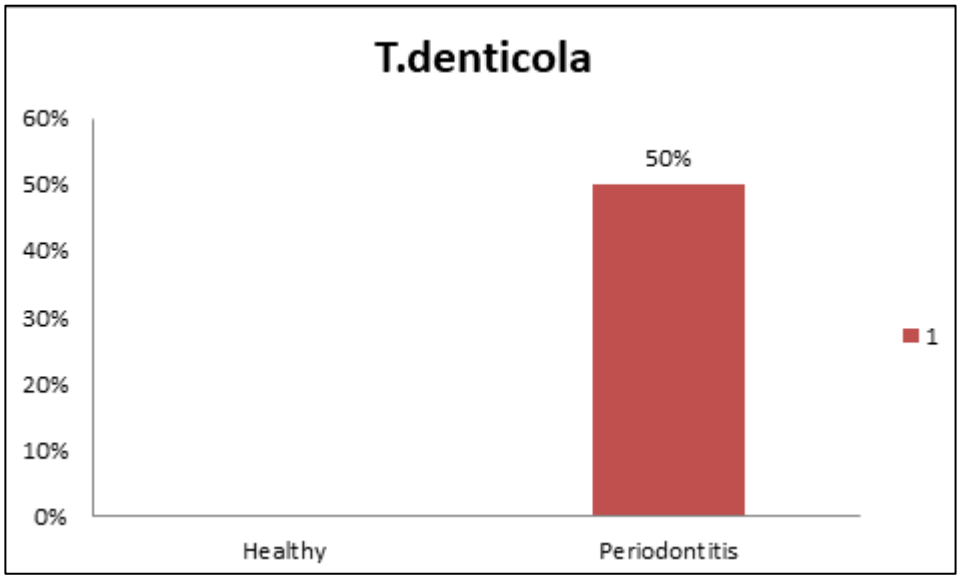
In health group frequency distribution of *P. gingivalis* is about 10% whereas in periodontitis group is about 70%

Graph no-2 FREQUENCY DISTRIBUTION OF *T. forsythia* IN HEALTH AND DISEASE



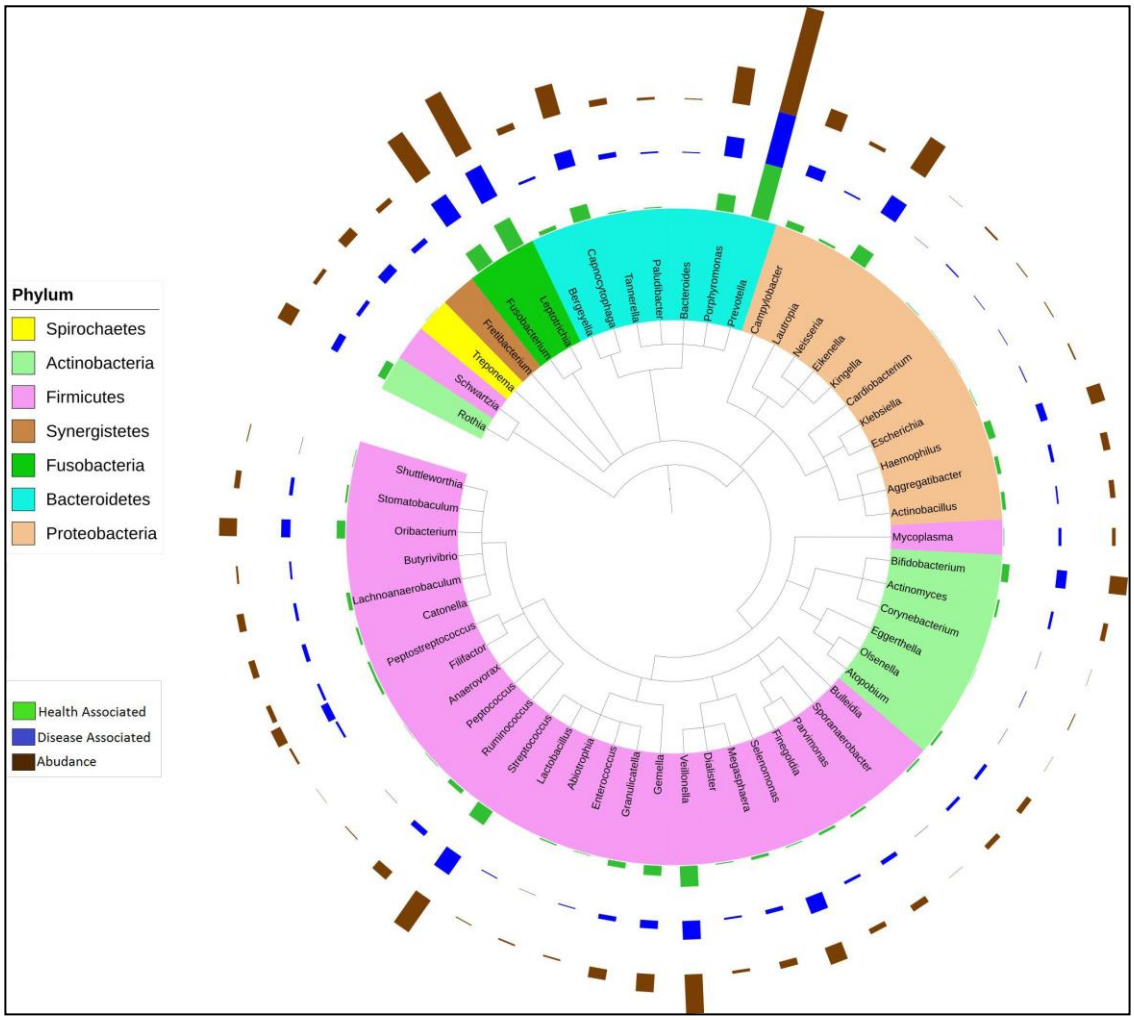
The frequency distribution of *T. forsythia* in health group is about 10% whereas in periodontitis group is about 80%

Graph no-3 FREQUENCY DISTRIBUTION OF *T.denticola* IN HEALTH &DISEASE



The frequency distribution of *T. denticola* in periodontitis group is about 50% whereas in health group is undetectable.

Graph.no-4 PHYLOGENETIC TREE AT GENUS LEVEL



Discussion

DISCUSSION

Periodontal disease is a polymicrobial chronic inflammatory disease associated with changes in the subgingival microbiome and characterized by tissue destruction and the loss of the supporting structure of the periodontium. In humans, a majority of the microbes live symbiotically and plays an important role in maintaining health and disease. Microbiome changes contribute to the pathogenesis of several diseases [18] and reflect the host's health or disease status. Therefore, monitoring the changes in the microbiome is a promising application for diagnosis and prognosis of disease.[56]

Dysbiotic changes in subgingival microbiome and community structures are thought contribute to periodontal disease pathogenesis**.** [162] The oral cavity can be divided into several ecological niches that may have distinct microbial determinants. It is thought that saliva may act as translocating medium that allows for communication between these ecological niches like the buccal epithelium, the dorsum of the tongue, the floor of the mouth, palate, tonsils and the periodontal pocket[117]**.** Saliva is an easily and inexpensively accessible biological fluid, that has been thoroughly analysed for biomarkers of health and disease over the past decade[42]**.**Salivary host derived proteins[64] and levels of proinflammatory cytokines [126] have been investigated as possible biomarkers of periodontitis. [64, 126, 33,118]

There is still inadequate literature relating to salivary microflora and its contribution to periodontal disease in our population. In this study therefore, we have attempted to characterize the salivary microflora using one of the newer detection methods.

In the current study, we have used the NGS- Next Generation Sequencing technology for high-throughput genomic analysis. This technique is advantageous as it can detect previously unknown microbes including those which are non-cultivable. This methodology is in accordance with previous studies of **Griffen [46], Kumar [69],** 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 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 MiSeq 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 in-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.

However, the Illumina sequencing remains almost prohibitively expensive for routine clinical and research use. We have therefore combined resources to use this technology and achieve a reasonable sample size. while other studies have focused on the orange, yellow and green complexes, this studies have evaluated the red complex bacteria.

Subjects were periodontally evaluated and allocated into two groups as healthy controls and periodontitis patients.

Periodontal examinations were performed to determine periodontal status of all subjects. The diagnosis of periodontitis was determined based on AAP classification parameters that includes PD ≥ 5mm in more than four sites. Healthy subjects were required to have no bleeding on probing and sites with probing depth ≤ 3mm.

The salivary microbiome as a whole is represented at the genus level using the phylogenetic tree. (Graph no-4)

In the present study *T. forsythia* was the most prevalent microorganism followed by *P. gingivalis, T. denticola.* Our results are consistent with those of **Liu B et al [83], Wang J et al [154]** whose whole-metagenomic data on subgingival plaque revealed a community dominated by the bacterial phyla *Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria and Proteobacteria.*

There was a statistically significant increase in the abundance of *T. forsythia, P. gingivalis, T. denticola* in periodontitis group when compared to the health group. Present study are consistent with those previously reported by **Fidan Bahtiar Ismail,1 Gener Ismail et al [36].** These authors used real time PCR analysis for identification of subgingival profile in a group of pre-dialysis chronic kidney disease patients with or without periodontal disease& reported that the most prevalent microorganism was *C. gingivalis,* followed by *P. micros , F. nucleatum , T. denticola , P. intermedia and T. forsythia , P. gingivalis E. nodatum* .

Several studies have revealed that in periodontitis communities there were higher proportions of Spirochaetes, Synergistetes and Bacteroidetes, whereas the Proteobacteria were found at higher levels in healthy controls. **[1], [51], [77], [72], [39], [46], [133], [156]**The results of our study too fall in line with these earlier studies.

Red complex bacteria were not identified in two of our disease samples. These results are somewhat in agreement with **Kumar P.S et al., Griffen A.L et al.-** [70] who stated that there was no significant difference in red complex bacteria between health and disease when the microbiome was studied as a whole.

The role of the red complex in the etiopathogenesis of periodontal disease has been extensively evaluated.*P. gingivalis* is one of the major colonizers in deep periodontal pockets as it provides a strict anaerobic ecological niche that promotes the bacterium's growth. *P. gingivalis* has been described as a "keystone pathogen".[48] It utilizes its major virulence factors, lipopolysaccharide, capsule, gingipains, and fimbriae, to establish the infection by interacting with the host. Although it has a high number of potent virulence factors, it has not yet been shown that its presence in a healthy periodontium predicts an increased risk of disease onset. This indicates that this bacterium is probably a late colonizer that invades already diseased tissues and contributes to an increased progression of periodontal breakdown.

P. gingivalis cells occasionally gain entry to the human circulatory system, and the resulting transient bacteremia may induce systemic effects as well [53]. Species- specific virulence factors like gingipain R produced by *P.gingivalis* induce platelet aggregation which leads to thrombus formation, Karilysin produced by *T.forsythia* degrade LL-37 antimicrobial peptide

,Dentilisin produced by *T. denticola* invade the epithelial &endothelial cells which leads to immune suppression**.** [76]

T. denticola level has been increased in ratio to the severity of periodontitis [129.] This microorganism may possess several pathogenic factors, including its major outer sheath protein and proteases. Dentilisin is a prolyl-phenylalanine specific surface protease. Dentilisin hydrolyses host bioactive proteins [90 &36] and is cytotoxic to periodontal ligament epithelial cells.

In *T. forsythia* trypsin-like and PrtH proteases, a leucine-rich repeat cell surface- associated and secreted protein BspA, a-D-glucosidase and N-acetyl-b-glucosaminidase, a hemagglutinin has been identified that may play roles in the degradation of host proteins, providing essential amino acids, peptides and heme for the growth of T. forsythia. [3]

It must be remembered that most of these studies have been performed in plaque samples and not in saliva.

Among salivary studies, our results are comparable with those of **Belstrøm D-**[10] who have found several putative periodontal pathogens including *Tannerella forsythia, Parvimonas micra and Filifactor alocis* were statistically more significant and at higher levels in saliva samples from periodontitis patients. These authors suggested that some anaerobic and facultative anaerobic

pathogens associated with periodontitis were undetectable in saliva because their natural habitat is in the deeper areas of the periodontal pocket.

The putative role of the salivary microflora in the etiopathogenesis of periodontal disease cannot be assessed from the results of our study.

It may be hypothesized that salivary red complex bacteria may involved in the development of generalized periodontitis by colonizing uninvolved sites from the affected one and recolonization of previously treated sites.[10]

However, the association of salivary red complex bacteria with periodontitis suggests they may be used as biomarkers. A longitudinal study with greater sample size will be required to confirm the findings of this study.

Summary and Conclusion

SUMMARY AND CONCLUSION

This study evaluated the salivary red complex species in periodontitis and in healthy individuals. Saliva was collected from total of twenty individuals, of which ten were periodontally healthy and ten were periodontitis patients. Analysis of red complex species was done with NGS technology using Illumina MiSeq sequencing.

There was a statistically significant increase in red complex bacteria in patients with periodontitis [P. *gingivalis* (P = 0.020), *T. forsythia* (P = 0.005), and *T. denticola* (P = 0.033)] when compared to healthy subjects. The strong association of red complex bacteria with periodontitis suggests that they may be used as microbial risk markers for periodontal disease. However, a longitudinal study with a greater sample size will be required to confirm the findings of this study.

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Annexures

ANNEXURE I

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 WHOMSOEVER IT MAY CONCERN

Date: 30.1.2019

Place: Chennai

From
The Institutional Review Board,
Ragas Dental College & Hospital,
Uthandi, Chennai – 600 119.

The dissertation topic titled "**ANALYSIS OF RED COMPLEX BACTERIA IN THE MICROBIOME OF WHOLE SALIVA IN PERIODONTAL HEALTH AND PERIODONTITIS INDIVIDUALS USING NEXT GENERATION SEQUENCING TECHNOLOGY**" submitted by **Dr. KAVIPRIYA. K** has been approved by the Institutional Review Board of Ragas Dental College and Hospital.

DR. N.S. AZHAGARASAN, MDS
Member Secretary,
Institutional Ethics Board,
Ragas Dental College & Hospital,
Uthandi, Chennai – 600 119.

ANNEXURE - II

URKUND

Urkund Analysis Result

Analysed Document:	K.KAVIPRIYA -THESIS.docx (D47312847)
Submitted:	1/28/2019 6:04:00 AM
Submitted By:	kavisanthosh08@yahoo.com
Significance:	4 %

Sources included in the report:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4100321/
https://www.sciencedirect.com/topics/medicine-and-dentistry/tannerella-forsythia
https://academic.oup.com/femsle/article/333/1/1/586464
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4746253/
http://etheses.whiterose.ac.uk/2866/1/Corrected_thesis_Abigail_att.pdf

Instances where selected sources appear:

14

ANNEXURE – III

CONSENT FORM

I ……S/o, w/o, d/o……
aged about ……. 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 Doc