“MICROBIAL ANALYSIS OF RED 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

MAY 2020

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

CHENNAI

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **MICROBIAL ANALYSIS OF RED 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 B Shiva Kumar M.D.S.,** Professor, Department of Periodontology, Ragas Dental college & Hospital, Chennai.

Date: 06.02.2020

Place: Chennai

Dr Ragha Malika S,
Post Graduate Student,
Department of Periodontology,
Ragas Dental College & Hospital.

CERTIFICATE

This is to certify that this dissertation titled **MICROBIAL ANALYSIS OF RED 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 Ragha Malika 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.

Guide

RAGAS DENTAL COLLEGE Chennai

Dr. B Shiva kumar M.D.S.,

Professor,
Department of Periodontology,
Ragas Dental College & Hospital,
Chennai.

Department of Periodontics,
Ragas Dental College & Hospital,
2/102, ECR, Chennai-119

Head of the Department

Dr. K V Arun M.D.S.,

Professor and Head,
Department of Periodontology,
Ragas Dental College & Hospital,
Chennai.

Dr.K.V.ARUN MDS
Head of the Department
Department of Periodontics
Ragas Dental College and Hospital
Chennai - 600 119.

Dr. N S Azhagarasan M.D.S.,

Principal,
Ragas Dental College & Hospital, Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI-600 119.

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

CHENNAI

PLAGIARISM CERTIFICATE

This is to certify that this dissertation work titled **MICROBIAL ANALYSIS OF RED COMPLEX ORGANISMS OF THE WHOLE SALIVA IN PATIENTS WITH GINGIVITIS AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING** of the candidate **Dr. Ragha Malika S** for the award of **MASTER OF DENTAL SURGERY** in the branch of **PERIODONTOLOGY.**

I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **12 percentage** of plagiarism in the dissertation as per the report generated.

Guide & Supervisor sign with seal:

Date: 06.02.2020

Place: Chennai

Dr. B Shiva Kumar, M.D.S.,

Professor,

Department of Periodontology,

Ragas Dental College, Chennai.

Department of Periodontics,
Ragas Dental College & Hospital
2/102, ECR, Chennai-119

ACKNOWLEDGEMENT

I would like to express my gratitude to all the people who supported me in the completion of this dissertation.

My sincere thanks to the principal, ***Dr.N.S.Azhagarasan****, MDS, Ragas Dental College and Hospital for his guidance throughout my post-graduation course.*

I extend my gratitude to my guide ***Dr B Shiva Kumar, MDS,*** *Professor for his continuous guidance and constant encouragement throughout my study period. I am deeply grateful to him for his patience and guidance during my dissertation.*

I express my sincere thanks to my most respected and beloved Professor and HOD ***Dr K V Arun MDS,*** *professor and head of the department of Periodontics, Ragas dental college Chennai, for his valuable advice, guidance, patience and insight throughout my post graduate course.*

I would like to express my gratitude to ***Dr G Sivaram MDS,*** *Professor and to* ***Dr Ramya Arun MDS,*** *Reader who has constantly inspired me and has unceasingly guided me throughout my study process with their constant support****.***

I would like to express my profound sense of gratitude to ***Dr. Swarna Alamelu, MDS,*** *Reader and* ***Dr. Archana Meenakshi, MDS,*** *Reader for their constant support and encouragement throughout my tenure.*

I would like to thank ***Dr. Deepavalli, MDS,*** *Senior Lecturer* ***Dr. Sreemohana, MDS,*** *Senior Lecturer for their continuous support and guidance. I would also like to thank* ***Dr. R.S. Pavithra, MDS,*** *Senior Lecturer,* ***Dr. J. Velkumar, MDS,*** *Senior Lecturer and* ***Dr. A.R. Akbar, MDS,*** *Senior Lecturer for their constant support*

I remain ever grateful to my batch mates ***Dr S Indhumathi*** *and* ***Dr Meenakshi R P,*** *for their constant support and encouragement.*

I thank all my seniors, especially ***Dr Krupa, Dr Cynthia and Dr Kavipriya****, and my juniors* ***Dr. Davis Navin, Dr.Gayathri, Dr. Shanmugapriya, Dr.Lakshmi Prasanna,*** *and* ***Dr. Aswathy.***

I would like to thank my friends ***Dr Priyadharini*** *and* ***Dr Priyadharshini*** *for putting up with me and being the constant source of enthusiasment through this journey.*

I extend my gratitude to ***Mr. Jotheeswaran, Mrs. Parvathi, Mrs. Rosamma, Mr. Chellapan*** *and* ***Mrs. Mala*** *for their timely help during the tenure.*

I would like to extend my heartfelt thanks to my mom ***Mrs U Ponbarani,*** *my dad* ***Mr S P Sivasankaran*** *& my brother* ***Mr Hari Prasath*** *for their constant support, love, care and encourage in all my ups and downs throughout these years.*

I thank the almighty God for guiding me always. He guided and helped me throughout my life in every endeavour and for that am ever grateful

"I'm blessed and I thank god for everyday

for everything that happens to me"

-Lil Wayne

LIST OF ABBREVIATION

PSD	POLYMICROBIAL SYNERGY AND DYSBIOSIS MODEL
LPS	LIPOPOLYSACCHARIDE
AHL	ACYLHOMOSERINE LACTONE
AI	AUTOINDUCER
HGT	HORIZONTAL GENE TRANSFER
LGT	LATERAL GENE TRANSFER
ECM	EXTRA CELLULAR MATRIX
rRNA	RIBOSOMAL RIBONUCLEIC ACID
PCR	POLYMERASE CHAIN REACTION
DNA	DEOXYRIBONUCLEIC ACID
NGS	NEXT GENERATION SEQUENCING
SOLID	SUPPORTED OLIGONUCLEOTIDE LIGATION AND DETECTION
Mseq	METAGENOMIC SEQUENCING
HOMD	HUMAN ORAL MICROBIOME DATABASE
BLAST	BASIC LOCAL ALLIGNMENT SEARCH TOOL
OSCC	ORAL SQUAMOUS CELL CARCINOMA

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF GRAPHS

LIST OF ANNEXURES

Abstract

ABSTRACT

BACKGROUND

The red complex bacteria which includes *Porphyromonas gingivalis, and Tannerella forsythia, Treponema denticola* are described as climax colonizers and are thought to play an important role in progression of periodontal disease. These bacterial species are usually found abundant in periodontal pockets, suggesting that they are associated with the dysbiosis of the periodontal environment. Technological developments in sequencing and identifying DNA and powerful bioinformatics tools have helped in characterization of microbiota of interest in relation to the total microbial load. The aim of this study was to evaluate the presence of the red complex bacteria in saliva using Next Generation Sequencing Technology in gingival health, gingivitis and gingival recession.

MATERIALS AND METHODS

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), 10 were patients with gingivitis (test group 1) and 10 were patients with gingival recession (Test group 2). 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 the current study suggest that the Red complex bacteria were statistically significantly higher levels in gingivitis and gingival recession groups [*Porphyromonas gingivalis* P<0.001, *Treponema denticola* P<0.001 and *Tanerella forsythia* P<0.001] when compared to healthy subjects, but there is no significant difference between the test groups [*Porphyromonas gingivalis* P=0.85, *Treponema denticola* P=0.70 and *Tannerella forsythia* present in all 20 samples]. Therefore salivary red complex bacteria may be used as potential microbial risk markers for periodontal diseases. However, a longitudinal study with a greater sample size will be required to confirm the findings of the current study.

CONCLUSION

Salivary red complex bacteria may be used a suitable candidate for the risk markers of gingivitis and gingival recession.

KEYWORDS

Illumina MiSeq Sequencing, Next Generation Sequencing, 16S rRNA, Gingivitis, Gingival recession, salivary microbiome, Red complex organisms

Introduction

INTRODUCTION

Periodontal disease is multifactorial disease in which bacteria is a necessary prerequisite for the disease to develop.[1] Gingivitis and Periodontitis are common oral disease that are inflammatory in nature. Gingivitis is a reversible inflammatory reaction of the marginal gingiva and is a non-specific 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.[2] However, existing evidence indicates that periodontitis is always preceded by gingivitis; however, not all gingivitis develops into periodontitis.[3]

Untreated periodontal disease may initially lead to increase in the pocket depth or pocket-free gingival recession, which have been recognized as two separate periodontal phenotypes.[4] Gingival recession refers to exposure of root surfaces caused by apical displacement of gingival margin beyond cemento-enamel junction. Gingival recession could be a result of faulty tooth brushing, mucogingival deformity or accumulation of local factors.

Various study established the association of socio-economic status with periodontal health.[5–7] Indian subcontinent is a population wherein increased accumulation of local factors are found due to inadequate oral hygiene practices. Gingival recession along with inflammation is the most common condition found among these population. Accumulation of local

factors in Indian population with respect to gingival recession is well established as in other countries.[8, 9]

Periodontitis is no longer considered a simple bacterial infection that leads to periodontal destruction. Rather, it represents a collection of complex diseases involving iterative interactions between the host inflammatory & immune systems, sub-gingival microbiota & modifying environmental factors.[10] It is also recognised that the periodontal microbiota is generally of a commensal nature & its relationship to the host is usually in a state of homeostasis. A ground-breaking experiment in humans suggested a cause–effect relationship between the aggregation of bacterial deposits in the area of the gingival crevice and gingival inflammation.[11] The microbial etiology of periodontal disease was being analysed over the past 5 decades now using various technique.[12, 13]

Over the last 15 years it has become clear that the overall diversity of the periodontitis-associated microbiota is very broad, with potential involvement of several hundred different species and subspecies.[14–16] Bacterial analysis of the disease started with the culture technique and evolved via checkerboard hybridization technique and have reached high-throughput approaches. High throughput approaches include microarray and next generation sequencing. Microarray is a closed ended while next generation sequencing is an open ended sequencing technique.[17, 18]

The current trend in sequencing of microbiome is based on Next-Generation Sequencing (NGS) that uses parallel sequencing of multiple small fragments of DNA to determine genetic sequences Since 2006, there have been a lot of studies that used next-generation sequencing devices to sequence whole genomes of different organisms, transcriptomes of single organisms and even communities of organisms.[19,20] NGS is an open ended sequencing technique based on 16S rRNA sequencing.

Microbial communities of periodontal disease condition involving deep periodontal pocket have been widely studied.[21,22] However, there is paucity of data regarding microbial communities in areas of gingival recession along with inflammation. There are a lot of studies that showed that there is a variation in gingival characteristic among developing and developed nations and among western and Indian population.[23, 24]

Although there are few data available on the microbiome of gingival recession, they are centered over the western population.[14,25] Acquiring similar data in our population would help us understand more about the disease progression and its microbiome in this population. Recent advances in periodontal diagnosis are more focussed on the personalised treatment rather than generalisation of the treatment plan. Since personalised medicine is the near future, acquiring this microbial data will give a helping hand in personalising the treatment.

Saliva is a biofluid comprising secretions of the salivary glands (the parotid, sub-mandibular, sublingual and other minor salivary glands), oral

mucosa cells, blood and gingival crevicular fluid. Similar to serum and other biofluids, saliva also contains biomolecules such as DNA, mRNA, microRNA, protein, metabolites and microbiota.[26] Since obtaining saliva can be low cost, simple, non-invasive and does not cause patient discomfort, it is a highly desirable body fluid for biomarker development for clinical applications. Hence, saliva is a widely used medium of analysis of metabolome, proteome, genome, epigenome, transcriptome and microbiome.[27–29]

Although saliva does not have a resident microflora, salivary microorganisms may play an important role in the etiology and propagation of periodontal diseases because of translocation.[26] Bacterial colonization of sub-gingival environment of various teeth translocate through saliva and has been proposed to play a role in transfer of sub-gingival bacteria from uninfected to infected sites and recolonization of treated sites in the periodontium.[28]

Aims and Objectives

AIMS 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 gingival inflammation and recession.

Objectives:

1. To evaluate the presence of the red complex bacteria in saliva using Next Generation Sequencing Technology in gingival health, gingivitis and gingival recession.

2. To compare the presence of the salivary red complex in gingivitis and gingival recession with those of healthy controls.

Review of Literature

REVIEW OF LITERATURE

MICROBIOME:

The human body is the habitat of various bacteria and other microorganisms. The microbiome is the genetic material of the aggregate of all microbiota that colonizes on or within various human tissues and body fluids. Every human being has a personalized set of microorganisms essential to maintain health which are also capable of causing disease under circumstances.[30] The 215 cm^2 surface area of the oral cavity presents a numerous surface of microbial colonization. As many as 700 bacterial species may colonize the surface of the oral cavity.[31]

The term microbiome was coined by **Joshua Lederberg** to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease. 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.[32]

Turnbaugh et al (2007)[33] described human microbiome to be classified into a core microbiome and a variable microbiome. Core microbiome comprises of the predominant species that exist under healthy conditions at different sites of the body, and it is shared by all individuals.[34,35] Variable microbiome is one that has evolved in response to unique lifestyle, phenotypic and genotypic

determinants; it is exclusive for an individual. Even though individuals share microbiota at similar sites of the body, varying differences are observed at species and strain level of the microbiome which may be as unique as a fingerprint of an individual.[36]

Core microbiome is capable of thriving in both health & diseased condition and have synergistic interactions with health and disease associated species as they successfully grow with both groups. They act as a metabolic cornerstone for the microbial community and that their presence is important for the microbial shifts that leads to disease in the periodontium. The normal flora of core microbiome helps maintaining the homeostasis of the niche.[35,37]

The most abundant core species is found to be *Fusobacterium nucleatum*. It is the bridging organism and is identified to be the essential micro-organism for the survival of other periodontopathic bacteria in anaerobic environment. Its ability to co-aggregate with diverse range of bacteria helps in shifting the microbial colonization in plaque biofilm. 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 bacteria like *Porphyromonas gingival*is can thrive, that are more related to periodontitis.[38]

HEALTHY MICROBIOME:

The ability to maintain homeostasis within a microbial community increases with the diversity of its species. The oral 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**.[39] **Stappenbeck** et al in a study discovered that the commensal microbiota induces angiogenesis there by contribute to the development of the complex vascular beds under the mucosal surface. He also found that intercellular adhesion marker-1 expression in these vessels is also regulated by the presence of the commensal microbiota.[37]

Dysbiosis is defined as change/perturbations in the structure and composition of resident commensal bacterial communities relative to the community found in healthy individuals. Dysbiosis results when a symbiotic relationship disappears due to a reduction in the number of beneficial symbionts and/or an increase in the number of pathobionts. Maintaining microbial stability is necessary for sustaining symbiotic environment and for prevention of dysbiotic state.[40]

PERIODONTITIS:

Health is defined as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" by the World Health Organization in 2018. In accordance with this definition,

periodontal health should be defined as "a state free from inflammatory periodontal disease that allows an individual to function normally and not suffer any consequences as a result of past disease".[41]

However, it seems an impractical and limiting definition to explain the periodontal health. In its **pristine** form, periodontal health would be defined as the absence of histological evidence of periodontal inflammation and no evidence of anatomical change to the periodontium but is unlikely in most adult. This is because of the presence of commensal bacteria and immune surveillance of the gingival epithelium.[42] Therefore, the term **clinically healthy** should be adopted to cover the absence of clinical periodontal disease.[41]

Periodontal disease is multifactorial in nature, in which bacteria is a necessary prerequisite for the disease to develop but not sufficient to cause the disease alone.[1] The microbial challenge is presented by subgingival plaque which results in upregulated host immune inflammatory response in periodontal tissue characterised by excessive production of inflammatory mediators. The periodontal inflammation is initiated by the components of subgingival biofilm.[10]

Periodontal disease starts as a gingivitis, which is the inflammation of the gingival tissues. The gingivitis progress to periodontitis in many cases when the necessary treatment is not instituted at the right time. Gingivitis is a reversible inflammatory reaction of marginal gingiva to plaque accumulation, whereas periodontitis is a destructive, non-reversible condition

resulting in loss of tooth connective-tissue attachment to bone, which ultimately leads to loss of the involved teeth.[1]

Existing evidence indicates that gingivitis precedes onset of periodontitis; however, not all gingivitis cases develop into periodontitis.[43] 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.

PLAQUE AS ETIOLOGY:

Periodontitis 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.[44]

Löe et al in 1978 demonstrated the natural progression of periodontal disease through plaque biofilm in a study population of Sri Lankan tea workers of which represented a relatively uniform population that had little to no dental care and also had extremely poor oral hygiene. His several studies on experimental gingivitis showed that complex dental biofilm which comprises about 600 microbial species but a limited periodontal pathogen is involved in the causation and progression of periodontal disease. Therefore, plaque plays a

major role in the etiology of periodontitis.[4, 22] 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**[46] bacteria were implicated in periodontal disease with the observation that administration of penicillin inhibited periodontitis in laboratory animals, and **Keyes and Jordan**[47] demonstrated the infectious nature of periodontitis by its transmissibility in animal models.

Socransky and Haffajee et al in 1992[48] proposed the criteria for identification of the bacterial species as periodontopathic bacteria stating 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;

- Treatment must eliminate the putative pathogen from the periodontal lesion leading to clinical improvement.

PLAQUE HYPOTHESES:

1. *Specific plaque hypothesis – Loesche 1976*
2. *Non-specific plaque Hypothesis – Theilade 1986*
3. *Ecological Hypothesis- Marsh 2003*
4. *Polymicrobial Synergy and Dysbiosis – Hajishengalis 2012*

Specific plaque hypothesis: (Loesche, 1976)

In 1976, **Walter J. Loesche**[49] proposed the "Specific Plaque Hypothesis" which stated that the specific pathogens are responsible for the periodontal disease to develop. The abundance of certain organisms in the periodontal disease affected sites lead to the acceptance of this hypothesis. This hypothesis was then discarded because of the presence of the periodontopathic bacteria even in the healthy sites.

The main limitation of this hypothesis is that it failed to prove that why not all gingivitis progress to periodontitis. Page RC et al[1] stated that it is applicable only for the development of gingivitis, however periodontitis being a multifactorial disease cannot be explained by this hypothesis.

Nonspecific plaque hypothesis: (Theilade, 1986)

Non-specific hypothesis was first proposed in early 20th century based on the bacterial association with periodontal lesions based on microscopy and culture methods. The latest non-specific hypothesis was again proposed by **Theilade** in 1986[50]. He stated that the pathogenicity was determined by the quantity of plaque than discriminating between the levels of virulence and the specificity of bacteria present.

The disease develops only when the microbial load increases more than the threshold of the host to neutralize the bacteria and its products. The condition earlier known as aggressive periodontitis presented with the severe periodontal involvement with the presence of only minimal local factors. Non-specific hypothesis was unable to explain this pathogenesis.

Ecological plaque hypothesis:

In 1994 **Philip D. Marsh**[51] 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.[51,52]

Keystone pathogen hypothesis:

Hajishengalis et al in 2011 introduced 'keystone pathogen hypothesis" which states that certain low-abundance microbial pathogens increase the quantity of the normal microbiota and by changing its composition thus causing the inflammatory disease.[53] Keystone pathogens are capable of triggering inflammation when present even in lower numbers, in contrast to the dominant species that can influence inflammation by their abundant presence. The keystone pathogen is detected in higher numbers in periodontitis and gingivitis.[54]

Relative to its abundance, a pathogen with a disproportionately large effect on its environment, for example low-abundance red complex bacteria such as *Porphyromonas gingivalis* or *Tanerella forsythia* or *Treponema denticola* remodels a commensal microbial community into a dysbiotic and disease-provoking microbiota. **Darveau in 2009** confirmed that *Porphyromonas gingivalis* is capable of manipulating the native immune system of the host.[55]

Polymicrobial Synergy and Dysbiosis:

Currently, pathogenesis of periodontal disease is explained by "Polymicrobial Synergy and Dysbiosis Model (PSD)" proposed by **Hajishengalis et al**.[53] This model states that "the periodontal pathogenesis caused by a broad dysbiotic, synergistic microbiota against the traditional view

that it is caused by a single or several pathogens" especially red complex bacteria. It means that the dysbiotic environment and polymicrobial synergy are the key events which led to development of periodontitis rather than individual bacterial species. Dysbiosis is a symbiotic relationship that has changed due to decrease in number of beneficial symbionts and/or an increase in number of pathobionts.[21]

This PSD model alters host-microbe homeostasis and facilitate its transition to a chronic inflammatory state. **Lamont and Hajishengallis**[56] 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. Research over the past decade has led to the recognition of these dysbiotic microbiomes residing in the various oral ecological niches including mucosal surfaces and saliva.[57]

BIOFILM:

Biofilms are the self-renewing and constitute a significant continuous bacterial load on the host. These self-renewing reservoirs of endotoxins (LPS) and other bacterial toxins that can gain access not only into surrounding periodontal tissues but the general circulation as well.[58] Biofilms consist of one or more communities of microorganisms, embedded in a glycocalyx, that are

attached to a solid surface. The bacteria adheres to the biofilm and depend on it for the nutrients. In addition to the nutrition, biofilm provides these bacteria with the advantage over the planktonic bacteria.[59]

Potera et in 1999 has put forth in his experiment that 65% of infections that affect the human are caused by organisms growing in biofilms.[60] Biofilm should consist of three components:

a. a surface on to which the bacterial attachment occurs;

b. the biofilm community itself;

c. the bulk fluid over the biofilm that provides nutrients for the bacteria. Dental plaque is regarded as the specialized example of microbial colonization.

FORMATION OF DENTAL BIOFILM:

1. Adsorption of salivary glycoprotiens (acquired pellicle)
2. Reversible adhesion between the microbial cell surface and the pellicle
3. Initial adhesion/permanent attachment involving the interactions between specific molecules on the microbial cell surface (adhesins) and complementary molecules (receptors) present on the pellicle
4. Co-adhesion in which secondary colonizers adhere to receptors on already attached bacteria leading to increase in microbial diversity
5. Multiplication of attached cells, leading to an increase in biomass and synthesis of exopolymers to form the biofilm matrix (plaque maturation)
6. Detachment of attached cells to promote colonization elsewhere.(Lindhe, 6th edition)

Biofilm formation initiates with the adsorption of the salivary pellicle on to the tooth surface.

First phase of initial adhesion involves the transportation of bacteria onto the tooth surface, followed by the reversible adhesion of the bacteria.

Initial adhesion occurs through the Vander Waal's attractive force and electrostatic repulsive force. These are the weak, long range force while strong, short range forces such as covalent and hydrogen bonds results in the irreversible adhesion of the bacteria on the pellicle on the tooth surface.[61]

Short range forces, involve specific stereochemical interactions between components on the microbial cell surface (adhesins) and receptors in acquired pellicle formation. These types of interactions contribute to the tropism of an organism with a particular surface/habitat. Streptococci, an early colonizer binds to the acidic proline-rich protein and other receptors in the pellicle such as α amylase and sialic acid. **Gibbons in 1988**[62] demonstrated that the Actinomyces species also function as primary colonizers ie., *Actinomyces viscosus* possesses fimbriae that contain adhesins that specifically bind to proline rich proteins of the pellicle**.**

The predominant early colonizers of the subgingival plaque biofilms are *Actinomyces species* and *streptococci species*. Within few days, a complex microbial community develops within the space and the secondary colonizers includes the climax colonizers 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.[63]

COAGGREGATION:

This primary colonization of the bacteria provide new receptors for the attachment by other bacteria. This process is called "coadhesion". This leads to the formation of the second colonization thereby plaque maturation occurs. The firm attachment is followed by surface colonization (secondary colonization) and biofilm formation that eventually reaches "Climax community of dental plaque.

According to **Koglenbrander** a specific cell-to-cell recognition that occurs between genetically distinct cell types is termed as coaggregation. Adhesins / receptors are coaggregation mediators. He found that in the oral cavity, atleast 18 genera are found to be capable of co-aggregating with other bacteria and with each other.[64] **Gibbons & Nygaard**[65] termed it as "interbacterial aggregation".

In early stages of biofilm formation, coaggregation appears to be in ROSETTE formation, wherein single coccus is surrounded by a number of cooci. In gingivitis patients, **Lisgarten et al, 1965**[66] showed CORN COB PATTERN of coaggregation resulting from the growth of cocci on the surface of filamentous microorganism.

According to **Teughels W, Quirynen M,**[63] TEST TUBE BRUSH appearance of bacteria composed of central axis of bacilli with perpendicularly associated with filamentous organism. It is present in subgingival plaque of teeth associated with periodontitis.

BACTERIAL COMMUNICATION:

Bacterial communication with different bacteria & cell to cell signalling is required to enhance biofilm formation, nutrition and growth of climax community. Bacteria interact synergistically to metabolize complex endogenous molecules (e.g. glycoproteins), and food webs can develop. Schematic representation of the types of interaction (inter-bacterial and bacterial–host) that occur in a microbial community, such as dental plaque, growing as a biofilm is given in fig 1.

Bacteria communicate with each other in a cell density-dependent manner via diffusible signalling molecules, and with host cells. Cells are more tolerant of antimicrobial agents either because of the physical attributes of the biofilm, via gene transfer of resistance genes, or through protection by neighbouring cells that produce neutralizing enzymes. Cells may also gain advantage by production of inhibitory molecules.[67]

QUORUM SENSING:

Quorum sensing in biofilm was 1st reported by Cooper et al 1995[68] and in oral biofilm by Lijemark 1997. The term "quorum" is used to describe a signal system, which require a certain number of micro-organisms to be present for the signal to be sensed and for the population to respond to the signal.

Quorum sensing is an intercellular communication through accumulation of signaling compounds that regulate the expression of specific genes which allow the bacteria to mount coordinated response to their environment.[69] **Bassler et al, 1997**[70] stated that in quorum sensing, bacteria release, detect and respond to accumulation of small signal molecules, in a cell density-dependent manner, thereby regulating the expression a set of target genes.

Bacteria release chemical substances called autoinducers into their surroundings. As the population density increases, so does autoinducer concentration. When the population density is sufficiently high (i.e., a quorum is achieved), autoinducer concentrations become high enough to bind to receptors on/within the source or nearby bacteria. The signal is then transduced into an intracellular biochemical signal or altered gene expression in the target bacteria. This induces a variety of adaptive physiological changes such as bioluminescence, production of antibiotics, and activation of biofilm formation.

Many classes of AIs have been described to date. The most intensely studied AIs are the N-acylhomoserine lactones (AHLs) of Gram-negative bacteria, the oligopeptides of Grampositive bacteria and a class of AIs termed AI-2, whose structures remain unknown in most cases.[71] **Kolenbrander** showed the peptides secreted by gram-positive organisms during growth and a "universal" signal molecule autoinducer 2 (AI-2) are the two types of signalling molecules detected in dental plaque.

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, in contrast to the strain-specific competence-stimulating peptides.(Fig 2) Wide-ranging changes in gene expression, in some cases affecting up to one-third of the entire genome in detection of AI-2.

In biofilm development, 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.

QUORUM QUENCHING:

It is the inhibition of quorum sensing (signaling molecules) by degradation enzymes for example, lactonase & Acylase. Quorum quenching can be done by drugs that are produced in the natural means or made synthetically

by chemical methods. It is also possible that quorum quenching is used as a defense mechanism against antibiotic-producing bacteria in the ecological niche. Quorum sensing disrupting compounds attenuate virulence of bacteria.

The genes encoding **AHL-lactonase and AHL-acylase** have been identified and characterized from *Agrobacterium tumefaciens*[72,73] and *Porphyromonas aeruginosa*[74], respectively. However, the expressions of these enzymes in the two bacterial species appear to be tightly regulated.

HORIZONTAL GENE TRANSFER:

Horizontal gene transfer (HGT), also lateral gene transfer (LGT) or transposition refers to the transfer of genetic material organisms other than vertical gene transfer is the transfer of gene from the parental generation to the offspring. Close cell-cell contact and mobile genetic element are required for the horizontal gene transfer. It is done by 3 methods namely

- Transformation
- Transduction
- Conjugation

TRANSFORMATION:

A process in which genetic material is taken up and maintained in a cell. The mobile genetic material is acquired from the live or dead bacteria.(Fig 3)

Oral bacteria Neiserria, Streptococcus,[75] Actinobacillus are naturally competent for DNA uptake. However, the longitivity of mobile DNA fragment act as a rate-limiting step in transformation process.[76] **Li et al in 2001**[77] showed that S. mutans is capable of uptaking DNA from dead cells and transform into erythromycin resistant.

TRANSDUCTION:

Transduction is the process in which whole bacterial DNA or a fragment of DNA gets incorporated into bacteriophage. Viruses that survive within the bacteria are called bacteriophages. 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 bacterium. Thus, the bacterial genetic material of primary cell can get transferred into that second cell.[78] Presence of bacteriophage is seen in periodontal bacteria, such as *A. actinomycetemcomitans, Fusobacteria and T. denticola.*[79]

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.[80] A set of specialized adhesives present on the surface of the cell membranes makes the bacterial interaction possible.

The tetracyclines resistant gene present in *Aggregatibacter actinomycetemcomitans* is a result of process of conjugative transfer of a plasmid between different strains of *Aggregatibacter actinomycetemcomitans* and also between different organisms. Conjugative transfer occurs between *Aggregatibacter actinomycetemcomitans* and non-pathogenic organism *Haemophillus influenzae* is the most studied, a result of which tet-B gene is made accessible to the organism. **Roe et al, 1995**[81] in his study showed that the major component of antibiotic resistance exhibited by *Aggregatibacter actinomycetemcomitans* to tetracycline is the Tet B gene**.** Thus, horizontal gene transfer aids in greater survival of pathogenic bacteria and increases virulence of pathogenic bacteria.

BACTERIAL SUCCESSION:

AUTOGENIC SUCCESSION:

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

ALLOGENIC SUCCESSION:

One type of community is replaced by another because the habitat is altered by non-microbial factors such as changes in the physical or chemical properties of the region or changes in the host.(Fig 4)

Loe et al and Theilade et al 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.[11]

The initial colonizers which includes yellow, green and purple complexes lead to the succession of the predominant members of orange and 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.

Elimination of all biofilm may be the primary step; this partially successful strategy is one of the most commonly employed target for treatment of periodontal therapy. 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 non-antimicrobial approach, which leads to decreased

plaque accumulation and possibly diminish red and orange complex development.

CLIMAX COMMUNITY:

The interaction between the microbial and non-microbial components of an ecosystem ultimately leads to a form of stabilisation in which microbial and non-microbial forms exist in harmony and equilibrium with their environment. This is a dynamic state in which cells are being replaced. The climax community can be modified from time to time by exogenous forces. The equilibrium tends to be restored as the habitat returns to its original state.(Fig 5)

Given the same initial physical and chemical site characteristics or identical hosts, the same general successional sequences will be initiated and fostered, giving rise to remarkably similar climax communities. Metatranscriptomic study revealed that periodontitis associated communities have augmented biological processes related to flagellar motility, peptide transport, iron acquisition, β-lactam degradation, lipid A biosynthesis and cellular stress responses. Most widely upregulated function is iron acquisition that the ability to compete for this nutrient may be an important determinant of which species are ultimately able to thrive as biomass accumulates.[82]

HEALTH TO GINGIVITIS: (Fig 6)

The classic experiments of **Loe et al.**[11] demonstrated that without doubt the accumulation of microbial plaque results in the development of

gingivitis 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 models.[2,2,83–86]

Most of the species depleted in gingivitis are characterised by their aerobic or facultative anaerobic metabolism, while most enriched species are anaerobes which suggests the formation of anaerobic niches during biofilm accretion. Most species enriched in gingivitis are gram negative. Species which are found to be depleted in gingivitis are Actinomyces naeslundi, Capnocytophaga sputigena, Haemophilus parainfluenza, Kingella oralis, Rothia aeria, streptococcus mitis, Streptococcus sanguinis.

GINGIVITIS TO PERIODONTITIS: (Fig 6)

Listgarten et al (1985)[87] performed a longitudinal study of the periodontal status of 69 adult subjects 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 **1970, Loe et al**[45] began a longitudinal study among tea laborers in Sri Lanka. with age, there was a significant increase in the amount of attachment loss and in the number of teeth affected. In 1982, Goodson et al. challenged the hypothesis that periodontal disease was a continuous, slowly progressive destructive disease and suggested that it existed as a dynamic condition of

disease exacerbation and remission as well as periods of inactivity for an unknown number of weeks or months.

Lindhe et al[88] observed that the loss of attachment between baseline, 3 and 6 years exhibited an annual rate of 0.2 mm 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.

Birkedal-Hansen et al (1994) published an excellent review of the views for and against the burst hypothesis. In healthy sites the microbial load is low. Isolates may be cultured from an individual healthy sulcus[89] consisting of mostly grampositive streptococci (e.g., Streptococcus gordonii) and Actinomyces with about 15% gram-negative rod species, including Fusobacterium nucleatum. In contrast, in periodontitis, the microbial load is higher, and there is an increase in the number of gram-negative organisms (15–50%)[22,89] when compared to clinically healthy sites.

Chronic periodontitis:

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.[44] **Keyes and Jordan, 1964** demonstrated the infectious nature of periodontitis by its transmissibility in animal models.[47]

Chronic periodontitis clinically present as the loss of clinical attachment of the periodontal ligament to the root surface. This clinical attachment loss can either present as a periodontal pocket or gingival recession alone. Periodontal pocket is the result of apical migration of the apical cells of junctional epithelium while the gingival margin remains unchanged or more coronal to the base of the pocket. Gingival recession in the apical migration of marginal gingival tissue along with the base of the sulcus.[90]

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 commonly associated with chronic periodontitis are *Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia, Campylobacter rectus, Eikenella corrodens, Fusobacterium 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 Fusobacterium nucleatum***.**[91]

Gingival recession:

According to **Wensstrom JL**[92] gingival recession refers to exposure of root surface caused by apical displacement of gingival margin beyond cemento-enamel 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 retention and aesthetic dissatisfaction.[93]

The main etiological factors are the accumulation of dental plaque biofilm with the resulting inflammatory periodontal diseases and mechanical trauma due to faulty oral hygiene technique. Three major factors are associated with increased susceptibility to gingival recession: (i) thin gingival tissue; (ii)mucogingival conditions; and/or (iii) a positive history of progressive gingival recession and/or inflammatory periodontal diseases in teeth presenting with either or both of the first two factors.[94]

Sarfati et al[95] reported that gingival bleeding was significantly associated with gingival recession severity and concluded that inflammatory reaction to dental biofilms is the predominant biologic feature shared by gingival recession and periodontitis. In his study, he found that gingival bleeding was significantly associated with severity of gingival recessions (P value = 0.010) and not with extent of gingival recessions, which suggested that extent of recession is related to plaque accumulation and that host response, i.e., inflammation conditions, is a key factor for recession severity.

The National Health and Nutrition Examination Survey (NHANES) III survey estimated that recession ≥3 mm affected 22.5% subjects and 6.5% teeth in United States adults 30 years and older.[96] The same study accessed that the teeth most affected with gingival recession ≥3 mm were the mandibular central incisors and the maxillary first molars.

Gingival recession resulting from well established, regular oral hygiene practices tends to be more frequent and severe in individuals with clinically healthy gingival tissue, little microbial plaque and good oral hygiene. However, there are conditions where patients present with the generalised clinical attachment loss without increase in probing depth accompanied by the accumulation of local factors and gingival inflammation.

Susin et al in 2004,[97] in his study on the prevalence of gingival recession among Brazilian population showed that the high level of gingival recession in this population is primarily related to destructive periodontal disease and is significantly associated with a higher levels of supra-gingival dental calculus and cigarette smoking. **Rios et al**,[98] assessed the risk indicators of gingival recession in Brazillian population and concluded that smoking and calculus are the major risk factora of gingival recession.

Mumghamba et al in 2009,[99] in his study showed that the occurrence of gingival recession was low and was associated with age, presence of calculus and gingival inflammation rather than with tooth cleaning practices since hygiene was very poor despite the high frequency of toothbrushing. However,

certain studies also found that the there is only weak association between the supra-gingival calculus and gingival recession.[100]

Palenstein-Helderman et al,[101] based on his findings, gave the working hypothesis that the longstanding calculus is an important determinant in the onset of gingival recession at sites exhibiting pronounced recession at a young age in populations deprived of prophylactic dental care. He showed that the plaque-associated gingival recession is more associated with the younger age deprived of oral hygiene habits. Lingual surfaces of the mandibular incisors, canine and first premolar and al the buccal surfaces of the mandibular incisors have the highest correlation with the plaque induced gingival recession.

VIRULENCE FACTORS

"Virulence" in Latin means "full of poison". Molecular components that are present in micro-organisms that can cause harm to host, in the absence of which their pathogenicity is impaired or reduced. Virulence factor means the properties of micro-organism that enable it to cause disease or interfere with metabolic or physiologic functions of the host.

Holt and Bramanti et al,[102] **in 1991** given the establishment of bacterial infection require 5 integrated events,

1. Initial colonization in tissue surface
2. Penetration of this surface directly or indirectly

3. Emergence and multiplication of invading bacteria
4. Eventual damage of host tissue
5. Survival of invading bacteria by evading host response.

According to **Holt and Ebersole,**[103] virulence factors can have a multitude of function.

1. Ability to induce microbe-host interaction (Attachment)
2. Ability to invade the host
3. Ability to grow in the confines of host cell
4. Ability to evade host defence

The early colonizers in the biofilm are chiefly involved in the formation of supragingival plaque and are mostly Gram positive cocci. They are involved in the development of initial immune response and in later stages, gingival inflammation. These organisms are however important in the pathogenesis of periodontal diseasesas they create an environment for the later organisms to colonize.[104]

These climax colonizers majorly consists of organisms that belong to red complex bacteria as described by **Socransky et al.**[54] These climax colonizers are considered to have virulent factors that lead to the inflammation. Red complex organisms includes *Prophyromonas gingivalis, Treponema denticola* and *Tanerella forsythia*. They possess these molecular factors that enable them to function as the putative pathogens.

ADHESINS:

These are molecular factors that are present in bacteria that helps it to adhere to host surface – tooth and gingival surface and to other micro-organisms present in the biofilm. Adhesion of a bacteria to other micro-organisms in the biofilm is called co-aggregation. Co-aggregation may occur among early colonizers or with the bridging organisms like *F. nucleatum* or with ther gram negative bacilli and late colonizers.

P. gingivalis possesses surface molecules like fimbriae, hidden receptors called 'cryptitopes' that play an important role in adhesion to host and co-aggregation. *T. denticola* has adhesins such as bacterial pole, collagen binding protein, major sheath protiens for its adhesion to the ECM protiens and leucine rich repeat protiens (LrrA) helps in co-aggregation. *T. forsythia* has lipoproteins for its adhesion.

INVASIN:

Invasion into the gingival tissue is the hallmark of putative periodontopathogens. Fimbriae and gingipains of *P. gingivalis* helps in invasion of the organisms into the host cell. Major sheath protiens act as an invasion for *T.* denticola and is a motile organism. Invasion of *T. forsythia* is aided by surface lipoproteins.

SUSTENINS

As larger number of organisms multiply within the tissues, the rate limiting step is the availability of nutrition to the multiplying bacteria. These demands are met by special molecular determinants collectively called sustenins. Therefore *P. gingivalis* is equipped with specific cysteine protease called gingipains, collagenases, and other peptidases. *Porphyromonas gingivalis* also contain siderophores that requires iron from the environment and *Porphyromonas gingivalis* ferritin for iron storage.

T. denticola contains cystalysin for heme regulation while *T. forsythia* is equipped with enzymes like proteinases, glycosidases and sialidase as a sustenin. The end product of these organisms viz volatile sulphur compounds and thereby contributes to tissue damage.

EVASIN

Evasion of host immune response is an important part of the strategy of micro-organisms to amplify its pathogenic mechanisms. *P. gingivalis* causes chemokine paralysis and other pathology with its sustenins such as gingipains, lipopolysaccharides, outer membrane protiens and vesicles. Lipopolysachharides of *Treponema denticola* and lipoproteins of *T. forsythia* act as evasins.

HOST-BACTERIAL INTERACTIONS:

The equilibrium between these bacterial components and the host have to be balanced for periodontal health. The host-bacterial interaction initiates the periodontal pathology. 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.

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.[105] 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.[106]

DETECTION METHODS

MICROSCOPIC STUDIES

Theodore Rosebury conducted a series of experiments to isolate bacteria of etiological importance in periodontal disease. **Coyler** had proposed use of dark field microscopy for evaluation of pocket microorganisms.[19] **Keyes**

proposed use of phase contrast microscopy in periodontal diagnosis to identify bacterial morphotypes.[47] Microscope techniques were reasonably rapid, but limited in the precision of identification of individual bacterial species.

CULTURE-DEPENDENT APPROACHES

Culture-dependent approaches to identify periodontal micro-organisms involve growing the microorganisms on defined media, followed by identification based on phenotypic and biochemical characteristics, differential staining methods, metabolic end-product analysis and cell-membrane composition of the organism.[19] **Moore & Moore** examined the composition of subgingival plaque samples in healthy periodontium and various sites with periodontal disease employing cultural techniques.[107] He examined over 600 periodontal sites with thousands of bacteria.

The main drawback of culture method is its narrow spectrum, and it is regarded as a time-consuming, labor-intensive, and expensive undertaking because only few plaque samples in small numbers of subjects can be examined. It have also been estimated that 50% to 60% of bacteria in oral cavity are still uncultivable.[108,109] However, cell culture is still essential to assess bacterial sensitivity to antibiotics and for verifying presence of known species.

Currently, the culture-dependent approach may involve extracting nucleic acid from a single colony, cloning the sequence into a plasmid vector, sequencing the ribosomal RNA genes and identifying the sequence using a

ribosomal RNA database. The extracted r-RNA sequences can be also used to identify microorganisms through terminal restriction fragment length polymorphisms, denaturing gradient gel electrophoresis, hybridization to micro and macroarrays and quantification by rt-PCR.[19]

CULTURE-INDEPENDENT APPROACH

- Checker board hybridization
- PCR
- Pyrosequencing
- Microarray
- NGS

POLYMERASE CHAIN REACTION

Kary Mullis first developed polymerase chain reaction (PCR) technique to amplify specific genes or parts of genes which are then used to identify bacterial species from which they originated.[12] PCR involves 3 steps namely DNA denaturation, primary annealing and extention of primed DNA sequence leading to the amplification of the required nucleic acid sequence using the appropriate primer.

Provided the appropriate primers, PCR is a rapid, simple technique that can detect small numbers of cells of a given species, and indicates the presence or absence of a species in the sample. However, it may not be cost effective for

large sample sizes, and for applications where relative levels of species are low.[110]

CHECKER BOARD HYBRIDIZATION

It is one of the closed ended approach for the molecular analysis to detech micro-organism based on hybridization of target species to labeled genomic DNA that has been attached to nylon membranes. Checkerboard technique is rapid, sensitive, and relatively inexpensive but is also dependent on culture technique to cultivate the target species for creating genomic probes. It provides a major benefit for studies of oral microbial ecology due to advantages like detection of multiple species from each sample simultaneously, and study of large sample size for large numbers of species.

Socransky et al[111] used DNA-DNA checker board analysis to enumerate the micro-organisms in periodontal ecosystem. The technique can detect only species for which DNA probes have been prepared. Studies by Loesche WJ et al,[49] Haffajee,[112] Ximenez-Fyvie et al,[113] Feres et al,[114] also reported bacterial species in a limited quantities in periodontal disease conditions.

OPEN-ENDED APPROACH

16S rRNA sequencing analysis

Next generation sequencing

16S rRNA sequencing analysis

In recent decades, in bacterial taxonomy 16Sr RNA gene sequencing & phylogenetic analyses have been increasingly applied. Universal distribution is seen in 16S r RNA. 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**.[115]

DNA and protein sequencing started in the 1970s when the virus Lambda (50,000 nucleotides) was sequenced by **Sanger et al**.[116] Sanger technique is use by **Frederick Sanger** and colleagues described the use of chain-terminating dideoxynucleotide analogs that caused base-specific termination of primed DNA synthesis.[110]

Next generation sequencing

Next generation sequencing methods employ a wide spectrum of technologies such as sequencing by synthesis, sequencing by ligation, single molecule DNA sequencing and colony sequencing. NGS is performed by repeated cycles of polymerase-mediated nucleotide extensions or by machinery automated cyclical ligation of oligonucleotides.

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**.**[117]

In a single machine run there will be an 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.[17]

Roche/454 FLX**,** the Illumina/ Solexa Genome Analyzer and the Applied Biosystems / SOLID are three main NGS technologies. HiSeq and the Ion Torrent Personal Genome Machine (PGM) are some NGS platforms that are being used for remarkable data output.

ILLUMINA SOLEXA GENOME ANALYZER

In 1997, British chemists Shankar Balasubramanian and David Klenerman conceptualized an approach for sequencing single DNA molecules attached to microspheres and founded Solexa in 1998. The Solexa Genome Analyzer, the first ―short read sequencing platform, was commercially launched and acquired by Illumina in 2006. Genome Analyzer uses a flow cell with bound oligonucleotide anchors wherein template DNA is fragmented into

several hundred base pairs and end-repaired.[17] Schematic representation of Solexa approach is given in fig 7.

The advantage of Solexa system is that it can generate 1.5 GB of sequence per run with read lengths that range from 35 to 100 bases and each run requires 3–5 days to complete.[118] A technical concern of Illumina sequencing is that base-call accuracy decreases with increasing read length primarily due to dephasing noise which occurs when a complementary nucleotide is not incorporated or when fluorophore is not properly cleaved at the end of cycle, thus blocking incorporation of next nucleotide base.[17] As a consequence, the sequence is out-of-phase for remainder of the template.[119] Another shortcoming is that short read lengths tend to produce biased sequence coverage that occurs in AT-rich repetitive sequences.[20]

NGS and periodontal disease:

Liu et al in 2012[120] have demonstrated that the subgingival microbiome can be effectively interrogated through high-throughput sequencing (NGS technology), and that the resulting data provide valuable insights into the molecular underpinnings of periodontal disease.

Bizzarro et al in 2013[15] studied the subgingival microbiome of smoker and non-smoker in periodontitis using next generation sequencing and found that genera *Fusobacterium, Prevotella* and *Selenomonas* were more abundant in smokers, while the genera *Peptococcus* and *Capnocytophaga* were more

abundant in non-smokers. He concluded that there is no significant difference in the microbial composition between the groups. Low taxonomic diversity was associated with higher disease severity, especially in smokers. This supports the hypothesis of the ecological microbial–host interaction in the severity of periodontal disease.

HUMAN ORAL MICROBIOME DATABASE

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.[121] 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.

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. Most uncultivable bacteria are identified via 16S rRNA technique and included to the database.

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.

The basic list of oral bacteria came from the literature works of **Sockransky,**[122] **Tanner,**[18,123] **Moore WE,**[107] and **Dzink JL.**[91] In 2010, **Dewhirst et al** identified 1,179 taxa which included cultivable, non-cultivable, named & unnamed phyla.[3] Upon validation, 434 novel non-singleton taxa were added to the HOMD.

ROLE OF SALIVA:

Saliva is a biofluid comprising secretions of the salivary glands, oral mucosa cells, blood and gingival crevicular fluid. Saliva contains a variety of biomolecules, including DNA, mRNA, microRNA, proteins, metabolites and microbiota; changes in the salivary concentration of these biomolecules can be used to develop dysregulated biomarkers to help identify early oral and systemic

diseases, evaluate disease prognosis and risk and monitor the response to treatment.[26] The knowledge about the various constituents in saliva, including the genome, epigenome, transcriptome, proteome, metabolome and microbiome is termed "Salivaomics".[124]

An argument made in UK biobank in the bid to include the collection of saliva was the possibility that mechanistic links exist between oral and systemic diseases. A hypothesis has emerged, in recent years, of a 'shared pro-inflammatory phenotype' that increases susceptibility to a whole range of inflammatory diseases.[125] As saliva is an easily accessible and economical biological fluid, saliva has been thoroughly analysed for biomarkers of health and disease over the past decade**.** Saliva also serves as a platform for personalized medicine.[126] A multi-centre study is currently validating a 'signature' of 7 mRNAs in saliva for the detection of oral cancer.[127]

Table 1. Major salivary gland secretion mediators associated with periodontal diseases

Marker	Relationship with periodontal disease	Type of periodontal disease
Specific		
Immunoglobulins (IgA, IgM, IgG)	Interfere in adherence and bacterial metabolism / increased concentration in saliva of periodontal patients	Chronic and aggressive
Nonspecific		
Mucins	Interfere with the colonization of *Aggregatibacter actinomycetemcomitans*	Aggressive
Lysozyme	Regulates biofilm accumulation	Chronic
Lactoferrin	Inhibits microbial growth / increased correlation with *A. actinomycetemcomitans*	Aggressive
Histatin	Neutralizes lipopolysaccharide and enzymes known to affect the periodontium	Chronic and aggressive
Peroxidase	Interferes with biofilm accumulation / increased correlation with periodontal patients	Chronic
Systemic		
C-reactive protein	Increased concentration found in serum and saliva of periodontal patients	Chronic and aggressive

(Giannobile, 2009)

Paju et al[28] studied 1198 adults of Finnish population with saliva as a diagnostic medium for oral microbiome analysis using PCR and found that the

periodontal pathogens including the red complex organisms along with *Prophyromonas intermedia, Aggregatibacter actinomacetemcomitans, Camphylobacter rectus* are increased in number and are more associated with the severity of periodontitis.

Mager et al[27] used checkerboard DNA–DNA hybridization to evaluate the oral microbiota in saliva from patients with oral squamous cell carcinoma and healthy subjects and found a combination of three microbiotas (*Capnocytophaga gingivalis, Prevotella melaninogenica* and *Streptococcus mitis*) that could be used as diagnostic biomarkers with 80% sensitivity and 82% specificity.

Mager and Haffajee et al in 2005[27] did the salivary microbial analysis of 40 common oral bacteria among the oral squamous cell carcinoma patients and the controls and found the elevated levels of *Capnocytophaga gingivalis, Porphyromonas melaninogenica* and *Streptococcus mitis* and concluded that these organisms can be used as the diagnostic indicators of OSCC. Diagnostic sensitivity and specificity in the matched group were 80% and 82% respectively.

B Shi et al[14] aimed to determine the dynamic changes in the subgingival microbiome in periodontitis patients before and after treatment at the same tooth sites can serve as a diagnostic and prognostic indicator. 38 genera that had an abundance of more than 1% were identified, Prevotella and Fusobacterium being the most abundant genera. Their results suggested that Synergistetes,

Filifactor and Mycoplasma should be considered expanded members of the red complex.

Hui Zheng et al[13] performed a study to analyze the microbial characteristics of oral plaque from peri-implant pockets of 10 healthy implants, 8 peri-implant mucositis sites and 6 peri-implantitis sites 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 *Porphyromonas gingivalis, Tannerella forsythia and Prevotella intermedia* were clustered into modules in the peri implant mucositis network.

Payungporn et al[128] conducted a study to identify potential bacterial species associated with periodontal disease in ten Thai patients within the age group of 43 to 53 years, of which 5 were from healthy controls and 5 were patients with chronic periodontitis. It was observed that *Porphyromonas gingivalis and Prevotella intermedia* were significantly associated with periodontal disease, whereas other bacteria like *Treponema denticola, Treponema medium, Tannerella forsythia, Porphyromonas endodontalis* and *Filifactor alocis* may be potentially associated with periodontal disease in Thai patients.

ROL Figures

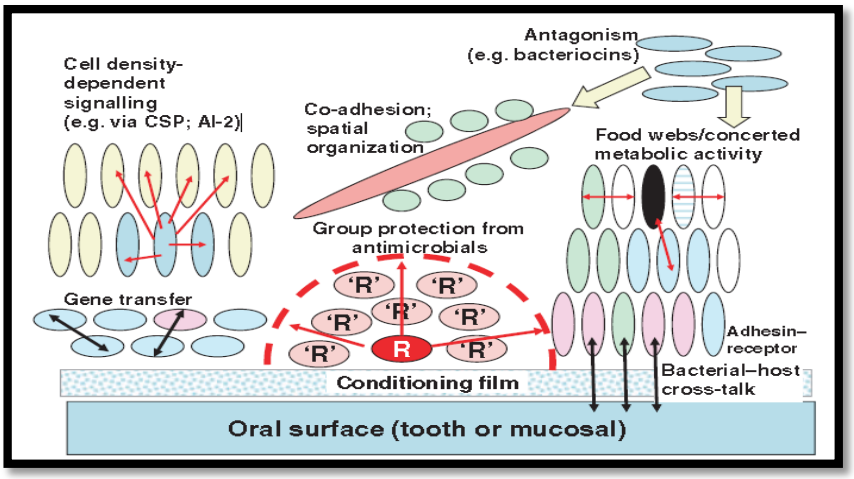


Figure 1: Schematic representation of the types of interactions in biofilm

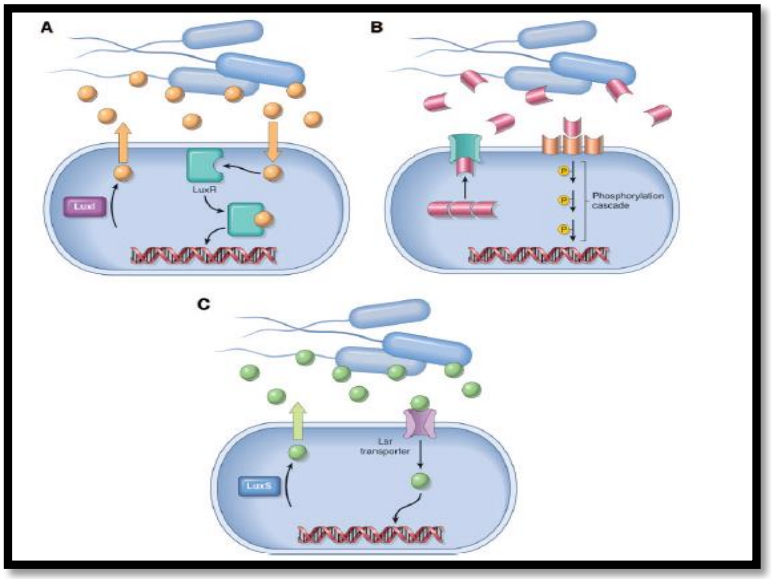


Figure 2: Summary of Quorum Sensing

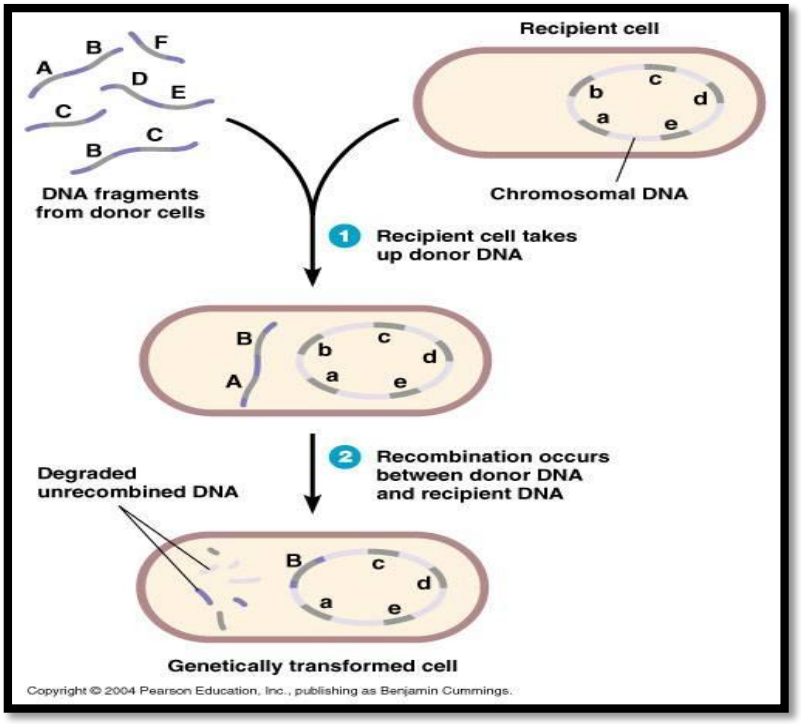


Figure 3: Schematic representation of transformation

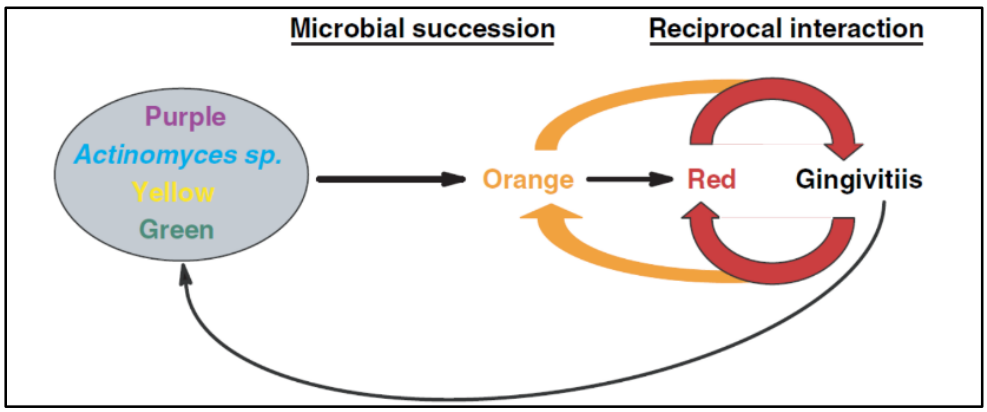


Figure 4: Microbial Succession

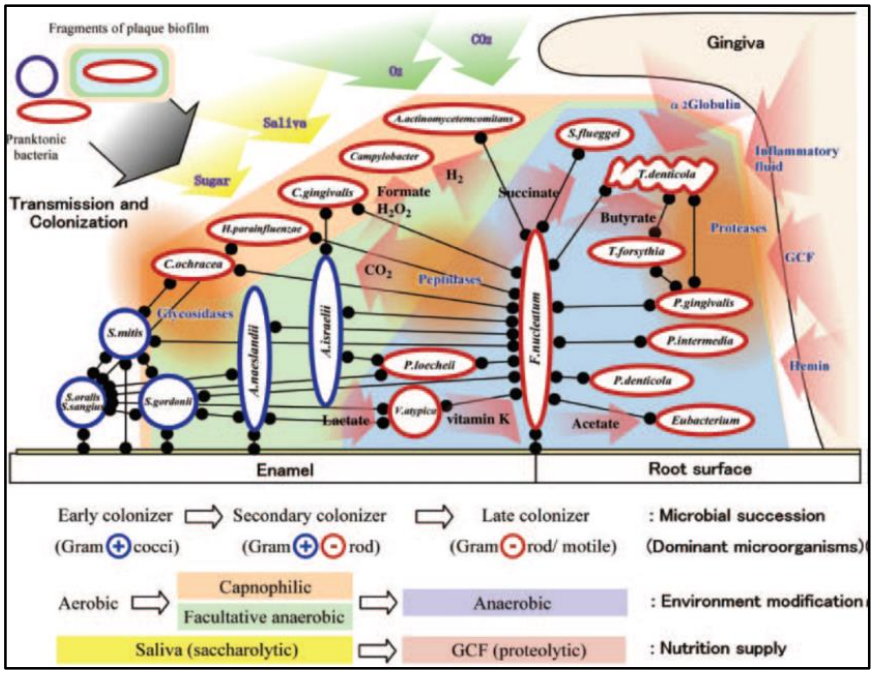


Figure 5: Early & Late colonization

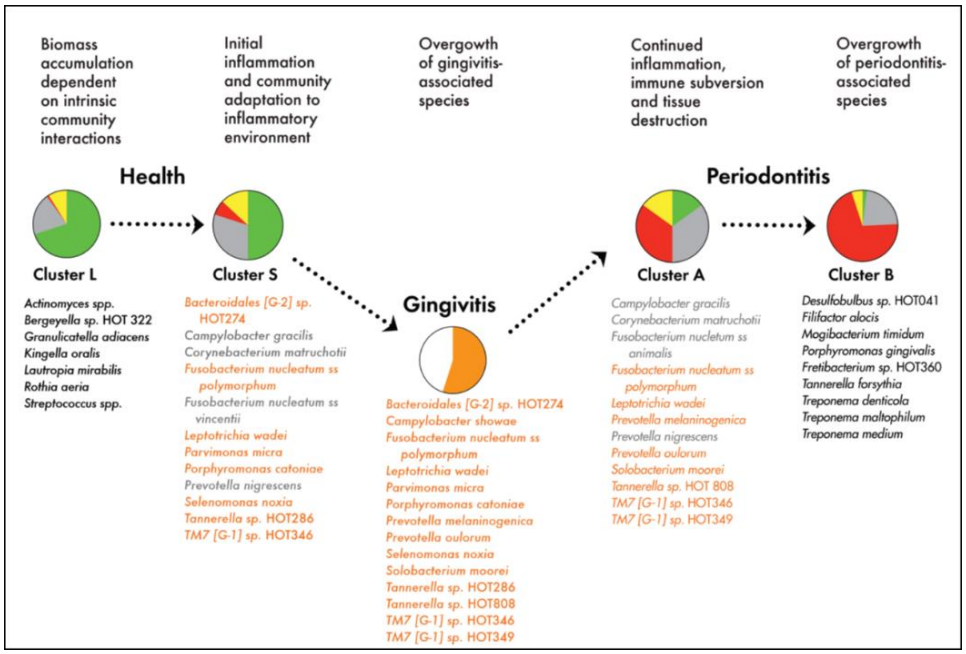


Figure 6: Bacterial communities in health, gingivitis & periodontitis – Hong et al

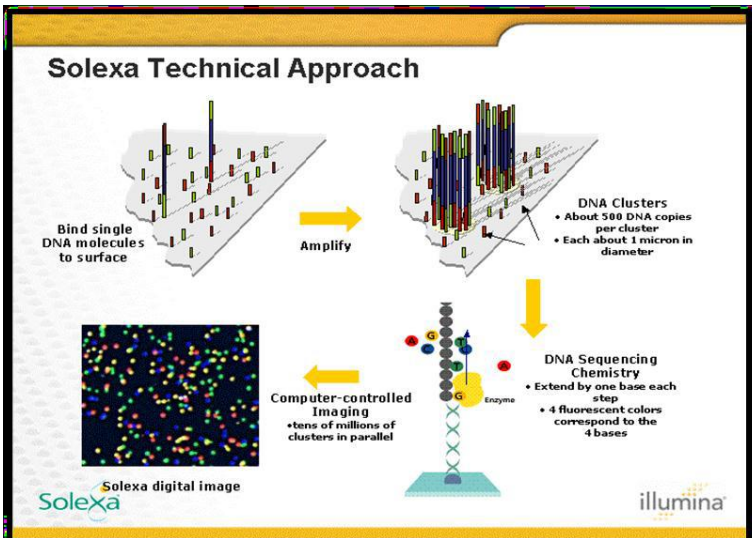


Figure 7: Illumina Solexa Genome Analyzer

Materials & Methods

MATERIALS & METHODS

A total of 30 individuals seeking dental treatment in Ragas Dental College and Hospitals, Chennai, were included in the present study. Certificate of ethical clearance for the study was obtained from Institutional Review Board of Ragas Dental College. 30 patients included were segregated into 3 groups of which 10 were periodontally healthy individuals (control group), 10 were patients with gingivitis on intact periodontium (test group 1) and 10 were patients who has gingival recession with a band of calculus (test group 2). A diagnosis of periodontal health, gingivitis and periodontitis was determined based on the American Academy of Periodontology parameters & new classification 2018.

INCLUSION CRITERIA

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 generalized bleeding on probing, PPD ≤ 3 mm, with no attachment loss or radiographical bone loss.

Test group 2 consisted of 10 subjects with PPD ≤ 3mm and heavy band of calculus along with attachment loss & radiographical bone loss 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.

EXCLUSION CRITERIA

- Patients 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 examiner using calibrated probe. The salivary samples, from the individuals included in all 3 groups, were collected in a sterile salivary tubs. Unstimulated whole saliva was collected in the morning and subjects were 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:

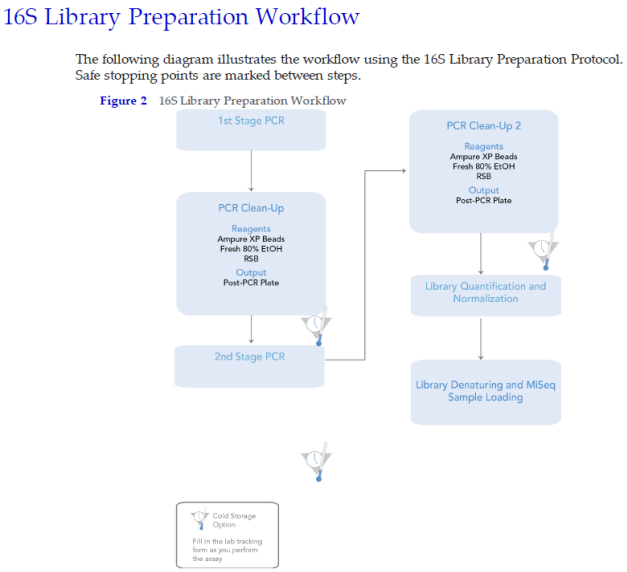
Genomic DNA was extracted from 30 saliva samples of gingival health and disease patients with the Qiagen powersoil kit according to manufacturer's recommendations.

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

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



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 were done. A comprehensive workflow for 16S rRNA amplicon sequencing is depicted as follows.

Workflow Summary:

1) Ordering amplicon primers–The protocol included the primer pair sequences for the V3 and V4 region that created a single amplicon of approximately ~460 bp. The protocol also included overhang adapter sequences that appended the primer pair sequences for compatibility with Illumina index and sequencing adapters.

2) Preparation of library–The protocol described the steps to amplify the V3 and V4 region and using a limited cycle PCR, addition Illumina sequencing adapters and dual-index barcodes are added to the amplicon target. Using the full complement of Nextera XT indices, up to 96 libraries were pooled together for sequencing.

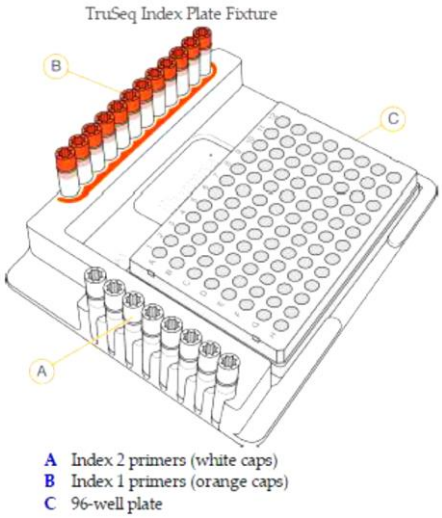
3) Sequencing on MiSeq–Using paired 300-bp reads, and MiSeq v3 reagents, the ends of each read were overlapped to generate high-quality, full-length reads of the V3 and V4 region in a single 65-hour run. The MiSeq run output was approximately > 20 million reads and assuming

96 indexed samples, could generate > 100,000 reads per sample, commonly recognized as sufficient for metagenomic surveys.

4) Analyzation 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 Greengenes database and showed 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:

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 were denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq sequencing. Each run included a minimum of 5% PhiX to serve as an internal control for these low diversity libraries. Illumina recommended using MiSeq v3 reagent kits for improved run metrics.

MiSeq Reporter Metagenomics Workflow:

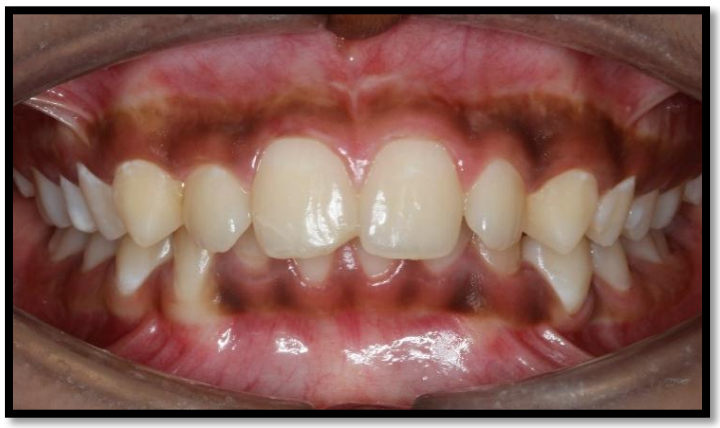
After loading the samples, the MiSeq system provided on-instrument secondary analysis using the MiSeq Reporter software (MSR). MSR provided several options for analysing MiSeq sequencing data. For this demonstrated 16S protocol, appropriate Metagenomics workflow was selected.

By following this 16S Metagenomics protocol, the Metagenomics workflow classified organisms from your V3 and V4 amplicon using a database of 16S rRNA data. The classification was based on the Greengenes database (http://greengenes.lbl.gov/). The output of this workflow was a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species.

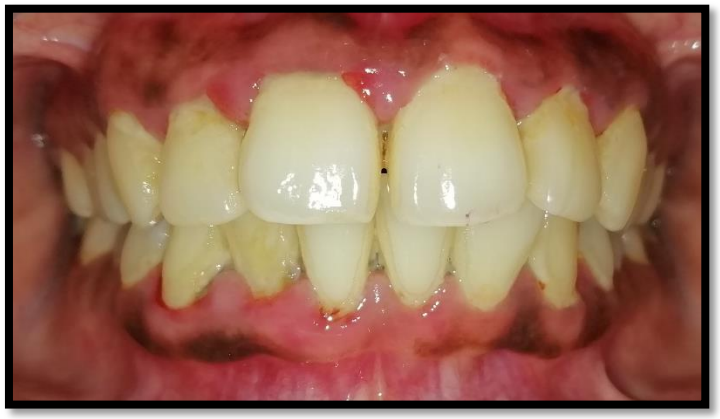
Data analysis was done by using 16s metagenomics tool from Base Space Onsite. Operational taxonomic units (OTUs) were assigned to each sequence using HOMD database.

Statistical analysis was performed for individual bacteria using frequency distribution and intergroup comparison was done using chi square test with statistical significance set as $P < 0.05$.

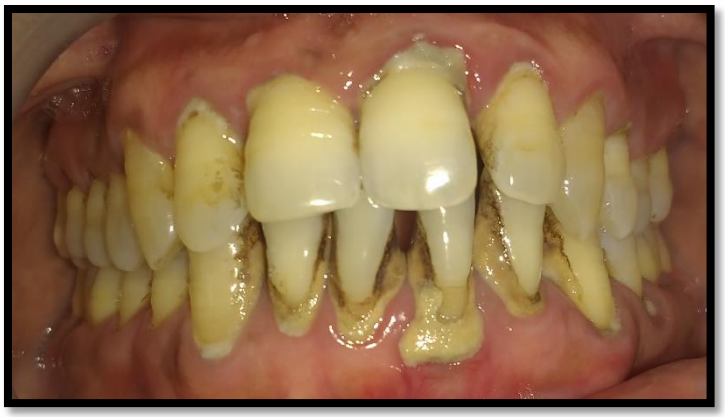
Photographs



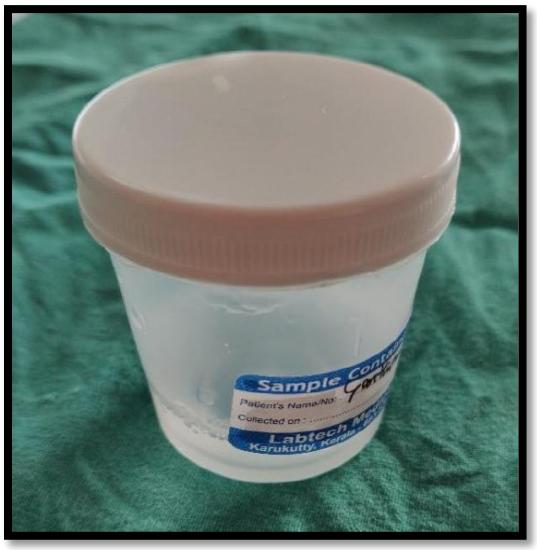
HEALTH



GINGIVITIS



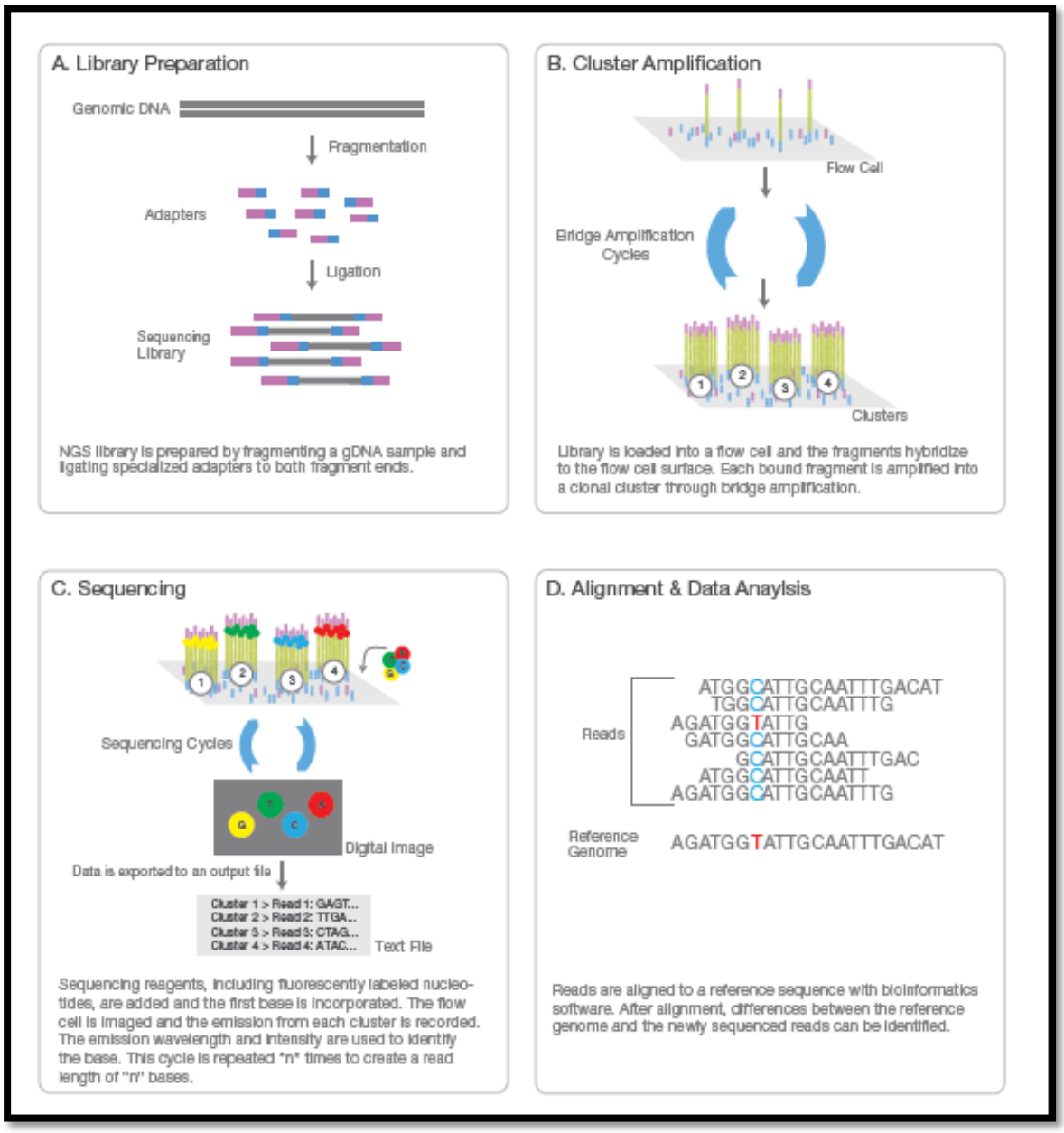
GINGIVAL RECESSION



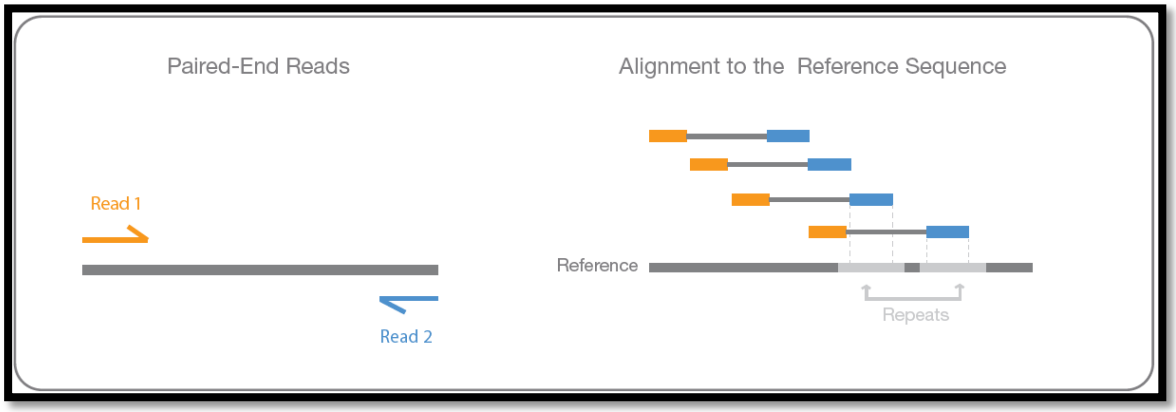
SALIVARY TUB

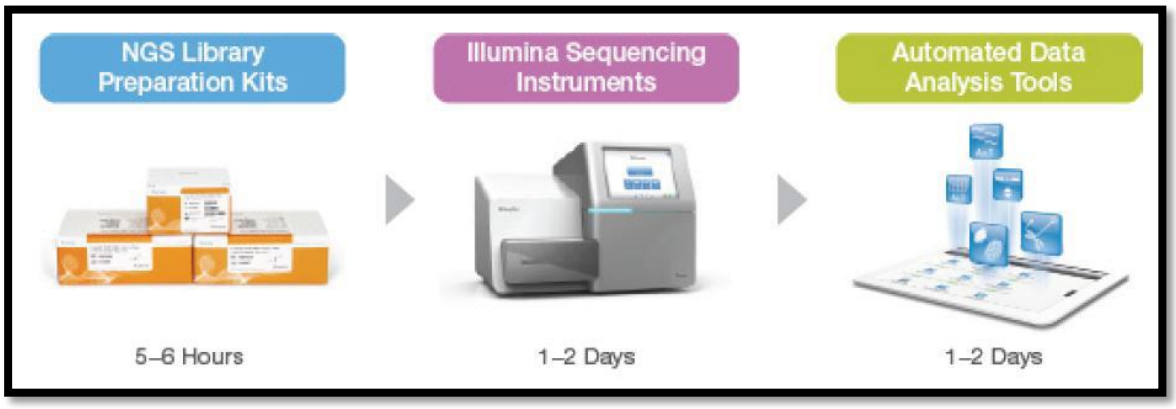
ILLUMINA-SOLEXA

4 BASIC STEPS IN ILLUMINA NGS WORKFLOW

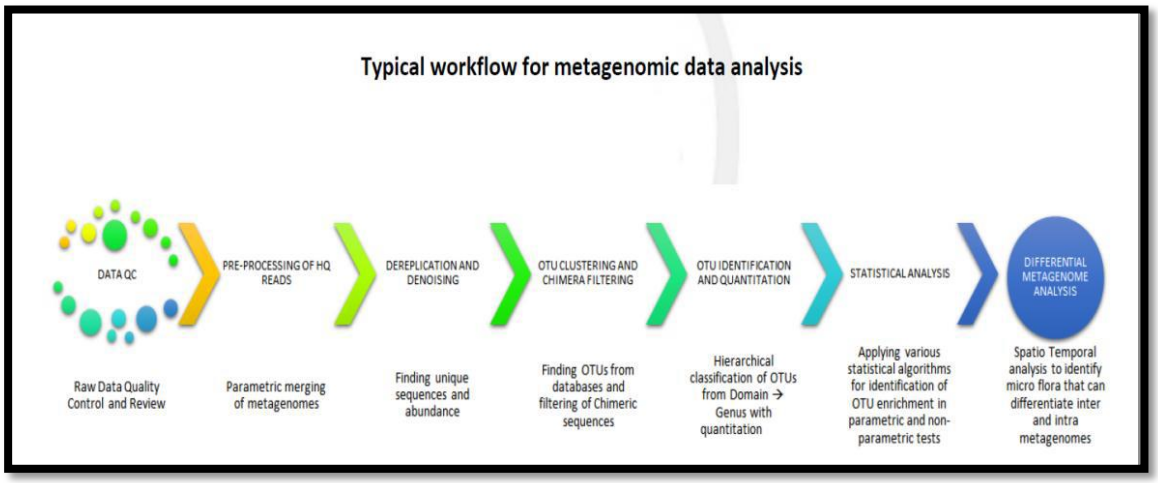


PAIRED-END SEQUENCING & ALLIGNING





WORKFLOW FOR METAGENOMICS DATA ANALYSIS



Results

RESULTS

The present study was carried out in a population of 30 individuals, that is 10 in health and 20 diseased individuals seeking dental treatment in Ragas Dental College and Hospital, Chennai. The age distribution of the study participants ranged from 20-45 years with a mean age of 32.5 years.

Saliva samples were collected in a saliva tub from periodontally healthy individuals (designated as H1 - H10), gingivitis patients (designated as G1 - G10) and from the patients with gingival recession (designated as P1 - P10). The collected samples are then subjected to 16SrRNA sequencing using NGS technology.

In health group, the results have shown that *Porphyromonas gingivalis* was present only in one health sample (H4) and *Tannerella forsythia* was present only in one sample(H7), whereas *Treponema denticola* was not identified in any of the samples. (Table 1)

In gingivitis group, the results have shown that *Porphyromonas gingivalis* was present in 9 of 10 samples obtained (ie., G1, G2, G3, G4, G6, G7, G8, G9, G10) and *Treponema denticola* was present in 8 of 10 samples (ie., G1, G2, G3, G4, G6, G7, G8, G10) while *Tannerella forsythia* was present in all 10 samples. (Table 2)

In periodontitis group, *Porphyromonas gingivalis, Tannerella forsythia* & *Treponema denticola* are present in all 10 samples obtained. (Table 3)

Comparison of frequency distribution of red complex bacteria in gingival health, gingivitis and gingival recession:

In the present study, all 3 red complex organisms had 100% detection frequency in test group 2 (Gingival recession). In the test group 1 (Gingivitis), *Tannerella forsythia* was the most prevalent organisms with a detection frequency of 100% followed by *Porphyromonas gingivalis* with a detection frequency of 90%, followed by *Treponema denticola* with a detection frequency of 80%. (Table 4)

There is a statistical significant increase in the distribution of all 3 red complex organisms in Group 2 - Gingivitis (Table 5) and Group 3 - Gingival Recession (Table 6) with **P value <0.001** when compared to group 1 (Gingival health) while there is no significant difference in the distribution of the organisms between the groups 2 and 3 with P value of *Porphyromonas gingivalis* 0.85 & *Treponema denticola* 0.70. (Gingivitis & Gingival recession). (Table 7)

Graph 1 shows the comparative bar representation of all 3 red complex organisms among 3 groups analysed. Graph 2 is the phylogenetic tree that depicted the salivary microbiome of all 3 groups in the genus level.

Tables and Graphs

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

S.No.	Red Complex	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
1	Porphyromonas gingivalis	-	-	-	1	-	-	-	-	-	-
2	Tannerella forsythia	-	-	-	-	-	-	1	-	-	-
3	Treponema denticola	-	-	-	-	-	-	-	-	-	-

Table-2: **EXPRESSION OF RED COMPLEXES IN GINGIVITIS GROUP**

S.No.	Red Complex	G1	G3	G4	G5	G6	G7	G8	G9	G10
1	Porphyromonas gingivalis	1	1	1	-	1	1	1	1	1
2	Tannerella forsythia	1	1	1	1	1	1	1	1	1
3	Treponema denticola	1	1	1	-	1	1	1	-	1

Table-3: **EXPRESSION OF RED COMPLEXES IN GINGIVAL RECESSION 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	1	1	1
2	Tannerella forsythia	1	1	1	1	1	1	1	1	1	1
3	Treponema denticola	1	1	1	1	1	1	1	1	1	1

Table 4:- **abundance of the red complex organisms in all 3 groups (Group 1 – Gingival Health, Group 2 – Gingivitis, Group 3 – Gingival recession)**

S.NO	Red complex	Abundance	Health	Gingivitis	Gingival recession
1	Porphyromonas gingivalis	1	10%	90%	100%
		0	90%	10%	00
2	Treponema denticola	1	00%	80%	100%
		0	100%	20%	00
3	Tannerella forsythia	1	10%	100%	100%
		0	90%	00	00

Table 5: **Abundance of RED complex organisms in Group 1 and Group 2**

S.NO	Red complex	Health	Gingivitis	Chi square	P value
1	Porphyromonas gingivalis	10%	90%	19.90	<0.001
2	Treponema denticola	00%	80%	23.33	<0.001
3	Tannerella forsythia	10%	100%	21.90	<0.001

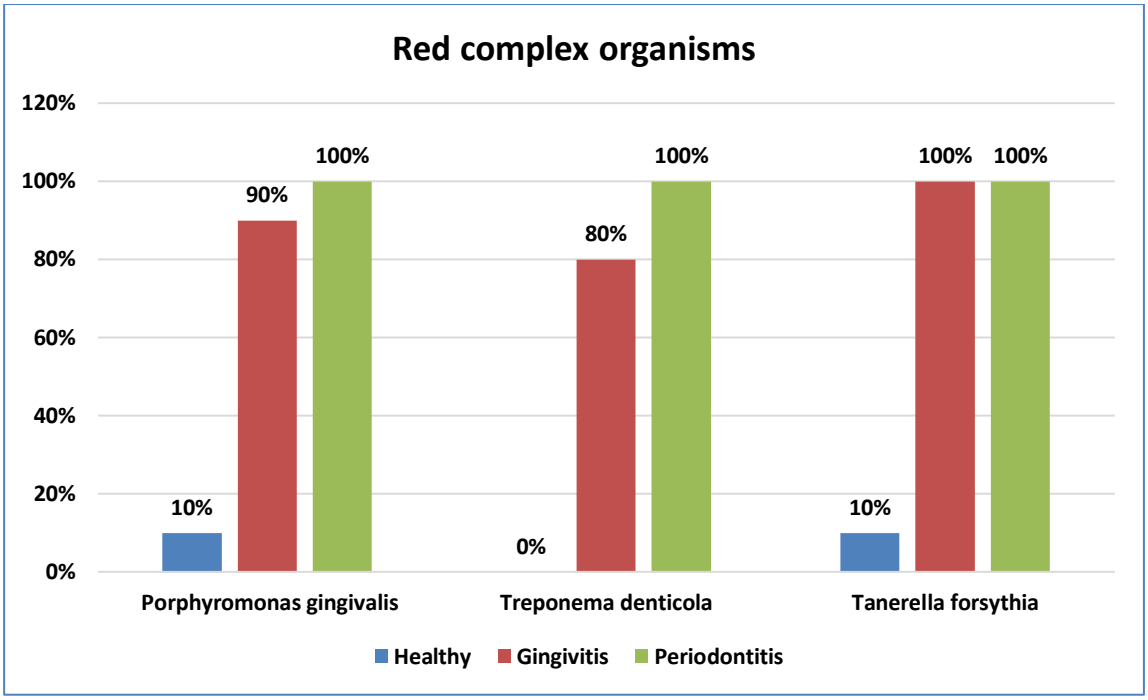
Table 6: **Abundance of RED complex organisms in Group 1 and Group 3**

S.NO	Red complex	Health	Gingival recession	Chi square	P value
1	Porphyromonas gingivalis	10%	100%	21.90	<0.001
2	Treponema denticola	0	100%	23.33	<0.001
3	Tannerella forsythia	10%	100%	21.90	<0.001

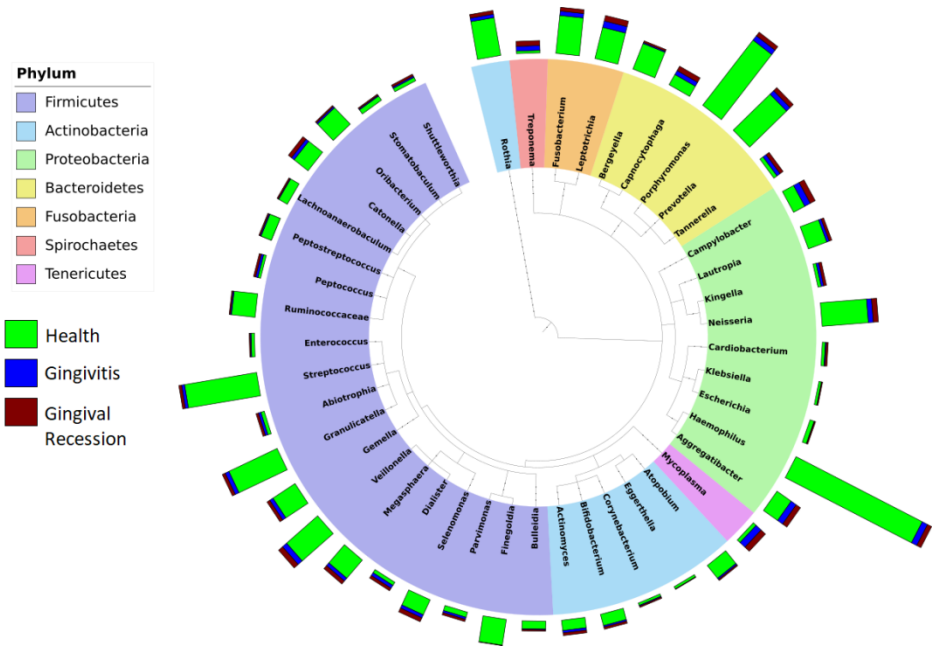
Table 7: **Abundance of RED complex organisms in Group 2 and Group 3**

S.NO	Red complex	Gingivitis	Gingival recession	Chi square	P value
1	Porphyromonas gingivalis	90%	100%	1.90	0.85
2	Treponema denticola	80%	100%	1.12	0.70
3	Tannerella forsythia	100%	100%	*	*

Graph 1: **Frequency distribution of RED complex organisms in Gingival health (Group 1), Gingivitis (Group 2) and Gingival recession (Group 3)**



Graph 2: **PHYLLOGENETIC TREE**



Discussion

DISCUSSION

Periodontitis is a polymicrobial disease characterized by chronic inflammation associated with changes in microbiome. The microbe survive in the subgingival environment in symbiosis. The disease sets in when there is a dysbiotic environment leading to increase in the pathologic microbial load and its by-products. Microbial changes contribute to the disease progression by altering the host-microbe symbiotic relationship.[129] Therefore, microbial analysis aids more in the understanding the disease progression and may be a promising approach for the diagnosis.

There is a voluminous amount of data regarding the microbiome in the periodontal pocket[21,22] while there is only minimal importance given to the microbiome present in gingival recession resulting from accumulation of local factors. The gingival margin migrates apically exposing the deposited subgingival calculus and its associated plaque, supragingivally. Our study analyses the microbial variation of red complex bacteria in gingival recession sites with that of healthy controls. This study also aims to detect the presence of salivary red complex bacteria in gingivitis and compare it with healthy and gingival recession sites.

In recent years, open ended methods like Sanger sequencing, pyro sequencing and deep sequencing of 16s rRNA has allowed the extensive characterization of the microbial communities in a high-throughput manner. Currently, the Next Generation Sequencing is a landmark in the development of

sequencing techniques and has led to significant improvements in the depth and scale of 16s rRNA sequencing.

Our study is done on a device of Next generation sequencing - Illumina/Solexa. It is the first generation NGS that requires the fixation of DNA for analysis. The strength of the Solexa system is that it can generate 1.5 gb of sequence per run. A weakness of the Solexa system is that it tends to produce biased sequence coverage that occurs in AT-rich repetitive sequences, presumably because of short read lengths. However, this technique is advantageous as it can detect previously unknown microbes including those which are non-cultivable.[19]

The major advantage of NGS are high throughput and the fact that specific taxa do not need to be targeted. The quality and the interpretation of the NGS data could be undermined at numerous steps. Making sense out of this data deluge is and will be the major challenge. This wide quality of microbial data also comes with the limitation of cost factor.

V3-V4 region of 16S rRNA was used in this study because though V4 region provides full overlap of two reads and reduces noise in sequencing data thus preventing OTU inflation, there is only less information contained in V4 region owing to its length (~255 base pairs). A longer fragment such as V3 which spans multiple hypervariable regions is most suitable for distinguishing all bacterial species to genus level.[17]

The earliest studies proposed that the development of periodontitis was associated with an increase in the microbial load.[128,114] The subgingival

microbial composition shifts from a population dominated by gram positive microorganisms to one with increased number of gram negative anaerobes. However, these studies have been based on microbiologic study methods like culture, immuno diagnostic methods, DNA-DNA hybridization technique etc. The DNA-DNA checkerboard analysis have shown the presence, levels and properties of certain specific species associated with the classic clinical sign of periodontal disease and were termed as red complex organisms.

Red complex organisms, which was coined by **Sockransky,** include *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*. These are gram negative, anaerobic bacteria that are known to produce a range of virulence factors that aid them in survival in the host and induce host inflammatory mediators leading to connective tissue breakdown and bone resorption.[130] The virulence factors produced by *P. gingivalis* include cysteine proteases such as gingipains, hemagglutinins, lipopolysaccharides, fimbriae, and heat shock proteins. Some of the factors responsible for the pathogenicity of T. forsythia are proteases, sialidases, hemagglutinins, and surface-layer associated glycoproteins.[131] T. denticola is known to produce dentilisin, major outer sheath protein OppA, cystalysin, and heme binding proteins as virulence factors.

These bacteria are not usually found alone, but in combination in the periodontal pockets, suggesting that some bacteria may cause the destruction of the periodontal tissue in a cooperative manner.[55] Studies have also shown that

interspecies interactions between these bacteria occur in vivo indicating nutritional interdependency and modulation of virulence factors of each other.[103,132]

In a study previously done in our department, we have established distinct microflora in health and periodontal pocket with respect to red complex organisms.[133] This study was aimed to establish if the change in red complex occurs in gingivitis level before the transition to periodontitis. This study was also aimed at detecting the red complex bacteria at recession sites that occur as a phenotypic expression of periodontitis (with the accumulation of local factors, gingival inflammation and loss of attachment), and not those caused due to developmental factors or toothbrush trauma.

The salivary microbial analysis is depicted in a phylogenetic tree (Graph 2). There was a distinct microbial profile observed in disease groups compared to health at genus level.

At species level, the present study results showed absence of red complex bacteria present in the health samples with the exception of *Tannerella forsythia* and *Porphyromonas gingivalis* in one sample each. These result are by and large comparable with the study by **B Leblebicioglu et al.**[134]

These bacteria were however present in almost all of the gingivitis samples examined in the study save for one or two. *Tannerella forsythia* is most prevalent red complex organism in gingivitis with 100% followed by *P.*

gingivalis with 90% and *T. denticola* with 80% prevalence. There was a statistically significant increase in the prevalence of red complex bacteria in the gingivitis group when compared to healthy controls [*Tannerella forsythia* p<0.001, *Porphyromonas gingivalis* p<0.001 and *Treponema denticola* p<0.001].This is similar to the results of that of **Liu B et al,**[120] **Wang J et al**[135] and **Belstrom.**[29]

The red complex bacteria were found in all 10 gingival recession samples examined. There was a statistically significant increase in the recession group when compared to health with the p value <0.001 for *T. forsythia, P. gingivalis* p<0.001, *T. denticola* p<0.001. However, there was no statistically significant difference in the prevalence of red complex bacteria between the test groups ie., gingivitis and gingival recession [*T. forsythia* present in all 20 samples of both groups, *P. gingivalis* p=0.85, *T. denticola* p=0,70].

Our results are in accordance with the results found in the plaque samples of gingival recession studied by **Bassir et al**[136] who reported *Porphyromonas gingivalis* and *Tannerella forsythia* at recession sites. However contrary to his study, our results showed that in addition to *Porphyromonas* and *Tannerella*, *Treponema denticola* was also found in most samples of gingival recession.

Porphyromonas gingivalis has been proposed to play a central role in progression of human periodontitis and thus is classified as a “keystone

pathogen" which influences composition of oral microbiome even when present at low levels.[55]

Many earlier studies showed the synergistic action of *Porphyromonas gingivalis* and *Tannerella forsythia* in the disease progression. *Porphyromonas gingivalis* and *Tannerella forsythia* were found to possess fimbriae with which it adheres to the other bridging organisms and a few initial colonizers. Pathogenesis synergism between *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in animal models was reported by **Ebersole et al,**[137] **Feuille et al,**[138] and **Metzer el al**[139] in animal models. They showed that *Porphyromonas gingivalis* with its trypsin-like enzyme activity is capable of causing abrogated tissue destruction either alone or in combination with other red complex organisms and *Fusobacterium nucleatum.*

The salivary red complex bacteria being significantly increased in the gingivitis group may lead to the hypothesis that the increase in salivary red complex bacteria in the gingivitis level itself may contribute to the Dysbiosis that is associated with pocket formation.

The lack of difference between the microflora of gingivitis & gingival recession may be a result of plaque environment being pretty much the same in both groups.

The microflora present in saliva may / may not directly contribute to the periodontal disease activity in individual sites. The red complex bacteria are by

nature Gram –ve anaerobic organisms, which potentially do not thrive well in aerobic environment such as those that are present in saliva. So, salivary organisms are thought to have originate from plaque.

Recent evidence suggests that translocation from plaque may not be in the form of individual planktonic organisms.[21] It may be possible that the migrating microbes exist in the saliva making it possible for the normally anaerobic bacteria to survive in salivary environment.[21] This translocation of the microbiome through saliva may lead to further disease activity increasing the aerotolerence of the red complex organisms.[21] However, further studies with greater sample size are required for the confirmation of this finding.

Limitations of the study:

This study considered only red complex organisms without its synergisms with other initial colonizers or bridging organism. This study is carried out in a small sample, in a cross-sectional manner. Within the limitations of this study, the red complex organisms were found to be significantly increased in the saliva of patients with gingivitis and gingival recession when compared to the controls with no significant difference between the disease groups.

Summary & Conclusion

SUMMARY AND CONCLUSION

The current study aims to evaluate the red complex organisms in saliva of individuals with gingivitis, gingival recession and healthy periodontium. 30 samples (10 samples each). Unstimulated whole saliva was collected from individuals with gingivitis, gingival recession and healthy periodontium. Analysis of red complex in the collected saliva samples was done with NGS technology using Illumina/Solexa sequencing.

There was a statistically significant increase in the prevalence of the red complex bacteria in the gingivitis when compared to the healthy controls [*Porphyromonas gingivalis* P<0.001, *Treponema denticola* P<0.001 and *Tannerella forsythia* P<0.001] and a statistically significant increase in gingival recession when compared to healthy controls [*Porphyromonas gingivalis* P<0.001, *Treponema denticola* P<0.001 and *Tannerella forsythia* P<0.001]. However, there was no statistically significant difference in the prevalence of these organisms between the gingivitis and gingival recession groups [*Porphyromonas gingivalis* P=0.85, *Treponema denticola* P=0.70 and *Tannerella forsythia* present in all 20 samples].

These results suggest that there is a strong association between the salivary red complex organisms and periodontal disease, perhaps making them suitable candidates as risk markers. However, a longitudinal study with greater sample size will be required to confirm the findings of this study.

Bibliography

BIBLIOGRAPHY

1. Page RC, Kornman KS. The pathogenesis of human periodontitis: an introduction. Periodontol 2000. 1997 Jun;14:9–11.

2. Schroeder HE, Lindhe J. Conversion of stable established gingivitis in the dog into destructive periodontitis. Arch Oral Biol. 1975;20(12):775–IN2.

3. Tatakis DN, Kumar PS. Etiology and pathogenesis of periodontal diseases. Dent Clin. 2005;49(3):491–516.

4. Hujoel PP, Cunha-Cruz J, Loesche WJ, Robertson PB. Personal oral hygiene and chronic periodontitis: a systematic review. Periodontol 2000. 2005;37:29–34.

5. Drury TF, Garcia I, Adesanya M. Socioeconomic disparities in adult oral health in the United States. Ann N Y Acad Sci. 1999;896(1):322–324.

6. Gundala R, Chava VK. Effect of lifestyle, education and socioeconomic status on periodontal health. Contemp Clin Dent. 2010;1(1):23.

7. Sakki TK, Knuuttila ML, Vimpari SS, Hartikainen MS. Association of lifestyle with periodontal health. Community Dent Oral Epidemiol. 1995;23(3):155–158.

8. Kadtane SS, Bhaskar DJ, Agali C, Punia H, Gupta V, Batra M, et al. Periodontal Health Status of Different Socio-economic Groups in Out-Patient Department of TMDC & RC, Moradabad, India. J Clin Diagn Res JCDR. 2014 Jul;8(7):ZC61–4.

9. Oberoi SS, Sharma G, Oberoi A. A cross-sectional survey to assess the effect of socioeconomic status on the oral hygiene habits. J Indian Soc Periodontol. 2016 Sep 1;20(5):531.

10. Preshaw PM. Host response modulation in periodontics. Periodontol 2000. 2008;48(1):92–110.

11. Loe H, Theilade E, Jensen SB. EXPERIMENTAL GINGIVITIS IN MAN. J Periodontol. 1965 Jun;36:177–87.

12. Kotsilkov K, Popova C, Boyanova L, Setchanova L, Mitov I. Comparison of culture method and real-time PCR for detection of putative periodontopathogenic bacteria in deep periodontal pockets. Biotechnol Biotechnol Equip. 2015;29(5):996–1002.

13. Zheng X-W, Yan Z, Robert Nout MJ, Boekhout T, Han B-Z, Zwietering MH, et al. Characterization of the microbial community in different types of Daqu samples as revealed by 16S rRNA and 26S rRNA gene clone libraries. World J Microbiol Biotechnol. 2015 Jan 1;31(1):199–208.

14. Shi B, Chang M, Martin J, Mitreva M, Lux R, Klokkevold P, et al. Dynamic Changes in the Subgingival Microbiome and Their Potential for

Diagnosis and Prognosis of Periodontitis. mBio [Internet]. 2015 Feb 27 [cited 2020 Jan 26];6(1). Available from: https://mbio.asm.org/content/6/1/e01926-14

15. Bizzarro S, Loos BG, Laine ML, Crielaard W, Zaura E. Subgingival microbiome in smokers and non-smokers in periodontitis: an exploratory study using traditional targeted techniques and a next-generation sequencing. J Clin Periodontol. 2013;40(5):483–492.

16. Mason MR, Preshaw PM, Nagaraja HN, Dabdoub SM, Rahman A, Kumar PS. The subgingival microbiome of clinically healthy current and never smokers. ISME J. 2015 Jan;9(1):268–72.

17. Voelkerding KV, Dames SA, Durtschi JD. Next-generation sequencing: from basic research to diagnostics. Clin Chem. 2009;55(4):641–658.

18. Tanner A, Maiden MF, Paster BJ, Dewhirst FE. The impact of 16S ribosomal RNA-based phylogeny on the taxonomy of oral bacteria. Periodontol 2000. 1994;5(1):26–51.

19. Pozhitkov AE, Beikler T, Flemmig T, Noble PA. High-throughput methods for analysis of the human oral microbiome. Periodontol 2000. 2011 Feb;55(1):70–86.

20. Harismendy O, Ng PC, Strausberg RL, Wang X, Stockwell TB, Beeson KY, et al. Evaluation of next generation sequencing platforms for population targeted sequencing studies. Genome Biol. 2009;10(3):R32.

21. Berezow AB, Darveau RP. Microbial shift and periodontitis. Periodontol 2000. 2011;55(1):36–47.

22. Tanner A, Maiden MFJ, Macuch PJ, Murray LL, Kent Jr RL. Microbiota of health, gingivitis, and initial periodontitis. J Clin Periodontol. 1998;25(2):85–98.

23. Haffajee AD, Bogren A, Hasturk H, Feres M, Lopez NJ, Socransky SS. Subgingival microbiota of chronic periodontitis subjects from different geographic locations. J Clin Periodontol. 2004;31(11):996–1002.

24. Bhute S, Pande P, Shetty SA, Shelar R, Mane S, Kumbhare SV, et al. Molecular characterization and meta-analysis of gut microbial communities illustrate enrichment of Prevotella and Megasphaera in Indian subjects. Front Microbiol. 2016;7:660.

25. Bassir SH, Chase I, Paster BJ, Gordon LB, Kleinman ME, Kieran MW, et al. Microbiome at sites of gingival recession in children with Hutchinson–Gilford progeria syndrome. J Periodontol. 2018;89(6):635–44.

26. Spielmann N, Wong DT. Saliva: diagnostics and therapeutic perspectives. Oral Dis. 2011;17(4):345–354.

27. Mager DL, Haffajee AD, Devlin PM, Norris CM, Posner MR, Goodson JM. The salivary microbiota as a diagnostic indicator of oral cancer: a descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. J Transl Med. 2005;3(1):27.

28. Paju S, Pussinen PJ, Suominen-Taipale L, Hyvönen M, Knuuttila M, Könönen E. Detection of multiple pathogenic species in saliva is associated with periodontal infection in adults. J Clin Microbiol. 2009;47(1):235–238.

29. Belstrøm D, Fiehn N-E, Nielsen CH, Kirkby N, Twetman S, Klepac-Ceraj V, et al. Differences in bacterial saliva profile between periodontitis patients and a control cohort. J Clin Periodontol. 2014;41(2):104–12.

30. Falony G, Vandeputte D, Caenepeel C, Vieira-Silva S, Daryoush T, Vermeire S, et al. The human microbiome in health and disease: hype or hope. Acta Clin Belg. 2019;74(2):53–64.

31. Singhal N, Maurya AK, Singh NS, Kumar M, Virdi JS. Antimicrobial resistance and its relationship with biofilm production and virulence-related factors in Yersinia enterocolitica biotype 1A. Heliyon [Internet]. 2019 May 22 [cited 2020 Jan 26];5(5). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6535577/

32. Marchesi JR, Ravel J. The vocabulary of microbiome research: a proposal. BioMed Central; 2015.

33. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007;449(7164):804.

34. Sonnenburg JL, Fischbach MA. Community health care: therapeutic opportunities in the human microbiome. Sci Transl Med. 2011;3(78):78ps12–78ps12.

35. Zaura E, Keijser BJ, Huse SM, Crielaard W. Defining the healthy" core microbiome" of oral microbial communities. BMC Microbiol. 2009;9(1):259.

36. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human–microbe mutualism and disease. Nature. 2007;449(7164):811.

37. Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc Natl Acad Sci U S A. 2002 Nov 26;99(24):15451–5.

38. Bradshaw DJ, Marsh PD, Watson GK, Allison C. Role of Fusobacterium nucleatum and coaggregation in anaerobe survival in planktonic and biofilm oral microbial communities during aeration. Infect Immun. 1998;66(10):4729–4732.

39. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. Science. 2008;320(5883):1647–1651.

40. Petersen C, Round JL. Defining dysbiosis and its influence on host immunity and disease. Cell Microbiol. 2014 Jul;16(7):1024–33.

41. Lang NP, Bartold PM. Periodontal health. J Clin Periodontol. 2018;45:S9–S16.

42. Bartold PM, Van Dyke TE. Host modulation: controlling the inflammation to control the infection. Periodontol 2000. 2017;75(1):317–329.

43. Bascones A, Gamonal Aravena JA, Gómez Carranza ML, Silva A, Gonzalez MA. New knowledge of the pathogenesis of periodontal disease. 2004;

44. Popova C, Dosseva-Panova V, Panov V. Microbiology of periodontal diseases. A review. Biotechnol Biotechnol Equip. 2013;27(3):3754–3759.

45. Löe H, Schiøtt CR. The effect of mouthrinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man. J Periodontal Res. 1970;5(2):79–83.

46. Mitchell DF, Johnson M. The nature of the gingival plaque in the hamster—production, prevention, and removal. J Dent Res. 1956;35(4):651–655.

47. Keyes PH, Jordan HV. Periodontal lesions in the Syrian hamster—III: Findings related to an infectious and transmissible component. Arch Oral Biol. 1964;9(4):377–IN6.

48. Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. J Periodontol. 1992 Apr;63(4 Suppl):322–31.

49. Loesche WJ, Grenier E. Detection of Streptococcus mutans in plaque samples by the direct fluorescent antibody test. J Dent Res. 1976 Jan;55:A87-93.

50. Theilade E. The non-specific theory in microbial etiology of inflammatory periodontal diseases. J Clin Periodontol. 1986;13(10):905–911.

51. Marsh PD. Microbial Ecology of Dental Plaque and its Significance in Health and Disease. Adv Dent Res. 1994 Jul 1;8(2):263–71.

52. Marsh PD. Are dental diseases examples of ecological catastrophes? Microbiology,. 2003;149(2):279–94.

53. Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. Nat Rev Microbiol. 2012;10(10):717.

54. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998;25(2):134–44.

55. Darveau RP. The oral microbial consortium's interaction with the periodontal innate defense system. DNA Cell Biol. 2009 Aug;28(8):389–95.

56. Lamont RJ, Hajishengallis G. Polymicrobial synergy and dysbiosis in inflammatory disease. Trends Mol Med. 2015;21(3):172–183.

57. Xu H, Hao W, Zhou Q, Wang W, Xia Z, Liu C, et al. Plaque bacterial microbiome diversity in children younger than 30 months with or without caries prior to eruption of second primary molars. PloS One. 2014;9(2):e89269.

58. Saini R. Autoinducer-2 signaling: Modulates cell-cell quorum sensing in oral biofilm. Dentistry. 2016;6(2):2161–1122.

59. Gurenlian JR. The Role of Dental Plaque Biofilm in Oral Health. Am Dent Hyg Assoc. 2007 Dec 31;81(suppl 1):116–116.

60. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. Periodontol 2000. 2002;28:12–55.

61. Newman MG, Takei H, Klokkevold PR, Carranza FA. Carranza's clinical periodontology. Elsevier health sciences; 2011.

62. Gibbons RJ, Hay DI. Human salivary acidic proline-rich proteins and statherin promote the attachment of Actinomyces viscosus LY7 to apatitic surfaces. Infect Immun. 1988 Feb;56(2):439–45.

63. Teughels W, Newman MG, Coucke W, Haffajee AD, Van Der Mei HC, Haake SK, et al. Guiding periodontal pocket recolonization: a proof of concept. J Dent Res. 2007 Nov;86(11):1078–82.

64. Kolenbrander PE, London J. Adhere today, here tomorrow: oral bacterial adherence. J Bacteriol. 1993 Jun;175(11):3247–52.

65. Gibbons M, Watkins DS. Innovation and the small firm. RD Manag. 1970;1(1):10–3.

66. Listgarten MA. Electron Microscopic Observations on the Bacterial Flora of Acute Necrotizing Ulcerative Gingivitis. J Periodontol. 1965;36(4):328–39.

67. Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, Palmer RJ. Communication among Oral Bacteria. Microbiol Mol Biol Rev. 2002 Sep 1;66(3):486–505.

68. Cooper M, Batchelor SM, Prosser JI. Is cell density signalling applicable to biofilms. Life Death Biofilm. 1995;93–96.

69. Prosser JI. Quorum sensing in biofilms. In: Dental Plaque Revisited: Oral Biofilms in Health and Disease. Bioline, Cardiff; 1999. p. 79–88.

70. Bassler BL, Greenberg EP, Stevens AM. Cross-species induction of luminescence in the quorum-sensing bacterium Vibrio harveyi. J Bacteriol. 1997;179(12):4043–4045.

71. Antunes LCM, Ferreira RBR. Intercellular communication in bacteria. Crit Rev Microbiol. 2009 May 1;35(2):69–80.

72. Zhang H-B, Wang L-H, Zhang L-H. Genetic control of quorum-sensing signal turnover in Agrobacterium tumefaciens. Proc Natl Acad Sci. 2002;99(7):4638–4643.

73. Zhang H-B, Wang C, Zhang L-H. The quormone degradation system of Agrobacterium tumefaciens is regulated by starvation signal and stress alarmone (p) ppGpp. Mol Microbiol. 2004;52(5):1389–1401.

74. Huang JJ, Han J-I, Zhang L-H, Leadbetter JR. Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and Pseudomonas aeruginosa PAO1. Appl Env Microbiol. 2003;69(10):5941–5949.

75. Cvitkovitch DG. Genetic competence and transformation in oral streptococci. Crit Rev Oral Biol Med. 2001;12(3):217–243.

76. Fujise O, Lakio L, Wang Y, Asikainen S, Chen C. Clonal distribution of natural competence in Actinobacillus actinomycetemcomitans. Oral Microbiol Immunol. 2004;19(5):340–342.

77. Li Y-H, Hanna MN, Svensäter G, Ellen RP, Cvitkovitch DG. Cell density modulates acid adaptation in Streptococcus mutans: implications for survival in biofilms. J Bacteriol. 2001;183(23):6875–6884.

78. Roberts AB, Tian F, Byfield SD, Stuelten C, Ooshima A, Saika S, et al. Smad3 is key to TGF-β-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. Cytokine Growth Factor Rev. 2006;17(1–2):19–27.

79. Sutherland IW. The biofilm matrix–an immobilized but dynamic microbial environment. Trends Microbiol. 2001;9(5):222–227.

80. Roberts AP, Johanesen PA, Lyras D, Mullany P, Rood JI. Comparison of Tn5397 from Clostridium difficile, Tn916 from Enterococcus faecalis and the CW459tet (M) element from Clostridium perfringens shows that they have similar conjugation regions but different insertion and excision modules. Microbiology. 2001;147(5):1243–1251.

81. Roe MT, Pillai SD. Monitoring and identifying antibiotic resistance mechanisms in bacteria. Poult Sci. 2003;82(4):622–626.

82. Teles RM, Graeber TG, Krutzik SR, Montoya D, Schenk M, Lee DJ, et al. Type I interferon suppresses type II interferon–triggered human anti-mycobacterial responses. Science. 2013;339(6126):1448–1453.

83. Payne W, Page RC, Ogilvie A, Hall W. Histopathologic features of the initial and early stages of experimental gingivitis in man. J Periodontal Res. 1975;10(2):51–64.

84. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. Lab Investig J Tech Methods Pathol. 1976;34(3):235–249.

85. Moore WE, Holdeman LV, Smibert RM, Good IJ, Burmeister JA, Palcanis KG, et al. Bacteriology of experimental gingivitis in young adult humans. Infect Immun. 1982;38(2):651–667.

86. Moore WE, Holdeman LV, Smibert RM, Cato EP, Burmeister JA, Palcanis KG, et al. Bacteriology of experimental gingivitis in children. Infect Immun. 1984;46(1):1–6.

87. Listgarten MA, Schifter CC, Laster L. 3-year longitudinal study of the periodontal status of an adult population with gingivitis. J Clin Periodontol. 1985;12(3):225–238.

88. Lindhe J, Haffaiee AD, Socransky SS. Progression of periodontal disease in adult subjects in the absence of periodontal therapy. J Clin Periodontol. 1983;10(4):433–442.

89. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. Periodontol 2000. 1997;14(1):12–32.

90. Lang NP, Lindhe J. Clinical periodontology and implant dentistry, 2 Volume Set. John Wiley & Sons; 2015.

91. Dzink JL, Gibbons RJ, Childs III WC, Socransky SS. The predominant cultivable microbiota of crevicular epithelial cells. Oral Microbiol Immunol. 1989;4(1):1–5.

92. Wennström JL. Mucogingival therapy. Ann Periodontol. 1996;1(1):671–701.

93. Chambrone LA, Chambrone L. Subepithelial connective tissue grafts in the treatment of multiple recession-type defects. J Periodontol. 2006;77(5):909–916.

94. Merijohn GK. Management and prevention of gingival recession. Periodontol 2000. 2016;71(1):228–242.

95. Sarfati A, Bourgeois D, Katsahian S, Mora F, Bouchard P. Risk assessment for buccal gingival recession defects in an adult population. J Periodontol. 2010;81(10):1419–1425.

96. Albandar JM, Kingman A. Gingival recession, gingival bleeding, and dental calculus in adults 30 years of age and older in the United States, 1988-1994. J Periodontol. 1999;70(1):30–43.

97. Susin C, Haas AN, Oppermann RV, Haugejorden O, Albandar JM. Gingival recession: epidemiology and risk indicators in a representative urban Brazilian population. J Periodontol. 2004;75(10):1377–1386.

98. Rios FS, Costa RS, Moura MS, Jardim JJ, Maltz M, Haas AN. Estimates and multivariable risk assessment of gingival recession in the population of adults from Porto Alegre, Brazil. J Clin Periodontol. 2014;41(11):1098–1107.

99. Mumghamba EGS, Honkala S, Honkala E, Manji KP. Gingival recession, oral hygiene and associated factors among Tanzanian women. East Afr Med J. 2009;86(3):125–132.

100. Chrysanthakopoulos NA. Gingival recession: prevalence and risk indicators among young greek adults. J Clin Exp Dent. 2014;6(3):e243.

101. van Palenstein Helderman WH, Lembariti BS, Van der Weijden GA, Van't Hof MA. Gingival recession and its association with calculus in subjects deprived of prophylactic dental care. J Clin Periodontol. 1998;25(2):106–111.

102. Holt SC, Bramanti TE. Factors in virulence expression and their role in periodontal disease pathogenesis. Crit Rev Oral Biol Med. 1991;2(2):177–281.

103. Holt SC, Ebersole JL. Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the 'red complex', a prototype polybacterial pathogenic consortium in periodontitis. Periodontol 2000. 2005;38(1):72–122.

104. Arun KV. Molecular Biology of Periodontium. Jaypee Brothers,Medical Publishers Pvt. Limited; 2010. 450 p.

105. Bosshardt DD, Lang NP. The junctional epithelium: from health to disease. J Dent Res. 2005;84(1):9–20.

106. Gemmell E, Yamazaki K, Seymour GJ. The role of T cells in periodontal disease: homeostasis and autoimmunity. Periodontol 2000. 2007;43(1):14–40.

107. Moore WEC, Moore LV. The bacteria of periodontal diseases. Periodontol 2000. 1994;5(1):66–77.

108. Vartoukian SR, Palmer RM, Wade WG. The division "Synergistes." Anaerobe. 2007;13(3–4):99–106.

109. Siqueira JF, Rôças IN. The oral microbiota: general overview, taxonomy, and nucleic acid techniques. In: Oral Biology. Springer; 2010. p. 55–69.

110. Socransky SS, Haffajee AD. Periodontal microbial ecology. Periodontol 2000. 2005;38(1):135–187.

111. Socransky SS, Haffajee AD, Smith C, Martin L, Haffajee JA, Uzel NG, et al. Use of checkerboard DNA–DNA hybridization to study complex microbial ecosystems. Oral Microbiol Immunol. 2004;19(6):352–362.

112. Haffajee AD, Socransky SS, Dzink JL, Taubman MA, Ebersole JL, Smith DJ. Clinical, microbiological and immunological features of subjects with destructive periodontal diseases. J Clin Periodontol. 1988;15(4):240–246.

113. Ximénez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra-and subgingival plaque in health and periodontitis. J Clin Periodontol. 2000;27(9):648–657.

114. Feres M, Haffajee AD, Allard K, Som S, Socransky SS. Change in subgingival microbial profiles in adult periodontitis subjects receiving either systemically-administered amoxicillin or metronidazole. J Clin Periodontol. 2001;28(7):597–609.

115. Spratt DA. Significance of bacterial identification by molecular biology methods. Endod Top. 2004;9(1):5–14.

116. Sanger JM, Sanger JW. Assembly of cytoskeletal proteins into cleavage furrows of tissue culture cells. Microsc Res Tech. 2000;49(2):190–201.

117. Mardis ER. Next-generation DNA sequencing methods. Annu Rev Genomics Hum Genet. 2008;9:387–402.

118. Rothberg JM, Hinz W, Davidson JF, Van Oijen AM, Leamon JH, Huber M. Method and Apparatus for Rapid Nucleic Acid Sequencing. 2011.

119. Dohm JC, Lottaz C, Borodina T, Himmelbauer H. Substantial biases in ultra-short read data sets from high-throughput DNA sequencing. Nucleic Acids Res. 2008;36(16):e105.

120. Liu B, Faller LL, Klitgord N, Mazumdar V, Ghodsi M, Sommer DD, et al. Deep Sequencing of the Oral Microbiome Reveals Signatures of Periodontal Disease. PLoS ONE [Internet]. 2012 Jun 4 [cited 2020 Jan 26];7(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3366996/

121. Chen T, Yu W-H, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database. 2010;2010.

122. Socransky SS, Haffajee AD. Evidence of bacterial etiology: a historical perspective. Periodontol 2000. 1994;5(1):7–25.

123. Tanner AC, Paster BJ, Lu SC, Kanasi E, Kent Jr R, Van Dyke T, et al. Subgingival and tongue microbiota during early periodontitis. J Dent Res. 2006;85(4):318–323.

124. Wong KV, Hernandez A. A review of additive manufacturing. Int Sch Res Not. 2012;2012.

125. Galloway KC, Becker KP, Phillips B, Kirby J, Licht S, Tchernov D, et al. Soft robotic grippers for biological sampling on deep reefs. Soft Robot. 2016;3(1):23–33.

126. Zhang H, Liu S, Zhu B, Xu Q, Ding Y, Jin Y. Composite cell sheet for periodontal regeneration: crosstalk between different types of MSCs in cell sheet facilitates complex periodontal-like tissue regeneration. Stem Cell Res Ther. 2016 Dec;7(1):168.

127. Li Y, Zhou X, St. John MAR, Wong DTW. RNA profiling of cell-free saliva using microarray technology. J Dent Res. 2004;83(3):199–203.

128. Payungporn S, Arirachakaran P, Poomipak W, Praianantathavorn K, Charalampakis G, Poovorawan Y. Identification of Bacteria Associated with a Periodontal Disease in Thai Patients Based on Next-Generation Sequencing. Jundishapur J Microbiol [Internet]. 2017 Jun [cited 2020 Jan 26];10(6). Available from: http://jjmicrobiol.com/en/articles/13646.html

129. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nat Rev Genet. 2012;13(4):260.

130. Offenbacher S. Periodontal diseases: pathogenesis. Ann Periodontol. 1996 Nov;1(1):821–78.

131. Sharma A. Virulence mechanisms of Tannerella forsythia. Periodontol 2000. 2010 Oct;54(1):106–16.

132. Kesavalu L, Sathishkumar S, Bakthavatchalu V, Matthews C, Dawson D, Steffen M, et al. Rat model of polymicrobial infection, immunity, and alveolar bone resorption in periodontal disease. Infect Immun. 2007 Apr;75(4):1704–12.

133. 240202319kavupriya.pdf [Internet]. [cited 2020 Feb 2]. Available from: http://repository-tnmgrmu.ac.in/10880/1/240202319kavupriya.pdf

134. Leblebicioglu B, Kulekci G, Ciftci S, Keskin F, Badur S. Salivary detection of periodontopathic bacteria and periodontal health status in dental students. Anaerobe. 2009 Jun 1;15(3):82–6.

135. Wang J, Qi J, Zhao H, He S, Zhang Y, Wei S, et al. Metagenomic sequencing reveals microbiota and its functional potential associated with periodontal disease. Sci Rep. 2013 May 15;3(1):1–10.

136. Bassir SH, Chase I, Paster BJ, Gordon LB, Kleinman ME, Kieran MW, et al. Microbiome at sites of gingival recession in children with

Hutchinson–Gilford progeria syndrome. J Periodontol. 2018 Jun;89(6):635–44.

137. Ebersole JL, Feuille F, Kesavalu L, Holt SC. Host modulation of tissue destruction caused by periodontopathogens: effects on a mixed microbial infection composed ofPorphyromonas gingivalisandFusobacterium nucleatum. Microb Pathog. 1997 Jul 1;23(1):23–32.

138. Feuille F, Ebersole JL, Kesavalu L, Stepfen MJ, Holt SC. Mixed infection with Porphyromonas gingivalis and Fusobacterium nucleatum in a murine lesion model: potential synergistic effects on virulence. Infect Immun. 1996 Jun 1;64(6):2094–100.

139. Metzger Z, Lin Y-Y, Dimeo F, Ambrose WW, Trope M, Arnold RR. Synergistic Pathogenicity of Porphyromonas gingivalis and Fusobacterium nucleatum in the Mouse Subcutaneous Chamber Model. J Endod. 2009 Jan 1;35(1):86–94.

Annexures

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

The Institutional Review Board

Ragas Dental College and Hospital

Uthandi, Chennai-600119.

Date: 09-01-2020

Place: Chennai

The Project topic titled "**MICROBIAL ANALYSIS OF RED COMPLEX ORGANISMS OF THE WHOLE SALIVA IN PATIENTS WITH GINGIVITIS AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING**" submitted by **Dr.RAGHA MALIKA S** 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:	Ragha malika thesis - Plagiarism check.docx (D63076698)
Submitted:	1/28/2020 4:24:00 AM
Submitted By:	raghamalika4@gmail.com
Significance:	12 %

Sources included in the report:

Plagiarism check 2 (1).docx (D47718899) 6.ROL.docx (D34269228)
Thesis toto.docx (D47313880) https://archive.org/stream/dental_DropBooks_386/Dental_Clinics_of_North_America_DropBooks_App_djvu.txt
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4753713/ https://www.ncbi.nlm.nih.gov/pubmed/29520806
https://www.ncbi.nlm.nih.gov/pubmed/25164479 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3177396/
https://core.ac.uk/download/pdf/18410844.pdf https://www.deepdyve.com/lp/wiley/management-and-prevention-of-gingival-recession-z0r0T04J6N
https://www.frontiersin.org/articles/10.3389/fmicb.2017.00340/full
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6213094/

Instances where selected sources appear:

47

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