COMPARATIVE ANALYSIS OF SALIVARY MICROBIOME IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS AND HEALTHY INDIVIDUALS USING 16S rRNA SEQUENCING

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In partial fulfillment for the Degree of

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BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

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CERTIFICATE

This is to certify that this dissertation titled **"COMPARATIVE ANALYSIS OF SALIVARY MICROBIOME IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS AND HEALTHY INDIVIDUALS USING 16S rRNA GENE SEQUENCING"** is a bonafide dissertation performed by **Ishwarya.S** under our guidance during the postgraduate period 2015 - 2018.

This dissertation is submitted to **THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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CONTENTS

Introduction

INTRODUCTION

Microbiome is defined as the collective genome and gene products of the microbiota within an organism. Every human body contains a personalized microbiome that is essential to maintain health but capable of eliciting disease. An imbalance or shift in the microbiota at a given body site results in dysbiosis. Shifts from the core microbiome to dysbiosis has been associated with various diseases[1].

Oral cancer is a serious and growing problem with more than half a million people affected worldwide of which 90% are Oral Squamous Cell Carcinoma (OSCC).The etiology of oral cancer is multifactorial. Ultraviolet radiation, areca nut, alcohol, tobacco usage, nutritional deficiencies and viral infections have been implicated. Around 25% of oral cancer do not have any known risk factors[2].

Microorganisms are associated with 20% of fatal cancers in humans. In the oral cavity chronic inflammation has been observed at various stages of OSCC which could result from persistent epithelial or mucosal cell colonization by microorganism[3]. Keeping in mind the increasing evidence of the involvement of oral bacteria in inflammation, it has been suggested that the shift in oral microbiome would be a factor in the etiology of chronic inflammation which would influence the pathogenesis of oral cancer.

A consortium of microbes rather than one species is usually involved in causing disease. In dental caries, the ecological shift favours growth of acidogenic and aciduric species, namely streptococci mutans, lactobacilli and Bifidobacteria. In

periodontal disease, proteolytic bacteria that challenge the host inflammatory response are in play. The leading bacteria at periodontal destruction sites include members of the "red complex", namely P. gingivalis, Tannerella forsythia and Treponema denticola , as well as the newly described taxa, Synergistetes and Saccharibacteria (TM7)[4]. Bacteria such as Exiguobacterium oxidotolerans, Prevotella melaninogenica, Staphylococcus aureus and Veillonella parvula were isolated from tumours while tumour associated saliva sample showed the presence of Capnocytophagia gingivalis, Prevotella melaninogenica and Streptococcus mitis. Streptococcus mutans, Lactobacilli, and Streptococcus sobrinus were found to be associated with dental caries. Microbes such as F.nucleatum, Spirochaetes (especially Treponema), Actinomyces, Lactobacillus, Enterococcus faecalis, Dialister species were implicated in the periapical diseases[5].

The bacteria that are involved in OSCC need to be identified to establish the role of the microorganism in carcinogenesis. In the background of the importance of microorganisms in dysbiosis, the present study was done to study the oral microbiota in the saliva of patients with OSCC and normal individuals.

Aim and objectives

AIM

To ascertain the oral microbiome in saliva samples of Oral Squamous Cell Carcinoma (OSCC) patients and healthy individuals using 16S rRNA sequencing of bacteria and BLAST (Basic Local Alignment Search Tool) analysis in NCBI (National Centre for Biotechnology Information) database.

OBJECTIVES

1. To ascertain the oral microbiome in saliva samples of OSCC patients using 16S rRNA sequencing of bacteria with BLAST analysis and NCBI database.
2. To ascertain the oral microbiome in saliva samples of healthy individuals using 16S rRNA sequencing of bacteria with BLAST analysis and NCBI database.
3. To compare the oral microbiome in saliva samples of OSCC patients and healthy individuals using 16S rRNA sequencing of bacteria with BLAST analysis and NCBI database.

HYPOTHESIS

There is no difference in the oral microbiome in the saliva of OSCC patients and healthy individuals.

Materials and methods

MATERIALS AND METHODS

The study was designed to analyze the oral microbiome in the saliva samples of Oral Squamous Cell Carcinoma (OSCC) patients and healthy individuals by 16S rRNA sequencing of bacteria with BLAST (Basic Local Alignment Search Tool) analysis in NCBI (National Centre for Biotechnology Information) database.

STUDY GROUP

Individuals who are diagnosed with OSCC by biopsy (n =10).

Inclusion criteria:

- OSCC patients.

Exclusion criteria:

- Patients under antibiotic therapy, antifungals and corticosteroids 3 months prior to the study.
- Patients with systemic diseases (Diabetic mellitus, Cardiovascular disorders, Gastroesophageal reflux disorder).
- Pregnant women.
- Patients who are not willing to participate.

CONTROL GROUP

Individuals who are healthy (n = 10).

Exclusion criteria:

- Patients under antibiotic therapy, antifungals and corticosteroids 3 months prior to the study.
- Patients with systemic diseases (Diabetic mellitus, Cardiovascular disorders, Gastroesophageal reflux disorder).
- Pregnant women.
- Patients who are not willing to participate.

STUDY DESIGN

Ten consecutive OSCC patients and ten healthy individuals satisfying the study criteria were enrolled. The unstimulated saliva samples of ten patients in each group were collected and analysed for oral microbiome using 16S rRNA sequencing.

STUDY SETTING

After receiving patient's consent (Annexure VII), the study was conducted at Ragas Dental College and Hospital and Madha Trust, a secular charitable institution for poor cancer patients in Chennai, South India. The laboratory techniques were carried out at Enable Biolabs Private Limited, Chennai (Annexure III).

SALIVA SAMPLE COLLECTION

A. Armamentarium

1. Pre-sterilized 50 ml graduated centrifuge tube
2. Saline
3. Gloves
4. Mask
5. Case sheet(Annexure VII)
6. Sharpie permanent marker
7. Consent form
8. Patient apron

B. Patient instruction

1. Do not eat or drink anything but water 1 hour prior to sample collection.
2. Rinse oral cavity with drinking water (room temperature) 1 hour prior to sample collection.

C. Collection

a. 5ml of saline to be swished around oral cavity for 30 seconds.

b. To spit the entire content into the sterile graduated centrifuge tube.

c. The containers with the samples to be labelled. Labels should include the following details:

- Name of the patient:
- Age/Gender:
- Case code:
- Time of collection:

D. Transport

To be carried in ice box. Temperature 3° to 5°C.

E. Storage

Refrigerated between 2°C to 8°C.

Methods:

The entire procedure from extraction of bacterial DNA to quantification of DNA and further amplification and sequencing of 16S rRNA was performed by the reagents, (Cat# 51304) from QIAamp ™ DNA minikit, Qiagen, Germany (Annexure III).

Bacterial DNA extraction

- Centrifuge 2 ml of oral saline rinse at 3000rpm for 5mins at room temperature to precipitate bacterial cells.
- The precipitated cells were suspended in 100µl cell lysis buffer containing 36% to 50% guanidine hydrochloride(RNA isolation).

- Incubated at 57°C for 2 hours to enable complete lysis of both gram positive and gram negative bacterial cells
- Following lysis, an equal volume of 100% ethanol was added to precipitate the genomic DNA.
- Transfer content to DNA spin columns containing silica membrane
- Centrifuge at 8000 rpm for 1min at room temperature.
- Precipitated DNA gets captured in the silica membrane
- Silica columns were washed twice with wash buffer (supplied by the manufacturer Qiagen)
- Degraded proteins and membrane lipid particles get washed off during the wash steps
- The captured DNA from the silica membrane was eluted with 50µl of elution buffer (supplied by the manufacturer Qiagen).

Quantification of DNA

- The DNA extracted from bacterial cells was quantified by QUBIT^{TM} Fluorometer to determine the total DNA concentration.

16S rRNA amplification and sequencing

- 50ng of total genomic DNA was subjected to polymerase chain reaction (PCR) amplification with 16S rRNA gene hypervariable region specific primers.

Forward: AGTTTGATC[A/C]TGGCTCAG

Reverse: GGACTAC[C/T/A]AGGGTATCTAAT.

- The following conditions were be used to amplify the 16S rRNA gene region: After an initial denaturation for 5 min at 95°C
- The DNA was subjected to 40 cycles of 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C, with a final extension for 10 min at 72°C.
- This results in amplification of an 800-bp 16S rDNA fragment, corresponding to Escherichia coli positions 10 to 806.
- The amplified rDNA product was subjected to gel electrophoresis to confirm the size of amplified product, which was then purified and sequenced with forward primer only.
- The sequenced data was then compared with reference bacterial gene sequences deposited in public database (ncbi.nlm.nih.gov) using BlastN program.
- The microbiome charts were generated using Kronas software™.

- The following values were noted from the results obtained:

BLAST (Basic Local Alignment Search Tool) is an algorithm for comparing primary biological sequence information.

Query: The input sequence to which all of the entries in a database are to be compared.

Score: The score is a numerical value that describes the overall quality of the alignment of base pairs between the query sequence and the database sequence. Higher numbers correspond to higher similarity.

Max score: Highest alignment score between query sequence and database sequence.

Score is different from max score if several parts of database sequence match different parts of query sequence.

Total score: Sum of alignment scores of all segments from the same database sequence that match the query sequence.

Query coverage: Percentage of the query length that is included in the aligned segments.

E value: Number of alignments expected by chance with a particular score. E is represented as the exponent of 10(eg; 1e-5=$1x10^{-5}$ =0.00001).

Bit score: Log representation of score.

Identity: The extent to which two sequences have the same residues at the same positions in an alignment, often expressed as a percentage.

Accession number: It is a unique identifier given to a DNA or protein sequence record to allow tracking of different versions of that sequence record and the associated sequence over time in a single data repository.

Max Identity: BLAST calculates the percentage identity between the query and the hit in the nucleotide to nucleotide alignment. If there are multiple alignment with a single hit, then only the highest percent identity is shown.

Individual sample results

- Top 100 sequences producing significant alignments were taken into consideration for the study. Lower the expected chance value better the significance of identified bacteria. A number close to 0 means that the hit has to be significant and not due to chance.
- When there are two or more identical E values, the Max score is then used to sort the hits. The Total score becomes important when BLAST finds multiple, but not joint section of similarity between query and hit.
- If Max score is equal to the Total score then only a single alignment is present. If Total score is larger than Max score then multiple alignment is present and their individual scores have contributed to the total score.

- When a bacteria was present in three or more samples, it was considered as predominant.

Uncultured bacterium

In certain samples(O-1, 0-6, 0-8) presence of uncultured bacterium was noted. The unculturable bacteria are bacterial sequences that have not been uploaded into the NCBI database as the method of identification in 16S rRNA gene sequencing technique involves comparing the sequences in the study sample with that available in the NCBI database. This is because bacteria maybe recalcitrant for culturing. This could be due to lack of necessary symbionts, nutrients or surfaces, excess inhibitory compounds, incorrect combinations of temperature, pressure or atmospheric gas composition, accumulation of toxic waste products from their own metabolism and intrinsically slow growth rate and rapid dispersion from colonies.

Low concentration of DNA:

DNA concentration can be decreased when extracted by non-commercial protocols. Other components of saliva such as enzymes, hormones, immunoglobulins and other biomolecules can interfere with the quality and quantitiy of the DNA extracted. The concentration of DNA extracted is not affected even when the saliva is frozen or stored for a longer duration.

Review of literature

INDEX

ORAL SQUAMOUS CELL CARCINOMA

Oral cancers ranks eleventh among the common malignancies globally. Forty percentage affected are in developing regions such as South-east Asia. Ninety percent of all oral cancers are squamous cell carcinoma originating from the mucosal epithelium.

If detected during its early stages, the 5 year survival rate of oral cancer is 60-80% [6].The etiology of Oral Squamous Cell Carcinoma(OSCC) is multifactorial and a combination of environmental risk factors and genetic predisposition. The risk factors can be grouped as established, strongly suggestive, possible and speculative factors based on the available global evidence[2]. Tobacco along with alcohol and betel quid usage are the most important etiological factors in South East Asia. Risk of oral cancer due to tobacco and alcohol is estimated to be more than 80%[7]. Human Papilloma Virus infection is involved in oro-pharyngeal carcinogenesis[2] (Annexure IV, Table 1).

The average delay time in diagnosing and treating oral cancers is about 2 to 5 months. Delayed detection may account for high morbidity rate of OSCC patients. Early detection and diagnosis lead to a greater survival rate and play a significant role in successful treatment of the disease[8,9]. Recently, factors such as the oral microbiome, are being explored for their role as significant risk factors.

TAXONOMIC CLASSIFICATION SYSTEM

A reliable classification system is a prerequisite for scientists and professionals dealing with microorganisms. The ultimate objective of biological classification is the characterization and orderly arrangement of organisms into groups. It is often confused with identification but, as a matter of fact, classification is prerequisite for identification[10].

The late 19th century saw the beginning of microbial taxonomy. Microbial taxonomy may be defined as the study and classification of the diverse microorganisms with the aim of organizing and prioritizing in an orderly manner. Two kinds of taxonomic and nomenclatural systems are of primary interest- A Linnaean system which is based on the Linnaean hierarchy and a phylogenetic system is a system based on the principle of descent.

Linnaen Hierarchy:

The Linnaean hierarchy is the series of ranked taxonomic categories based on those adopted by Linnaeus (1758) to which taxa (named groups of organisms) are assigned to seven principal categories-Kingdom, Division/Phylum, Class, Order, Family, Genus, and Species. The Linnaean system of taxonomy has since been complimented with the highest taxonomic rank for prokaryotes, called a "domain". All prokaryotes are placed within the domains Bacteria or Archaea. Successively lower ranks follow as non-overlapping subsets of the domain:

"phylum"; "class", "order", "family", "genus", "species" and "subspecies". The "phylum", "family" and "subspecies" ranks, as well as "suborder" and "subclass", sometimes used for classification of prokaryotes, were added to the original Linnaean classification scheme. The "species", assigned to a "genus", in a binomial combination, is considered to be the basic unit of microbial taxononomy[11].

Strain - a "population", derived from a clonal variant /A group of presumed common ancestry with clear-cut physiological but usually not morphological distinctions.

Species –comprising related organisms or populations potentially capable of interbreeding. It is the basic unit of *biological* classification.

Genus - a category of taxonomic classification ranking above the species and below the family comprising structurally or phylogenetically related species.

Family - a category of taxonomic classification ranking above the genus and below the order and comprising several related genera.

Order - a category of taxonomic classification ranking above the family and below the class comprising several related families.

Class - a major category in biological taxonomy ranking above the order and below the phylum comprising several related orders.

Phylum - a primary category in biological taxonomy that ranks above the class and below the kingdom comprising related classes.

Kingdom - a major category in biological taxonomy that ranks above the phylum and below the domain.

Domain - the highest taxonomic category in biological classification ranking above the kingdom.

Phylogenetic classification:

The second change involving concepts of taxa was associated with Phylogenetic Systematics or Cladistics. Concepts of higher taxa as groups of similar species were replaced with concepts of higher taxa as clades, that is, monophyletic (holophyletic) groups of species. This new concept of the higher taxon was derived directly from the principle of descent in that it equated higher taxa with units of exclusive common ancestry.

Clade - A clade is a group of organisms that includes an ancestor species and all of its descendants

Cladogram - A cladogram shows how species may be related by descent from a common ancestor. (Annexure IV, Figure 1)

Node-based: The clade stemming from the most recent common ancestor of a and b (Where a and b are organisms, species, or clades).

Stem-based: The clade composed of c and all members of x that share a more recent common ancestor with c than with d.b. (Where c and d are organisms, species, or clades, and x is a clade that includes both c and d).

Apomorphy-based: The clade stemming from the first ancestor of y to evolve character e.(Where y is an organism, a species, or a clade, and e is a derived character)[11].

The comparison between the Linnaen and Phylogenetic classification is given in Annexure IV, Table 2.

MICROBIOME

Microbiome refers to "the totality of microbes, their genetic information, and the milieu in which they interact"[12]. 'Microbiome' is a terminology coined by Joshua Lederberg to signify the ecological community of commensal, symbiotic and pathogenic microorganisms that share our body space[13]. These microbial organisms that contribute to microbiome are termed as 'Microbiota'[12]. The human cells are out numbered by the microbes that occupy the body by several folds, thus earning humans the name of 'supraorganisms'[14]. The microbiota's composition can vary according to the environmental sites and the host status[8]. In health, the microbiome is in a state of homeostasis wherein the majority of the microorganisms act as commensals or symbiotics[15]. When this relatively stable state of microbial homeostasis is disrupted, dysbiosis takes place[12].

The anatomical location is a primary determinant for community composition: interpersonal variation is substantial and is higher than the temporal variation seen at most sites in a single individual. Also, there are greater interpersonal similarities than a snap shot view indicates since the microbial system is dynamic in nature[12].

Diet inventories and 16*S* rDNA sequencing characterization of 98 fecal samples have shown that the fecal communities are clustered into enterotypes distinguished primarily by levels of *Bacteroides* and *Prevotella*. Enterotypes are strongly associated with long-term diets, particularly protein and animal fat (*Bacteroides*) versus carbohydrates (*Prevotella*). The substantial intestinal metagenomic changes is caused by dietary changes and the enterotypes are known to cluster based on dietary abundance of animal protein or carbohydrate[16].

Characterization of nasopharyngeal microbiota of 96 healthy children was done in 2011 by barcoded pyrosequencing of the V5–V6 hypervariable region of the 16S-rRNA gene, and compared microbiota composition between children sampled in winter/fall with children sampled in spring. The approximately 1000000 sequences generated represented 13 taxonomic phyla and approximately 250 species-level phyla types (OTUs). Microbiota profiles varied strongly with season, with in fall/winter a predominance of Proteobacteria (relative abundance (% of all sequences): 75% versus 51% in spring) and Fusobacteria (absolute abundance (% of children): 14% versus 2%

in spring), and in spring a predominance of Bacteroidetes (relative abundance: 19% versus 3% in fall/winter, absolute abundance: 91% versus 54% in fall/winter), and Firmicutes. This study reveals that there is seasonal variation of nasopharyngeal microbiota in young children which is independent of antibiotic use or viral co-infection[17].

The vaginal bacterial communities of 396 asymptomatic North American women who represented four ethnic groups (white, black, Hispanic, and Asian) and the species composition was characterized by pyrosequencing of barcoded 16S rRNA genes. The communities were clustered into five groups: four were dominated by *Lactobacillus iners, L. crispatus, L. gasseri*, or *L. jensenii*, whereas the fifth had lower proportions of lactic acid bacteria and higher proportions of strictly anaerobic organisms, indicating that a potential key ecological function, the production of lactic acid, seems to be conserved in all communities. The proportions of each community group varied among the four ethnic groups, and these differences were statistically significant [$P < 0.0001$]. Moreover, the vaginal pH of women in different ethnic groups also differed and was higher in Hispanic (pH 5.0 ± 0.59) and black (pH 4.7 ± 1.04) women as compared with Asian (pH 4.4 ± 0.59) and white (pH 4.2 ± 0.3) women[18].

A microarray was designed to detect and quantitate the small subunit ribosomal RNA (SSU rRNA) gene sequences of most currently recognized species and taxonomic groups of bacteria. They used this microarray, along

with sequencing of cloned libraries of PCR-amplified SSU rDNA, to profile the microbial communities in an average of 26 stool samples each from 14 healthy, full-term human infants, including a pair of dizygotic twins, beginning with the first stool after birth and continuing at defined intervals throughout the first year of life. To investigate possible origins of the infant microbiota, they also profiled vaginal and milk samples from most of the mothers, and stool samples from all of the mothers, most of the fathers, and two siblings. Most of the breast milk and maternal vaginal samples clustered perfectly by anatomic site of origin. The composition and temporal patterns of the microbial communities varied widely from baby to baby.

Despite considerable temporal variation, the distinct features of each baby's microbial community were recognizable for intervals of weeks to months. The strikingly parallel temporal patterns of the twins suggested that incidental environmental exposures play a major role in determining the distinctive characteristics of the microbial community in each baby. By the end of the first year of life, the idiosyncratic microbial ecosystems in each baby, although still distinct, had converged toward a profile characteristic of the adult gastrointestinal tract. The similarity of the microbial community profiles of stool samples from babies 1 year of age and older, to each other and to those of the adult stool samples suggested that the infant gastrointestinal communities converged over time toward a generalized "adult-like" microbiota.

The infants' gastrointestinal microbiota was not significantly more similar to that of their parents than to that of other adults. The transition to an "adult-like" profile was found to often follow the introduction of solid foods[19].

The shift in gut microbial communities was studied following antibiotic therapy using a mouse model to control the host genotype, diet, and other possible influences on the microbiota. They employed a tag-sequencing strategy targeting the V6 hypervariable region of the bacterial small-subunit (16S) rRNA combined with massively parallel sequencing to determine the community structure of the gut microbiota. Inbred mice in a controlled environment harbored a reproducible baseline community that was significantly impacted by antibiotic administration. The ability of the gut microbial community to recover to baseline following the cessation of antibiotic administration differed according to the antibiotic regimen administered. Severe antibiotic pressure resulted in reproducible, long-lasting alterations in the gut microbial community, including a decrease in overall diversity[20].

Thus, according to the review on microbiota by Cho and Blaser *et al*, each human over a lifetime develops a densely populated microbiome that is recapitulated in every individual and in every generation[12].

Microbiome variation and pathology:

Cutaneous microbiome:

In psoriasis, Firmicutes are over represented and Actinobacteria are significantly under-represented in the psoriatic lesions compared to both the unaffected skin in psoriasis patients and normal controls[21].

Cutaneous microbiome shifts, such as an increased abundance of Pseudomonaceae in individuals with chronic ulcers treated with antibiotics and an abundance of Streptococcaceae in diabetic ulcers have been reported[22].

Propionibacterium acnes have been implicated in the dermatological condition, acne[23].

Gastric microbiome:

Gastric microbiota diversity was found to be high in Helicobacter pylori (H.pylori) negative individuals with abundance of prominent gastric phylotypes (Streptococcus, Actinomyces, Prevotella,Gemella) in the oropharynx which indicates that either many constituents are swallowed from more proximal sites, or that close relatives of the oral microbiota colonize more distally.

In contrast, in H.pylori positive persons, H.pylori accounts for > 90% of sequence reads from the gastric microbiota, thus reducing the overall microbial diversity of this microbiota[24].

H.pylori presence is strongly associated with particular diseases and important age-related differences. Its presence increases the risk for developing peptic ulcer disease, gastric Mucosa Associated Lymphoid Tissue (MALT) tumors, and gastric adenocarcinoma but also is associated with decreased reflux esophagitis and childhood-onset asthma; demonstrating the complex biological interactions with microbiota[12].

Colon microbiome:

Inflammatory Bowel Disease susceptibility is associated with host polymorphisms in bacterial sensor genes such as nucleotide-binding oligomerization domain-containing protein 2 (NOD 2) and toll-like receptor 4 (TLR-4)[25].

Early childhood antibiotic exposure has been associated with increased risk for Crohn's disease and significantly diminished microbial diversity has been seen.

Crohn's disease patients have over-representation of E.faecium and of several Proteobacteria compared to controls[26].

Gut microbiome associated pathology:

Liver:

Gut microbiota may be involved in hepatologic conditions, including Non-Alcoholic Fatty Liver Disease (NAFLD), alcoholic steatosis and

hepatocellular carcinoma. Patients with cirrhosis have community-wide changes at multiple taxonomic levels, with enrichment of Proteobacteria and Fusobacteria (phyla), and Enterobacteriaceae, Veillonellaceae, and Streptococacceae (family)[27].

Obesity:

In humans, obesity is associated with decreased Bacterioidetes and diminished bacterial diversity (Ley RE et al, 2006). Antibiotic use in human infants, before the age of 6 months was related to obesity development while perinatal administration of a Lactobacillus rhamnosus GG-based probiotic decreased excessive weight gain during childhood[28.]

Rheumatoid arthritis:

Dysbiosis within gut lumen can cause dysregulation of host immune responses (local expansion of Th17 cells that activate B cells to produce antibodies) leading to increased antibody production against joints[29].

The complexity of dysbiosis and disease is best defined by Hill's criteria which states that "The criteria include the strength of association, its consistency, specificity, temporality, and biological plausibility, and whether biological gradients are present, experimental support exists, and support can be extrapolated from known causal relationships"[30].

ORAL MICROBIOME

In humans, oral microbiome is one of the most complex microbiome[31]. It is highly diverse, and includes bacteria, virus, fungi, archaea and protozoa[15]. More than 600 bacterial species have been detected, of which 50% have not been cultivated. A majority of 96% of bacteria belong to the phylum Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes and Fusobacteria; while the remaining 4% belong to Euryarcheota,Chlamydia, Chloroflexi, Synergistetes, Tenericutes and candidate phyla.(divisions SR1 AND TM7). A candidate phylum is a lineage of prokaryotic organisma for which until recently no cultured representatives have been found[32].

Due to the continuum of the oral cavity with the external environment, the oral bacterial flora undergoes dynamic changes in immeasurable rates[33]. This diversity varies from birth to adulthood due to various external and internal influences. Throughout childhood, the oral microbial load is found to increase but the microbial diversity seems to decrease[31]. The initial colonizers depend on:

1. Type of delivery:

   Babies born by vaginal delivery have bacterial communities quite similar to the mother's vagina – predominantly Lactobacillus, Prevotella, and Sneathia spp but babies born by cesarean section have bacteria similar to those present in the mother's skin –

predominantly Staphylococcus, Corynebacterium, and Propionibacterium spp[34].

2. Personal relationships:

   The infants show microflora according to the frequency of contact with the surrounding adults and children, domestic animals[31].

3. Hygiene habits and diet:

   Presence of Streptococcus species in edentulous children have been demonstrated thus disproving the fact that these species colonize only during the eruption of teeth. Hence oral hygiene practices become even more important right from birth[35].

   An increased diet of fermentable carbohydrates can favour the growth of acidogenic and aciduric species.

4. Development of teeth:

   Primary dentition: Higher prevalence of bacteria belonging to the class Gammaproteobacteria (Pseudomonaceae, Moraxellaceae, Enterobacteriaceae, Pateurellaceae) are present.

   Permanent dentition: Higher prevalence of bacteria belonging to Veillonellaceae family and Prevotella are seen[36].

Other factors that can influence oral microbiome composition are genetics, host defences, microbial interactions (Quorum Sensing), receptors for attachment, temperature, atmosphere, pH, and salivary flow[37].

Genetics: Genetic polymorphisms associated with interleukin (IL)-1, or other cytokines, can increase the likelihood of detecting certain key periodontal pathogens, and pre-dispose individuals to periodontitis.

Host-defences and microbial cross-talk: The host defence system is actively engaged in cross talk with its resident microbiota in order to effectively maintain a constructive relationship. Host cell pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like *receptors (*NOD-like receptors) are strategically deployed in tissues to sample the extracellular and intra-cellular environments and recognize microbe-associated molecular patterns (MAMPS), such as lipopolysaccharide, lipoteichoic acid, nucleic acid. They activate multiple signalling pathways many of which converge on nuclear factor κB (NF-κB). MAMPs are present on, or are released from, all microbial cells. The host has evolved systems to enable them to tolerate resident microorganisms without initiating a damaging inflammatory response, while also being able to mount an efficient defence against pathogens.

Environmental factors: Nutrients such as amino acids, proteins, and glycoproteins are obtained from endogenous supplies, and mainly from saliva, although gingival crevicular fluid (GCF) is another potential source. Saliva contains amino acids, peptides, proteins, and glycoproteins, vitamins and gases, and it also provides the main buffering capacity for the mouth. The catabolism of the more complex host molecules, such as host glycoproteins,

requires the sequential or concerted action of consortia of bacteria, in which their metabolic capabilities are combined.

Importantly for the stability of the microbial consortium, the metabolism of these substrates leads to only minor and slow changes to the local pH, which are well tolerated by the normal resident microbiota. In contrast the main impact of diet is the provision of fermentable carbohydrates that leads to ecologically devastating falls in pH, which if repeated frequently enough, lead to the selection of acidogenic and acid-tolerating bacteria and a greater risk of dental caries. Even a small change in pH can alter the growth rate and pattern of gene expression in subgingival bacteria, for example, the expression of proteases by P. gingivalis increases at alkaline pH, and thereby can increase the competitiveness of some of the putative pathogens. This could favour the growth of periodontal pathogens, such as P. intermedia, P. gingivalis, and A. actinomycetemcomitans that have alkaline pH optima for growth. If sustained, the combined selective pressures of the environmental factors will lead to a re-arrangement of community structure and an enrichment of the proportions of the anaerobic and proteolytic component of the microbiota[38].

As the child develops into an adult there is a shift in the bacterial population from aerobic or facultative gram positive cocci to anaerobic fastidious gram negative bacteria i.e; from a greater proportion of bacteria from phyla Firmicutes and Actinobacteria to Bacteroidetes, Fusobacteria, Spirochaetes, and Candidatus Saccharibacteria[37,38].

The set of initial colonizers seems to influence the subsequent colonization, thus setting the base for the complexity and stability of the microbial ecosystem in the adulthood[30]. Not only postpartum exposure influences the development of microflora but maternal health and hygiene also plays a role. A study has reported that there is 70% intrauterine colonization in amniotic fluid by oral microorganisms.

Pathogenic bacteria such as Fusobacterium nucleatum contributes to the risk of low birth weight and preterm babies[38]. The oral microbial flora's complexity depends on oxygen tension, nutrient availability, temperature and host immunological factor exposure[39]. The proportion of the oral microorganisms may vary according to the colonizing sites. It was found in 2009 that teeth and tongue present a higher microbial load compared to oral mucosa and saliva[40]. The interplay of all the above mentioned factors is responsible for the development of the oral microbiome and is significant in the determination of health and disease[31].

ORAL MICROBIOME – CLINICAL SIGNIFICANCE

When microbial homeostasis is disrupted by external or internal factors, oral diseases such as dental caries, pulpal disease, periapical disease, and oral cancer may occur[33].

Dental caries:

When there is an increased dietary carbohydrate intake, bacteria that ferment the carbohydrates such as Streptococcus mutans, Lactobacilli, and Streptococcus sobrinus adhere to the tooth surface and increase the acidity of the biofilm. This in turn increases the load of these acidogenic bacteria and out-competes the resident flora such as Streptococcus sanguis and Streptococcus gordonii[41]. Recent studies have shown that Firmicutes, Actinobacteria, and Proteobacteria are the 3 most abundant phyla in patients with caries using Next Generation Sequencing[42].

The difference in oral microbial diversity between children with severe early-childhood caries (S-ECC) and caries-free (CF) controls was evaluated in a study by means of a cultivation-independent approach called denaturing gradient gel electrophoresis (DGGE). Pooled dental plaque samples were collected from 20 children aged 2 to 8 years. Differences in DGGE profiles were distinguished on the basis of a cluster analysis. The microbial diversity and complexity of the microbial biota in dental plaque were found to be significantly less in S-ECC children than in CF children[43].

Periodontitis:

A dysbiotic microenvironment has been observed in periodontal inflammation, which is triggered mainly by Porphyromonas gingivalis. This bacteria exerts a keystone effect via host modulation to breakdown

homeostasis by remodeling the regular microbiome into a disease-provoking one[44].

Endodontic disease:

(i) Pulpal disease:

P.micra, F.nucleatum and Viellonella species have been implicated in endodontic pulpitits while Atopio genomo species C1, P.alactolyticus, Streptococcus species were found in deep dentinal caries. Rocaset et al noted this shift in microbial population suggesting the change in environment as the cause[45].

(ii) Periapical disease:

Periapical disease includes apical periodontitis and apical abcess. Gram negative saccharolytic rods such as Fusobacterium or Bacteroides are predominantly found in root canal spaces associated with periapical disease. Microbes such as F.nucleatum, Spirochaetes (especially Treponema), Actinomyces, Lactobacillus, Enterococcus faecalis, Dialister species have been implicated in the periapical diseases by recent studies so far which degrade the nitrogenous compounds into short chain fatty acids, ammonia, sulfur compounds, and indole that induce tissue inflammation by modulating immune response and promote apoptosis[46.47.48].

Halitosis:

Actinomyces, Viellonella, and Fusobacterium which are tongue-coating bacteria degrade the nutrients present on the tongue surface to produce short-chain fatty acids, ammonia, sulfur compounds and indole[49]. These molecules are also present in periodontitis, thus a positive relationship exists between halitosis and periodontitis[50].

Microbiome and Cancer

Even though bacteria were implicated as a potential cause of cancer in the microbial literature in the 19th century, the idea was dismissed[51]. Various propositions have been put forth recently which have revealed evidence based cancers associated with specific bacterial etiology[5] (Annexure IV, Table 3).

Khajuria *et al* in 2017, state that chronic infections triggered by bacteria can facilitate tumor initiation or progression because, during the course of infection, normal cell functions can undergo the control of factors released by the pathogen. These bacterial factors, namely
virulence factors, can directly manipulate the host regulatory pathways and the inflammatory reaction[51].

In many studies, it has been reported that smoking and alcohol consumption are commonly associated with carcinoma of the palate, while that of chewing tobacco is commonly associated with carcinoma of the alveolus and buccal mucosa. Alcohol is not carcinogenic, but there is

increasing evidence that a major part of the tumor promoting action of alcohol might be mediated via its first, toxic and carcinogenic metabolite, acetaldehyde. Acetaldehyde is produced from ethanol in the epithelia by mucosal alcohol dehydrogenases, but much higher levels are derived from microbial oxidation of ethanol by the oral microbial flora.

Thus, subjects consuming alcohol are at increased risk of developing cancer because of this synergistic action. Gram positive bacteria and yeasts are associated with higher acetaldehyde production, which could be a biologic explanation for the observed synergistic carcinogenic action of alcohol and smoking on upper gastrointestinal tract cancer. This may open a new microbiologic approach to the pathogenesis of the cancer of the oral cavity and upper gastrointestinal tract. Streptococcus intermedius, Prevotella, Capnocytophaga and Candida albicans have been isolated in increased numbers at carcinoma sites[51].

Paradigms proposed on role of microbiome in carcinogenesis:

1. Several bacteria cause chronic infections or produce toxins which can cause disturbances in the cell cycle and lead to alterations in the cell growth[52].
2. Genetic mutation: Chronic infections induce cell proliferation through Mitogen Activated Phosphotidyl Kinase (MAPK) pathways and cyclin D1 that increase the rate of cell transformation and tumor development by increased genetic mutation[53.]

3. Several infections cause intracellular accumulation of the pathogen, leading to suppression of apoptosis primarily through modulation of the expression of Bcl-2 family proteins or by inactivation of retinoblastoma protein, pRb[54]. This strategy provides a niche in which the intracellular pathogen can survive in spite of the attempts of the host immune system to destroy the infected cells by apoptosis. Thus, it allows the partially transformed cells to evade the self-destructive process and progress to a higher level of transformation, ultimately becoming tumorogenic[51].
4. Many pathogenic bacteria causing chronic infection with intracellular access subvert host cell signaling pathways, enhancing the survival of pathogen[54]. The regulation of these signaling factors is central to the development or inhibition of tumor formation. The precancerous lesion formed in such infections can regress with antibiotic treatment and clearance of bacteria[51].
5. Metabolism of potentially carcinogenic substances by the bacteria. Local microflora may facilitate tumourogenesis by converting ethanol into its carcinogenic derivative, acetaldehyde to levels capable of inducing DNA damage, mutagenesis and secondary hyperproliferation of the epithelium[55, 56].
6. Nitrosation - In which microbial cells catalyze the formation of N-nitroso compounds from the precursor's nitrite and amines, amides

or other nitrosatable compounds.eg; Escherichia coli[51] (Annexure IV, Figure 2).

Oral microbiome and Oral Squamous Cell Carcinoma:

The bacteria present in the tumor area can be causal, coincidental or potentially protective. They bind to and colonize the mucosal surfaces via a "lock and key" mechanism. Adhesins on bacteria bind specifically to complementary receptors on the mucosal surfaces of the host. These adhesins differ from species to species, leading to specificity in attachment to different surfaces. The bacteria that are involved in oral squamous cell carcinoma need to be identified to establish the role of the microorganism in carcinogenesis. The specificity of the bacterial species adhering to tumor mucosa could be due to the presence of their complementary receptors or simply due to the irregular and altered surface of the lesion favouring microbial retention[57].The Table 4 represents the microorganisms isolated from tumor patients in various studies[5].

Table 4

Microorganisms associated with oral cancer	
Bacteria isolated from tumor specimen	Exiguobacterium oxidotolerans, Prevotella melaninogenica, Staphylococcus aureus, Veillonella parvula.
Bacteria isolated with the tumor associated saliva sample	Capnocytophaga gingivalis, Prevotella melaninogenica, Streptococcus mitis.

Recently a number of studies have been taking place on the microbes involved in OSCC:

It was investigated in 2005 whether the salivary counts of 40 common oral bacteria in subjects with an OSCC lesion would differ from those found in cancer-free (OSCC-free) controls. Unstimulated saliva samples were collected from 229 OSCC-free and 45 OSCC subjects in 2005 by Hooper *et al* and evaluated for their content of 40 common oral bacteria using checkerboard DNA–DNA hybridization. It was concluded that high salivary counts of Capnocytophaga gingivalis, Prevotella melaninogenica and Streptococcus mitis may be diagnostic indicators of OSCC[58].

Certain bacterial infections may evade the immune system or stimulate immune responses that contribute to carcinogenic changes through the stimulatory and mutagenic effects of cytokines released by inflammatory cells. Bacterial toxins can kill cells or, at reduced levels, alter cellular processes that control proliferation, apoptosis and differentiation. These alterations are associated with carcinogenesis and may either stimulate cellular aberrations or inhibit normal cell controls[59].

The microbial populations on the oral mucosa differ between healthy and malignant sites and certain oral bacterial species have been linked with malignancies, but the evidence is still weak in this respect. Nevertheless, oral microorganisms inevitably up-regulate cytokines and other inflammatory

mediators that affect the complex metabolic pathways and may thus be involved in carcinogenesis[60].

With the primary objective to identify any bacterial species within the OSCC tissue a study was conducted in 2006 using a standard microbiological culture approach. At the time of surgery, a 1 cu.cm portion of tissue was harvested from deep within the tumor mass using a fresh blade for each cut. Diverse bacterial taxa were isolated and identified, including several putatively novel species. Most isolates were found to be saccharolytic and acid-tolerant species. Notably, some species were isolated only from either the tumour or the non-tumor tissue, indicating a degree of restriction. Successful surface decontamination of the specimens indicates that the bacteria detected were from within the tissue. Diverse bacterial groups have been isolated from within the OSCC tissue. The significance of these bacteria within the tumor warrants further study[61].

In another study the bacterial microbiota present within the oral cancerous lesions, tumorous and non-tumorous mucosal tissue specimens (approximately 1 cm^3) were harvested from ten OSCC patients at the time of surgery. Bacteria were visualized within sections of the OSCC by performing fluorescent in situ hybridization with the universal oligonucleotide probe, EUB338. DNA was extracted from each aseptically macerated tissue specimen using a commercial kit. This was then used as a template for polymerase chain reaction (PCR) with three sets of primers, targeting the 16S rRNA genes of Spirochaetes, Bacteroidetes and the domain bacteria. Differences between the

composition of the microbiotas within the tumoros and nontumoros mucosae were apparent, possibly indicating selective growth of bacteria within the carcinoma tissue. Most taxa isolated from within the tumor tissue represented saccharolytic and aciduric species[62].

The frequency of Streptococcus anginosus infection was assessed in oral cancer tissues and its infection route was investigated where Streptococcus anginosus DNA was frequently detected in squamous cell carcinoma (19/42), but not in other types of cancer (lymphoma and rhabdomyosarcoma) or leukoplakia samples[63].

The most prevalent genera in the OSCC library were concluded as Streptococcus, Gemella, Rothia, Peptostreptococcus, Porphyromonas and Lactobacillus by Pushalkar *et al* in 2011**.** To understand the role of bacteria in the development of oral cancer, the first step is to identify both cultured and uncultured organisms in the saliva as these organisms have the potential to cause inflammation that may support OSCC progression[64].

The microbial flora using cultured saliva and oral swabs from subjects was assessed with OSCC and healthy controls, wherein Metgud *et al* concluded that the median number of colony forming units (CFUs)/mL at the carcinoma site were significantly greater than that at the contralateral healthy mucosa. Similarly, in the saliva of carcinoma subjects, the median number of CFUs/mL were significantly greater than in the saliva of healthy controls[65].

The bacterial spectra on the surface of OSCC was identified in comparison with the oral mucosa of patients with a higher risk to emerge an

OSCC and control group to determine their susceptibility to various common antibiotics by Bolt *et al* in 2014. They concluded from their study that the prominent pathogens of the normal healthy oral mucosa were aerobes. The ratio between aerobes and anaerobes was 2:1, balanced in risk patients and inverted in the OSCC group[3].

SALIVA AS A DIAGNOSTIC TOOL:

Whole saliva is a mixture of fluids produced and secreted by major and minor salivary glands in the mouth and throat. It contains proteins, microorganisms, cellular debris, gingival crevicular fluid, and serum components[66].

The advantage of using saliva is that:

(i) It is non-invasive and many unnecessary biopsies can be avoided.

(ii) Decreases the number of hospital visits for the patient.

Oral cavity provides a diversity of environments for bacterial communities and consequently microbiome profiles differ for various intraoral surfaces. Given that saliva is in direct contact with the oral mucosa and cancerous lesions, the screening and detection of early OSCC lesions using saliva shows promise[67]. Also, salivary microbial profiles tend to reflect the prevalence of bacterial pathogens in adherent oral biofilms. A decrease in the salivary count of pathogens can serve as an indicator of therapeutic effectiveness in the treatment of oral disease[68]. An important advancement in salivary diagnostics is the development of omics-based markers. The term

salivaomics was coined to reflect the rapid development of translational and clinical tools based on salivary biomarkers[69].

There are several molecular techniques that can be used to identify oral microbiota:

Whole genomic checkerboard DNA-DNA hybridization: Hybridization of a selection of labeled whole-genomic DNA probes to sets of sample DNA fixed on a membrane. The specificity of whole-genomic probes is low due to shared genomic sequences with other bacteria. This technique requires cultivable bacteria for constructing the probes and is thus not suitable for studying not-yet cultured- bacteria.

Reverse capture oligonucleotide hybridization: Hybridization of a selection of labeled PCR amplified 16S rDNA segments from sets of samples to species-species oligonucleotide probes fixed on a membrane. The probes have low sensitivity when the target bacteria are present at low levels in the sample.

Fluorescent In-Situ hybridization(FISH): In-situ hybridization of fluorescent labeled 16S rDNA oligonucleotide probes to bacterial cell rRNA in the sample. The oligonucleotide probes have low sensitivity when the target bacteria are present at low levels in the sample.

DNA Microarray: Hybridization of labeled DNA sequences in the sample to target-specific oligonucleotides fixed on a membrane/glass slide. When there are a multitude of unknown bacteria that still lack probes in the

arrays it is difficult to obtain, without DNA amplification, enough material from target bacteria found at low levels in a background of other bacteria.

16S rRNA gene sequencing: The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes[70].

METAGENOMIC SEQUENCING:

Metagenomics is analysis of microorganisms by direct extraction of DNA from all genomes within a sample[71]. Currently 16S rRNA sequencing has been solely used as a research tool. The ubiquitous and phylogenetically stable bacterial 16S rRNA which is 1500 bp (base pairs) long offers a very useful target for the identification of bacteria down to species level. The 16S ribosomal subunit has highly conserved regions between all bacterial species between which highly variable regions (V1-V9) are present that are used to identify specific bacteria. Universal primers are designed to amplify a specific variable region, of which the most commonly targeted regions are V3, V4 and V6[72]. After sequencing all bacteria are clustered based on their genetic similarity thus representing an Operational Taxonomic Unit (OTU). Grouping at 97% similarity allows identification at species level, while 94% allows for

genus level identification of bacteria[71,73]. This potential to use rapid sequencing in order to understand the impact of bacteria on diseases is huge and becoming increasingly relevant[73].

Results

RESULTS

Ten saliva samples from Oral Squamous Cell Carcinoma patients and saliva samples from ten healthy individuals (controls) were obtained. All samples were subjected to 16S rRNA gene sequencing.

DISTRIBUTION OF AGE IN THE STUDY GROUPS: (TABLE 1 & GRAPH 1):

The distribution of age of the patients was divided into 3 groups: 20-40 years, 41-60 years and those above 61 years of age. OSCC group consisted of 3 (30%) patients in the age group 20-40 years, 5(50%) patients in the age group of 41-60 and 2 (20%) patients in the age group above 61 years. Control group consisted of 4 (40%) cases in 20-40 years, 5 (50%) cases in 41-60 years and 1 (10%) cases above 61 years (p=0.788).

DISTRIBUTION OF GENDER IN THE STUDY GROUPS: (TABLE 2 &GRAPH 2):

In OSCC group, 8 (80%) were males and 2 (20%) were females. In Control group, 1(10%) was male and 9(90%) were females.

DISTRIBUTION OF HABITS IN THE STUDY GROUPS (TABLE 3 & GRAPH 3):

Based on the prevalence of habits in the study groups, they were categorized in to five groups. They were those without any habits, those with

habit of, chewing tobacco alone, chewing tobacco and consuming alcoholic beverages, smoking alone, consuming alcoholic beverages alone. In group I (control group) none of them had any habits. In OSCC group, there were 2(20%) who had no habits, 5 (50%) with habit of chewing tobacco alone, 1 (10%) had the habit of chewing tobacco and consuming alcoholic beverages, 1 (10%) had the habit of smoking & consuming alcoholic beverages and 1(10%) had the habit of consuming alcoholic beverages alone (p=0.010).

DISTRIBUTION OF SITE OF THE LESION IN THE OSCC GROUP (TABLE 4 & GRAPH 4):

In the OSCC group, 3(30%) had the lesion in buccal mucosa, 2(20%) had the lesion in tongue, 2(20%) had the lesion in maxillary alveolus, 1(10%) had the lesion in mandibular alveolus, 1(10%) had the lesion in oropharynx and 1(10%) had the lesion in hard palate.

SEQUENCING RESULTS OF THE STUDY:

CHART 1- Master microbiome chart represents the distribution of overall phyla of microbes present in all the samples.

CHART 2 – OSCC group microbiome chart represents the distribution of phyla of all the microbes present in OSCC group.

CHART 3 – Control group microbiome chart represents the distribution of phyla of all the microbes present in the control group.

TABLE 5 - Most predominant bacterial genera present under the major phyla.

GRAPH 5 (a, b, c) - Frequency of bacterial genera occurence common in the OSCC and control group.

TABLE 6 - Most predominant bacterial genera in the OSCC and control group.

TABLE 6A - Distribution of predominant bacterial genera in the OSCC group with respect to site.

All twenty samples were subjected to 16S rRNA gene sequencing in the variable region V5-V6. A total of 1900 sequences were obtained for all the samples with an average of 100 sequences per sample. One of the sample (control) did not show any sequences due to low DNA concentration.

A total of nineteen phyla (Chart 1) were identified of which Proteobacteria (39%), Firmicutes (22%), Actinobacteria (15%) and Bacteroidetes(12%) were the major phyla. The most predominant genera present under the four above mentioned major phyla are represented in Table 5. Other phyla include Euryarchaeota, Spirochaetes, Tenericutes, Cyanobacteria, Verrucomicrobia, Planctomycetes, Nitrospirae, Fusobacteria, Chloroflexi, Vulcanisaeta, Desulfurobacterium, Deinococcus, Flexistipes, Caldithrix, Solemya. Proteobacteria was the major phyla present in both OSCC patients and healthy individuals (Chart 2 and Chart 3). The combined groups (OSCC and healthy individuals) showed 569 bacterial genus with

299 bacterial genus in OSCC group and 270 bacterial genus in healthy individuals (control) group (Annexure V).

The most prevalent bacteria present in OSCC patients were Bacillus, Bacterium, Buchnera, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Flavobacterium, Gemmata, Hymenobacter, Lactobacillus, Listeria, Lysinibacillus, Marinifilum, Ruminococcus, Streptococcus, Streptomyces, and Thermoanaerobacter.

In healthy individuals the prevalent bacteria were Bacillus, Enterococcus, Lactobacillus, Massilia, Paenibacillus, Streptococcus. The predominant bacteria that are common in OSCC patients and in healthy individuals are Bacillus, Enterococcus, Lactobacillus and Streptococcus. (Table 6 and Graph 5 (a,b,c)).

Based on the site of the lesion, Streptococcus was the predominant bacteria present in all the sites (tongue (10%), buccal mucosa (20%), alveolus (10%), palate (20%). In OSCC patients, Streptomyces was seen both in alveolus (20%) and tongue (20%) whereas Bacillus and Listeria were seen only in the alveolar lesions (30%) (Table 6A)

INDIVIDUAL SAMPLE RESULTS (ANNEXURE VI):

SAMPLE O-1

- In the OSCC sample (O-1) top 100 sequences producing significant alignments were taken into consideration for the study in which the

bacterial sequences showing minimum identity of 70% and more were selected.

- The 100 sequences majorly belonged to Uncultured bacteria and Neisseria.
- We found that all the bacterial strains present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 1e-82 and ends at a value of 9e-04.

SAMPLE O-2

- In the Control sample (O-2) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Listeria, Bacillus and Streptococcus.
- We found that the bacterial species present in the sample belong to the phyla Firmicutes . The Expectation value (E) starts from 0.004 and ends at a value of 8e-04.

SAMPLE O-3

- In the OSCC sample (O-3) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Listeria, Gemmatta, Colwellia and Mycobacterium.
- We found that the bacterial species present in the sample belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Calditrichaeota, Euryarchaeota, Mollusca,

Cyanobacteria, Nitrospirae, Verrucomicrobia . The Expectation value (E) starts from 0.004 and ends at a value of 8e-04.

SAMPLE O-4

- In the Control sample (O-4) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Vitreoscilla, Neisseria, Acidovorax, Lampropedia, Simonsiella, Herbaspirillum and Parabulkholderia.
- We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 4e-15 and ends at a value of 3e-05.

SAMPLE O-5

- In the OSCC sample (0-5) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Shewanella, Candidatus, Serratia, Buchnera, Marinomonas, Pantoea and Methylomicrobium.
- We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 3e-49 and ends at a value of 9e-43.

SAMPLE O-6

- In the Control sample (0-6) top 100 sequences producing significant alignments were taken into consideration for the study in which the

bacterial sequences showing minimum identity of 70% and more were selected.

- The 100 sequences majorly belonged to uncultured bacteria.
- We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 1e-82 and ends at a value of 2e-80.

SAMPLE O-7

- In the OSCC sample (0-7) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Lactobacillus, Streptococcus, Enterococcus, Weissella, Carnobacterium and Listeria.
- We found that all the bacterial species present in the sample belong to the phylum Firmicutes. The Expectation value (E) starts from 1e-54and ends at a value of 8e-50.

SAMPLE O-8

- In the Control sample (0-8) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to uncultured bacteria and Neisseria.
- We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 1e-95 and ends at a value of 6e-95.

SAMPLE O-9

- In the OSCC sample (0-9) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Neisseria, Kingella and Vitreoscilla.

  We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 1e-91 and ends at a value of 9e-81.

SAMPLE O-10

- In the Control sample (0-10) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Coprococcus, Ruminococcus and Lachnospiraceae.
- We found that all the bacterial species present in the sample belong to the phylum Firmicutes. The Expectation value (E) starts from 0.001 and ends at a value of 6.9.

SAMPLE O-11

- In the OSCC sample (O-11) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Flavobacterium, Clostridium, Enterococcus, Lacinutrix, Streptomyces and Carnobacterium.

- We found that the bacterial species present in the sample belong to 12 phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Tenericutes, Euryarchaeota, Planctomycetes, Defferibacteres, Aquificae, Crenarchaeota, Deinococcus thermus . The Expectation value (E) starts from 0.37 and ends at a value of 16.

SAMPLE O-12

- No results were obtained due to very low DNA concentration in the sample.

SAMPLE O-13

- In the OSCC sample (O-13) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Flavobacterium, Clostridium, Enterococcus, Lacinutrix, Streptomyces and Carnobacterium.
- We found that the bacterial species present in the sample belong to 12 phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Tenericutes, Euryarchaeota, Planctomycetes, Defferibacteres, Aquificae, Crenarchaeota, Deinococcus thermus . The Expectation value (E) starts from 0.003 and ends at a value of 7e-04.

SAMPLE O-14

- In OSCC sample (O-14) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.

- The 100 sequences majorly belonged to Flavobacterium, Clostridium, Enterococcus, Lacinutrix, Streptomyces and Carnobacterium.
- We found that the bacterial species present in the sample belong to 12 phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Tenericutes, Euryarchaeota, Planctomycetes, Defferibacteres, Aquificae, Crenarchaeota, Deinococcus thermus . The Expectation value (E) starts from 0.003 and ends at a value of 9e-08.

SAMPLE O-15

- In the Control (O-15) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Geodermatophilus, Streptomyces and Microbacterium.
- We found that the bacterial species present in the sample belong to the phylum Firmicutes . The Expectation value (E) starts from 0.26 and ends at a value of 7.0.

SAMPLE O-16

- In the Control sample (O-16) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Paenibacillus, Bacillus and Lactobacillus.
- We found that the bacterial species present in the sample belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, Planctomycetes. The Expectation value (E) starts from 0.33 and ends at a value of 33.

SAMPLE O-17

- In the Control sample (O-17) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Actinoplanes, Janibacter, Marinomonas, Kitasatospora.
- We found that the bacterial species present in the sample belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, , Tenericutes, Euryarchaeota, Planctomycetes . The Expectation value (E) starts from 0.098 and ends at a value of 42.

SAMPLE O-18

- In the Control sample (O-18) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Flavobacterium, Clostridium, Enterococcus, Lacinutrix, Streptomyces and Carnobacterium.
- We found that the bacterial species present in the sample belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Euryarchaeota, Cyanobacteria, Nitrospirae, Spermatophyta and Fusobacteria. The Expectation value (E) starts from 1.1 and ends at a value of 25.

SAMPLE O-19

- In the OSCC sample (O-19) top 100 sequences producing significant alignments were taken into consideration for the study in which the

bacterial sequences showing minimum identity of 70% and more were selected.

- The 100 sequences majorly belonged to Mycoplasma, Gemmatta and Listeria.
- We found that the bacterial species present in the sample belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Euryarchaeota, Tenericutes, Planctomycetes and Fusobacteria . The Expectation value (E) starts from 0.003 and ends at a value of 6e-04.

SAMPLE O-20

- In the OSCC sample (O-20) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected.
- The 100 sequences majorly belonged to Listeria, Gemmatta, Colwellia and Mycobacterium.
- We found that the bacterial species present in the sample belong to 12 phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Tenericutes, Euryarchaeota, Planctomycetes, Chloroflexi, Nitrospirae, Verrucomicrobia . The Expectation value (E) starts from 0.004 and ends at a value of 8e-04.

Tables, graphs and charts

TABLE 1: DISTRIBUTION OF AGE IN STUDY GROUPS (N=20)

AGE GROUPS IN YEARS	OSCC GROUP	CONTROL GROUP	p-value
20- 40	3 (30%)	4 (40%)	0.788
41-60	5 (50%)	5 (50%)	
ABOVE 61	2 (20%)	1 (10%)	

GRAPH 1: DISTRIBUTION OF AGE IN STUDY GROUPS (N=20)

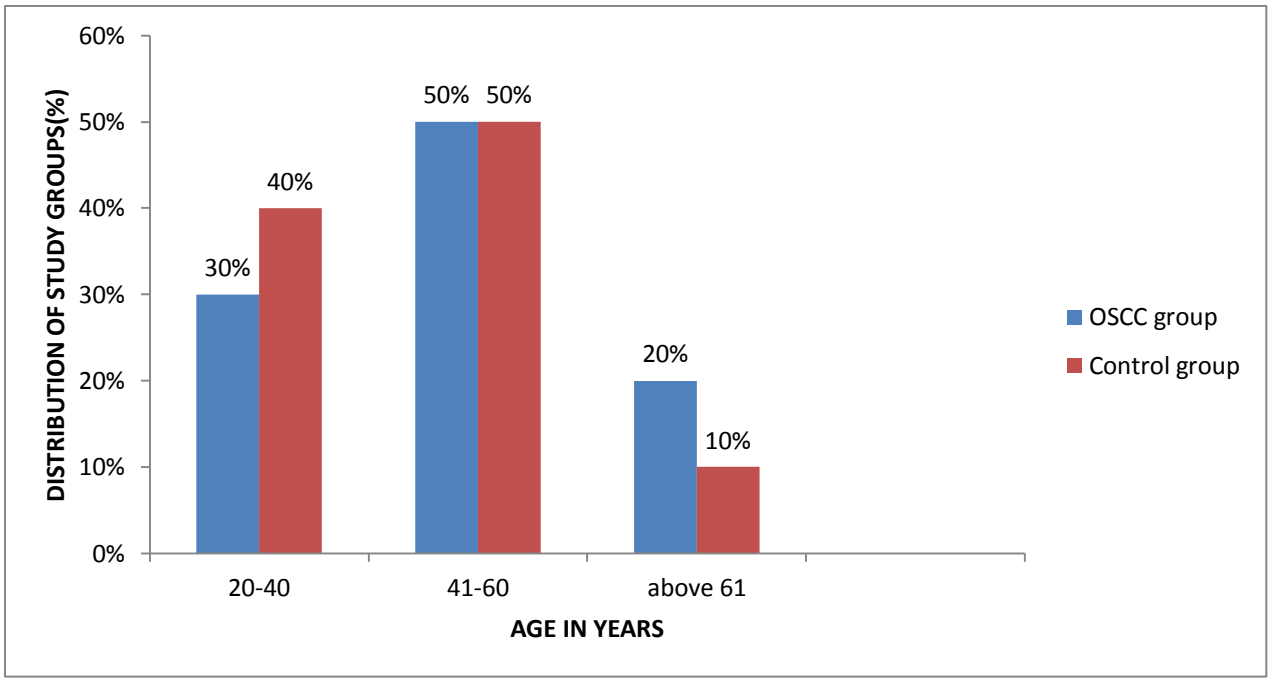


TABLE 2: DISTRIBUTION OF GENDER IN STUDY GROUPS (N=20)

GENDER	OSCC GROUP n=10	CONTROL GROUP n=10
MALE	8 (80%)	1 (10%)
FEMALE	2 (20%)	9 (90%)

GRAPH 2: DISTRIBUTION OF GENDER IN STUDY GROUPS (N=20)

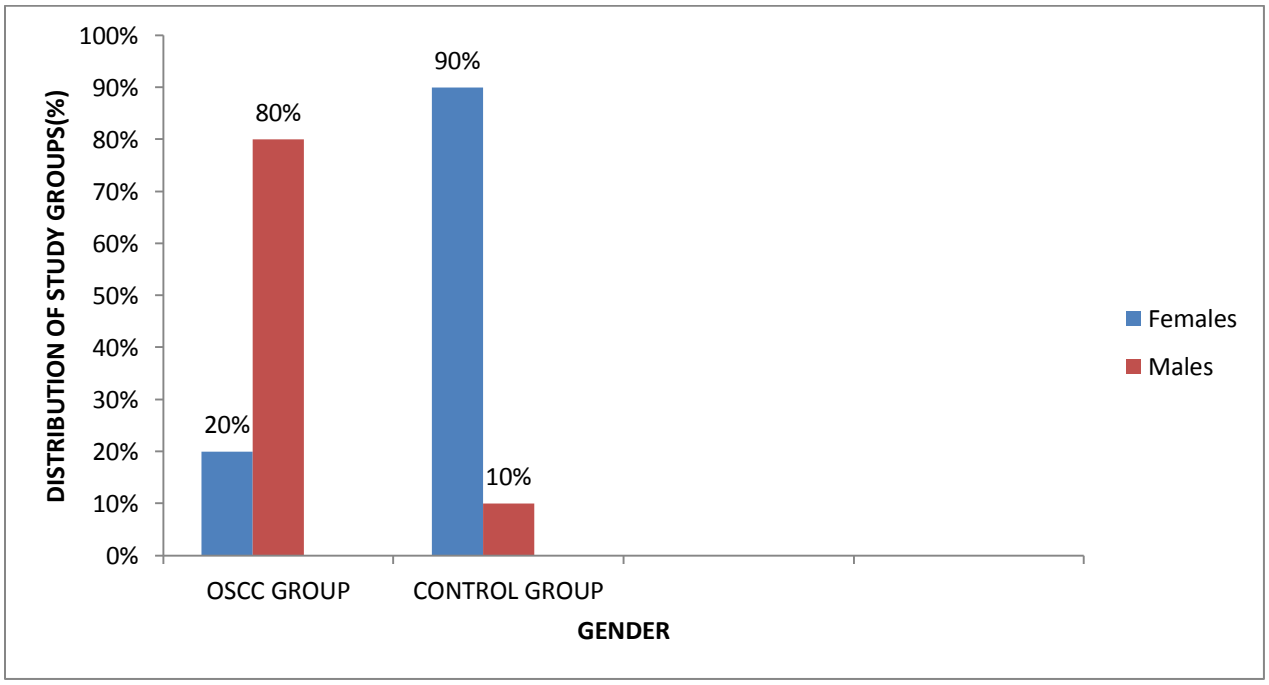


TABLE 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS (N=20)

HABITS	OSCC GROUP n=10	CONTROL GROUP n=10	p-value
NO HABITS	2 (20%)	10 (100%)	0.010*
CHEWING ALONE(C)	5 (50%)	0	
CHEWING+ALCOHOL(C+A)	1(10%)	0	
SMOKING+ALCOHOL(S+A)	1(10%)	0	
ALCOHOL ALONE(A)	1(10%)	0	

*** p<0.05 is significant**

GRAPH 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS (N=20)

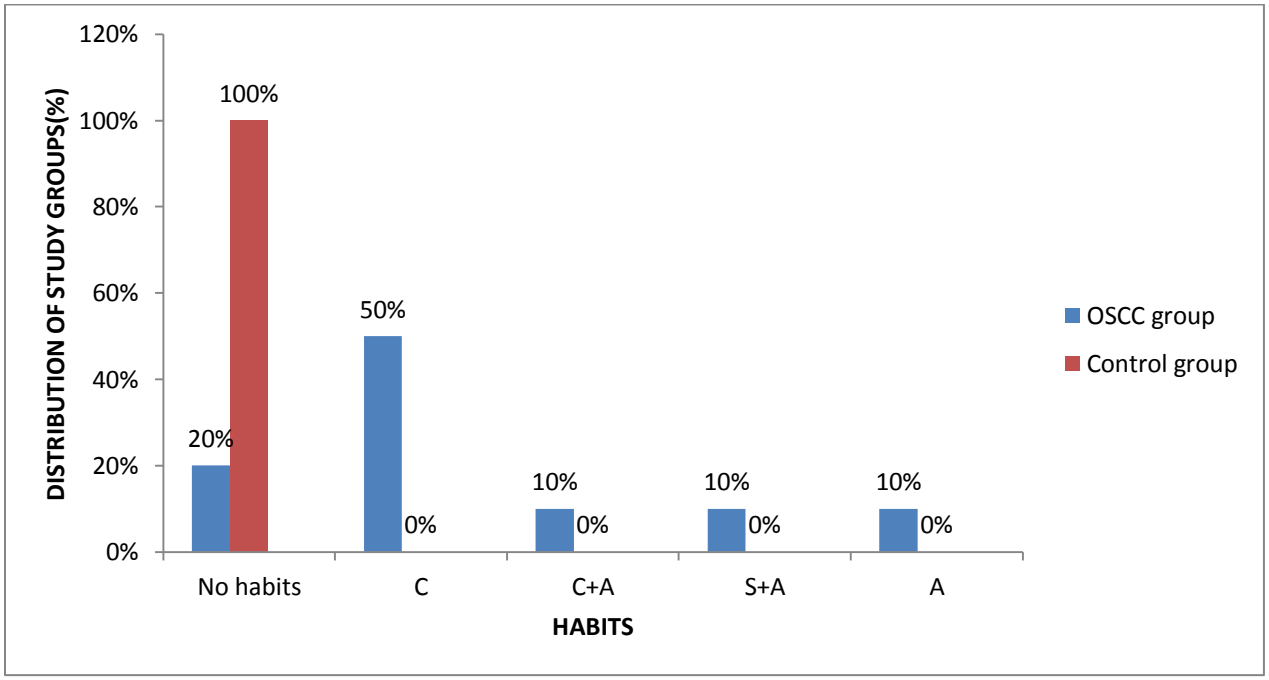


TABLE 4: DISTRIBUTION OF SITE OF LESION IN THE OSCC GROUP (N= 10)

SITE OF LESION	OSCC GROUP
BUCCAL MUCOSA	3 (30%)
TONGUE	2 (20%)
MANDIBULAR ALVEOLUS	2 (20%)
MAXILLARY ALVEOLUS	1 (10%)
OROPHARYNX	1 (10%)
HARD PALATE	1 (10%)

GRAPH 4: DISTRIBUTION OF SITE OF LESION IN THE OSCC GROUP (N= 10)

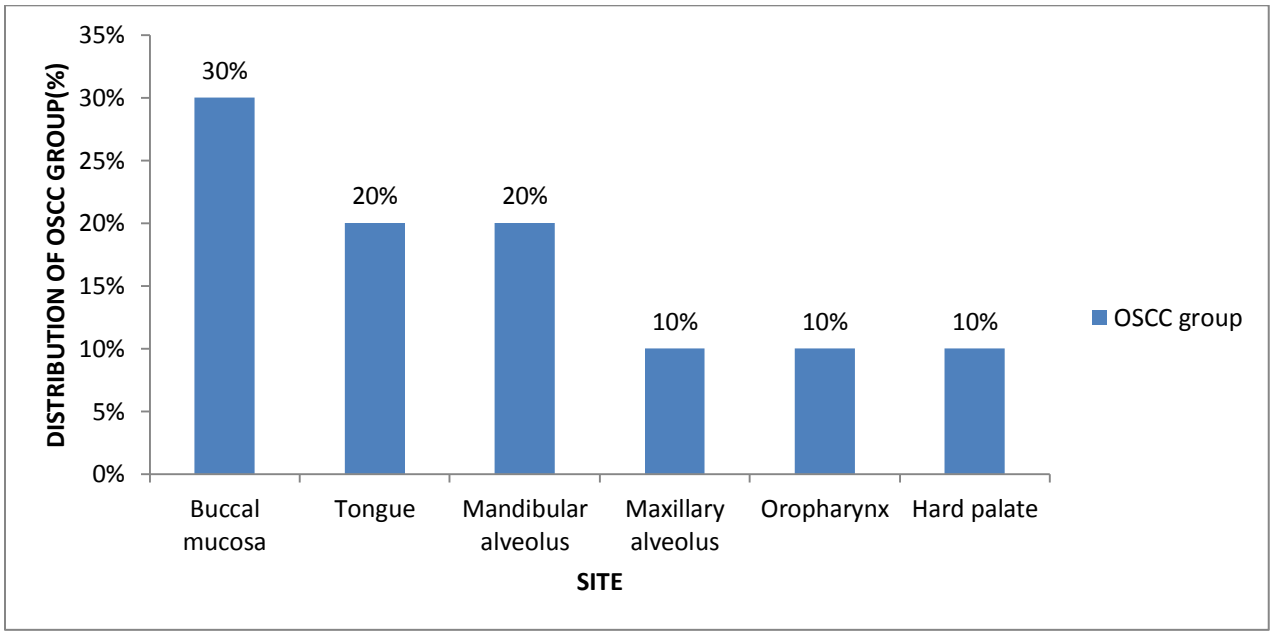


CHART 1: MASTER MICROBIOME CHART

DISTRIBUTION OF OVERALL PHYLA OF MICROBES PRESENT IN ALL THE SAMPLES (N=20)

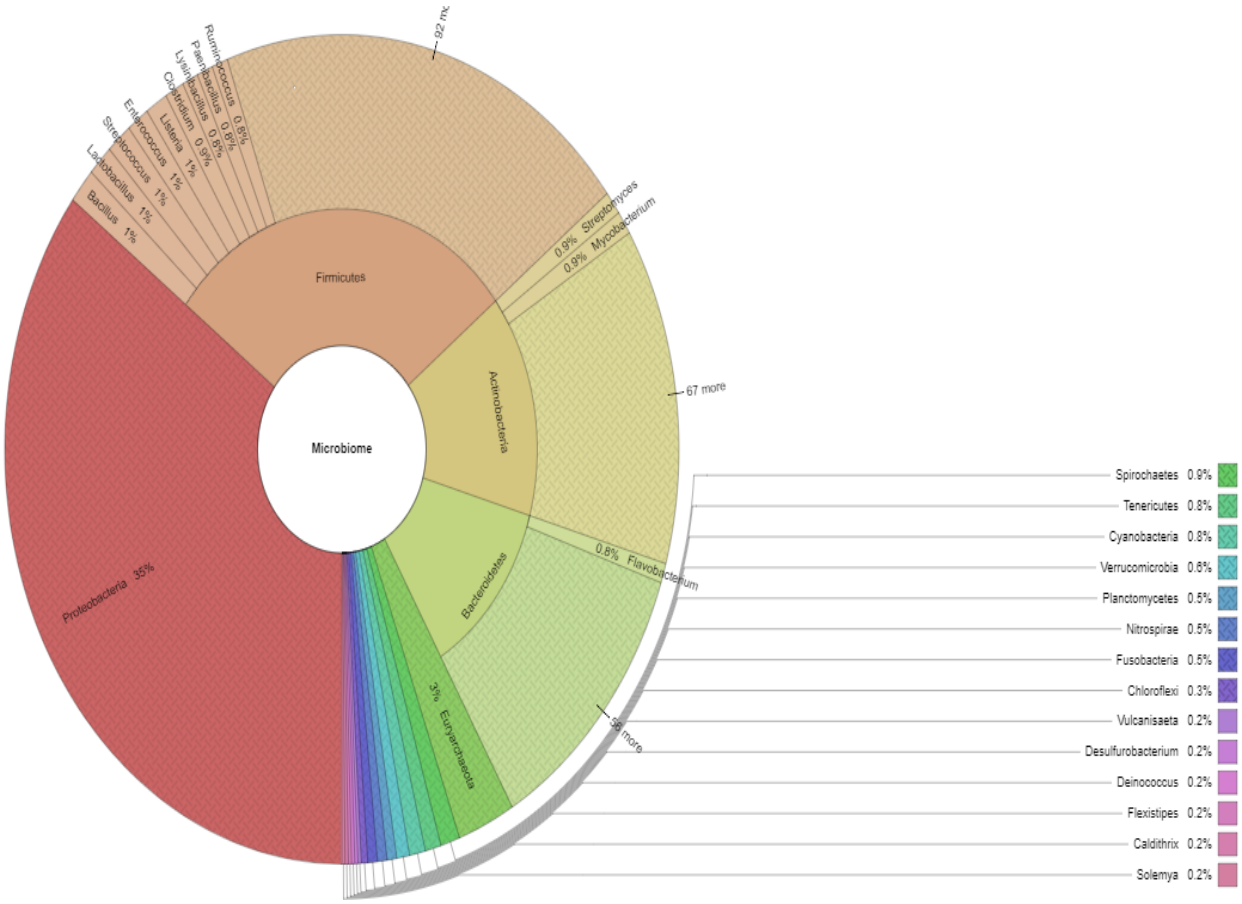


TABLE 5: MOST PREDOMINANT BACTERIAL GENERA PRESENT UNDER THE MAJOR PHYLA

Proteobacteria		**Firmicutes**		**Actinobacteria**		**Bacteroidetes**	
Acinetobacter	*	Aerococcus	$	Actinoplanes	*	Flavobacterium	$
Buchnera	*	Aphanizomenon	#	Corynebacterium	*	Hymenobacter	*
Campylobacter	$	Bacillus	$	Microbacterium	*	Marinifilum	$
Chryseobacterium	$	Carnobacterium	#	Mycobacterium	*	Prevotella	#
Mannheimia	*	Clostridium	#	Streptomyces	*		
Massilia	*	Enterococcus	$				
Moraxella	*	Granulicatella	$				
Photobacterium	$	Lactobacillus	$				
Rhizobium	*	Listeria	$				
Xenorhabdus	*	Lysinibacillus	*				
		Melisococcus	#				
		Paenibacillus	*				
		Ruminococcus	*				
		Streptococcus	$				
		Tetragenococcus	$				
		Thermoanaerobacter	#				
		Vagococcus	$				

*** - Aerobe**

#- Anaerobe

$ - Facultative anaerobe

CHART 2: OSCC GROUP MICROBIOME CHART

DISTRIBUTION OF PHYLA OF ALL THE MICROBES PRESENT IN THE OSCC GROUP (N=10)

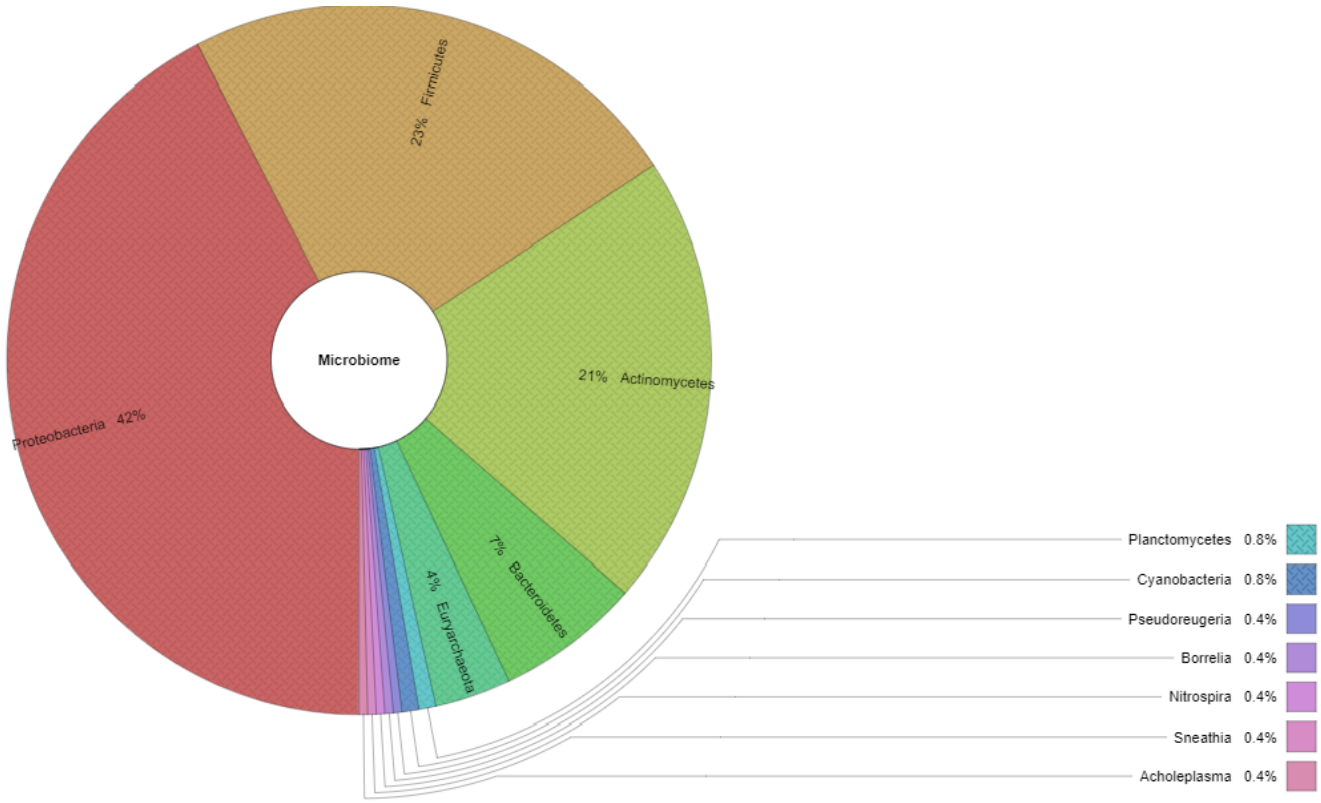
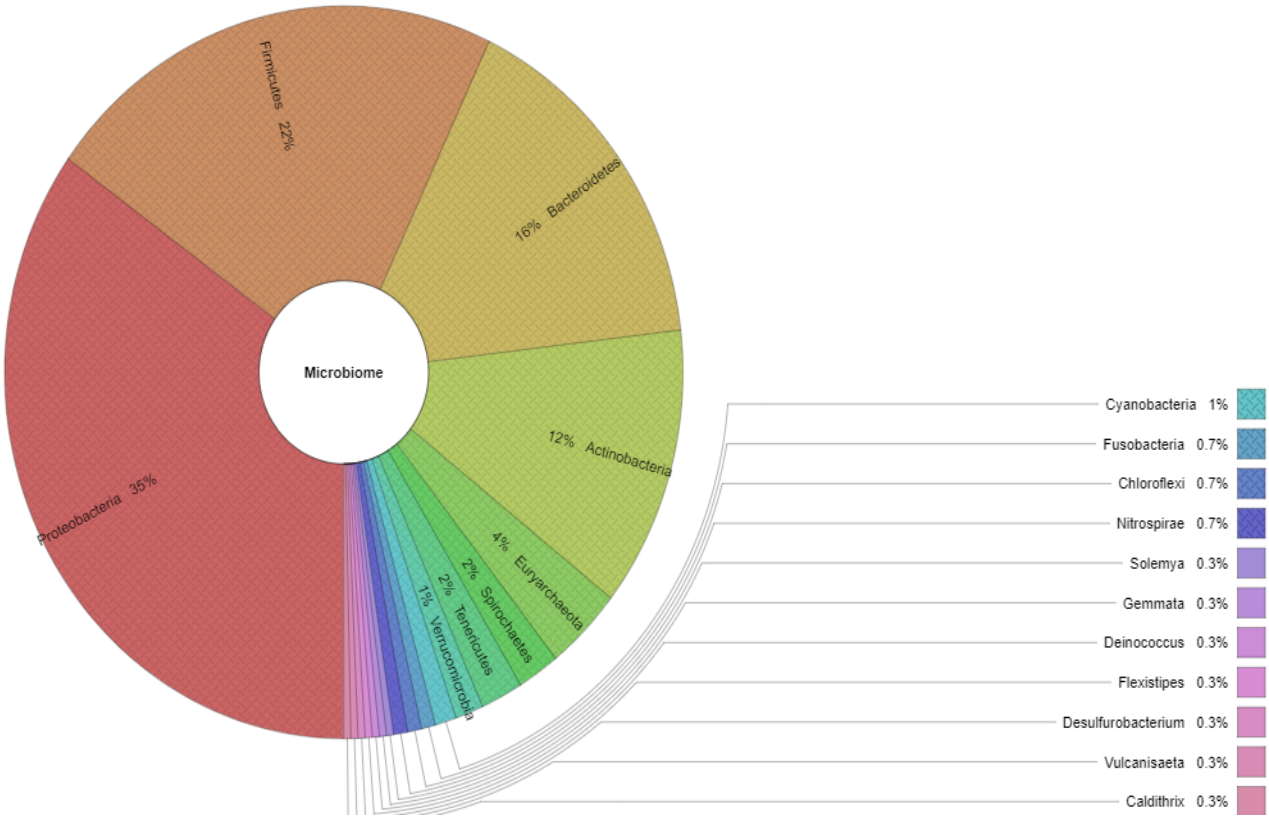
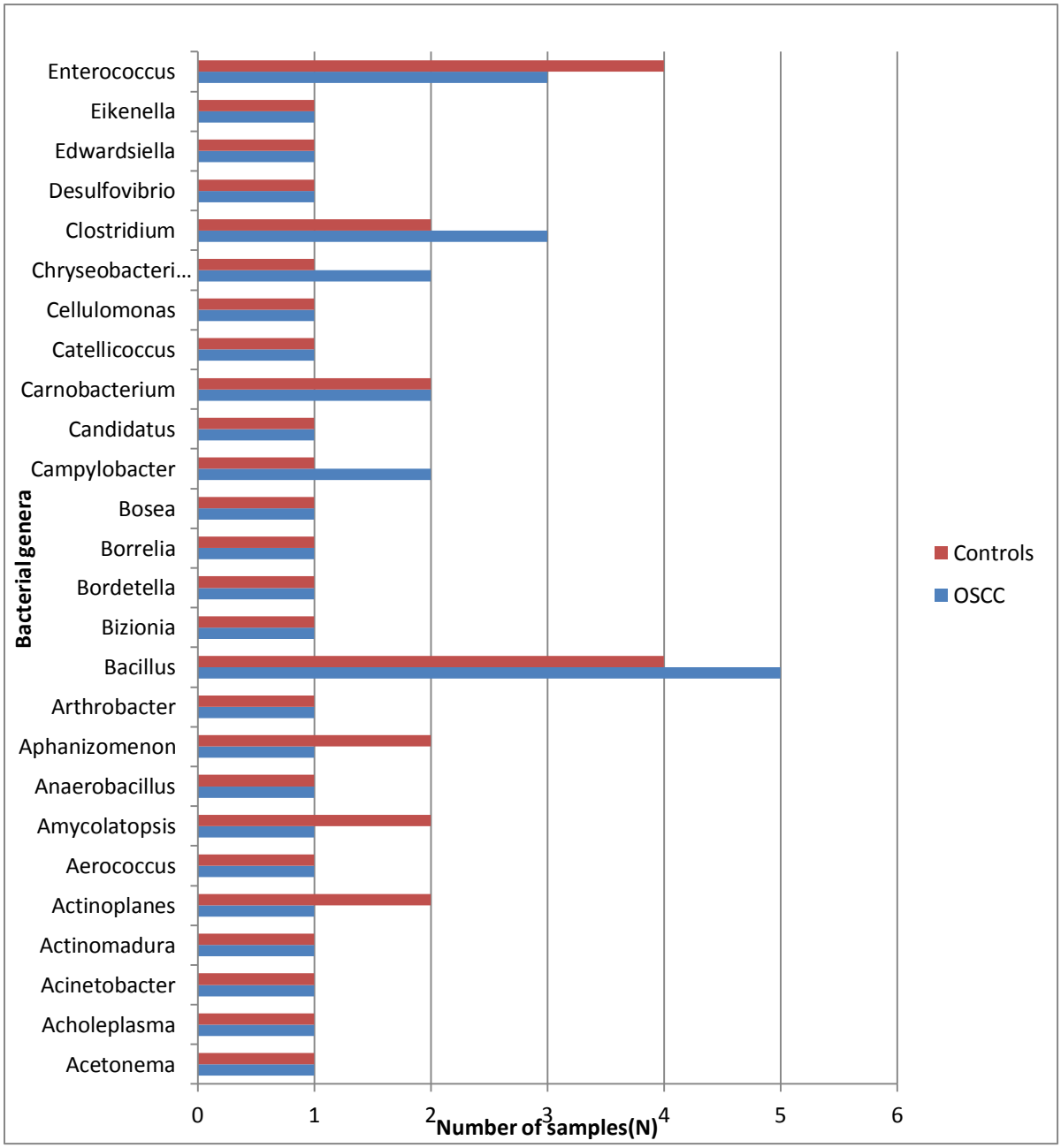


CHART 3: CONTROL GROUP MICROBIOME CHART

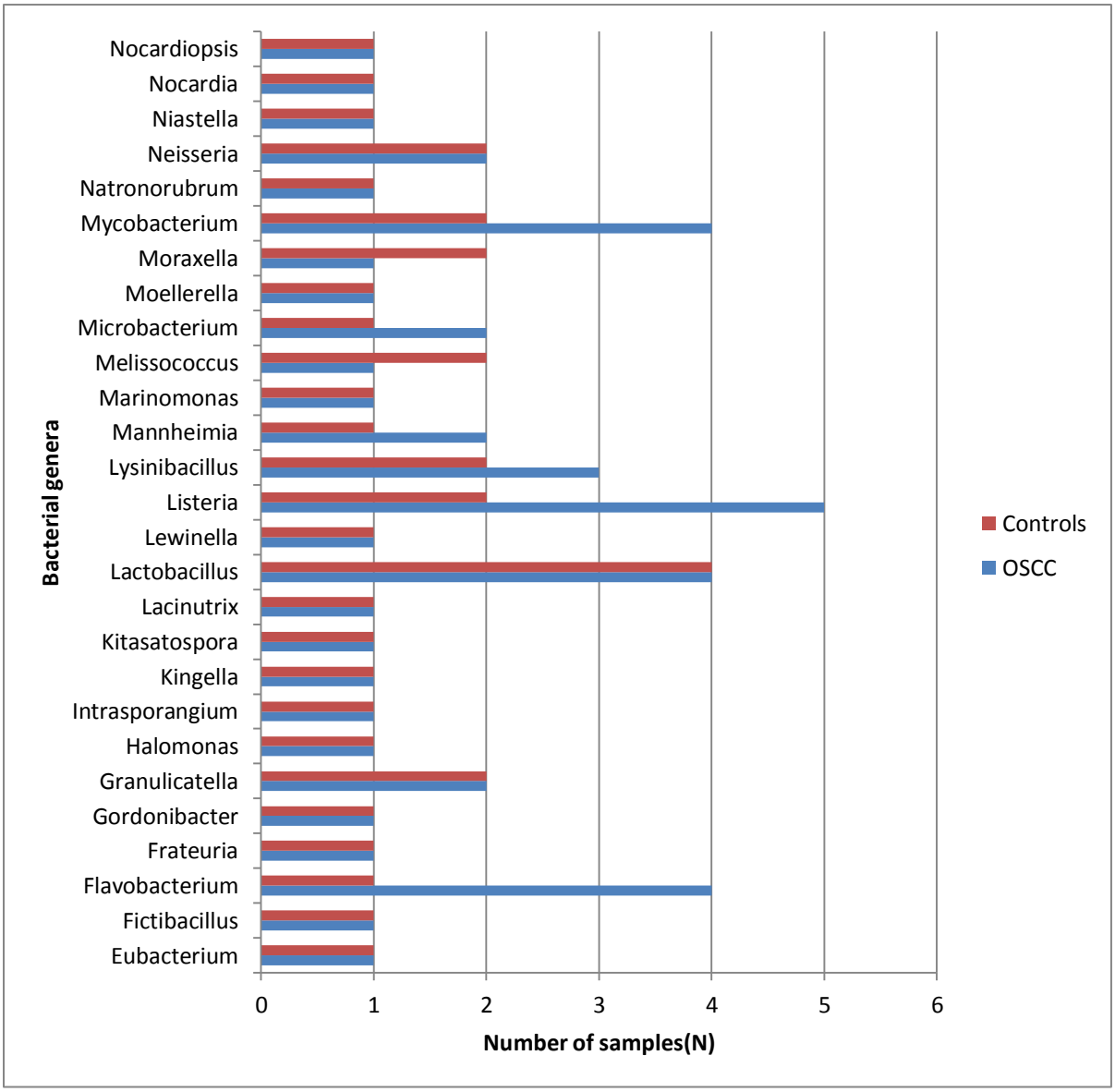
DISTRIBUTION OF PHYLA OF ALL THE MICROBES PRESENT IN THE CONTROL GROUP (N=10)



GRAPH 5 (a): FREQUENCY OF BACTERIAL GENERA OCCURENCE COMMON IN THE OSCC AND CONTROL GROUP (N=20)



GRAPH 5 (b): FREQUENCY OF BACTERIAL GENERA OCCURENCE COMMON IN THE OSCC AND CONTROL GROUP (N=20)



GRAPH 5 (c): FREQUENCY OF BACTERIAL GENERA OCCURENCE COMMON IN THE OSCC AND CONTROL GROUP (N=20)

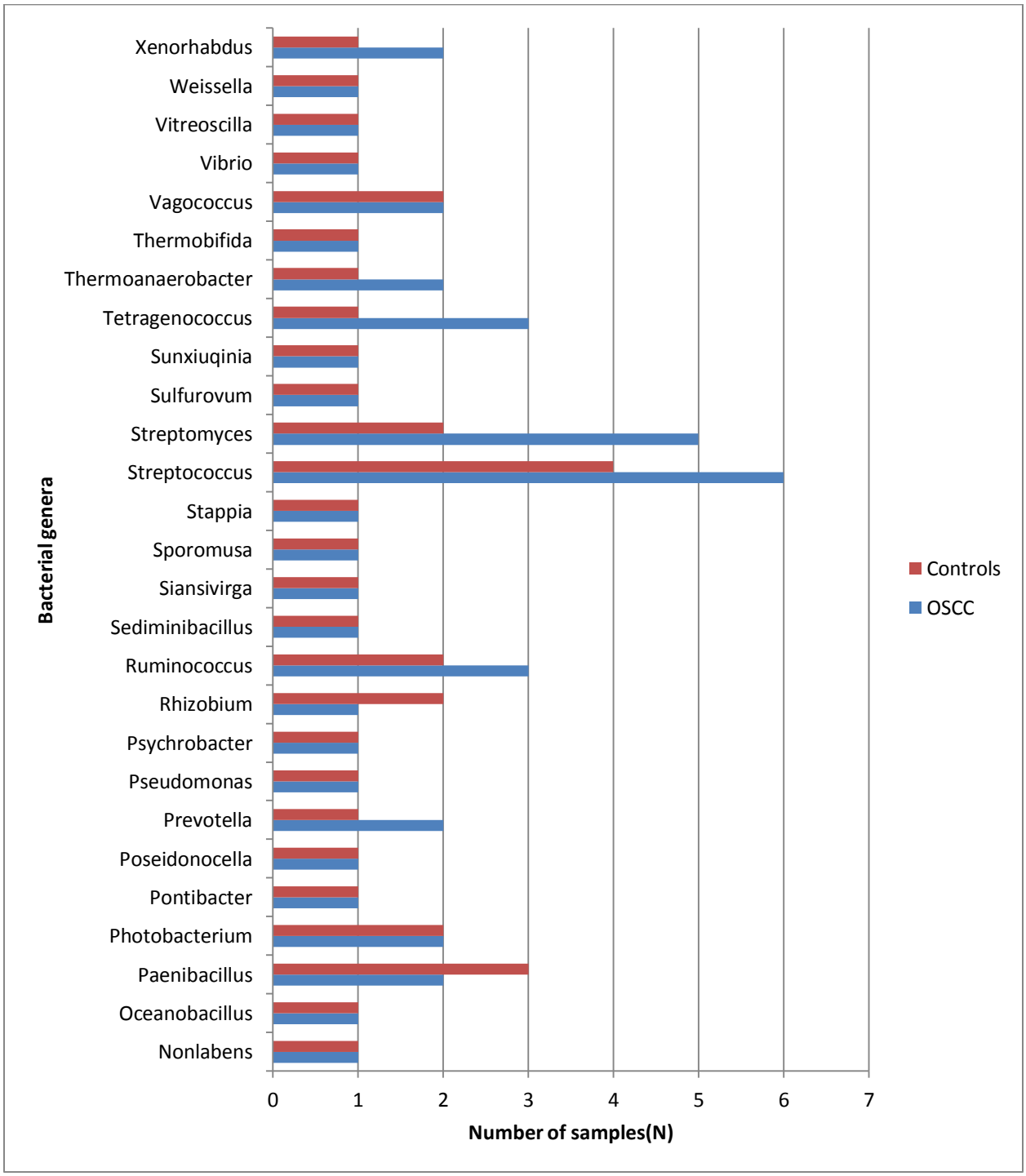


TABLE 6: MOST PREDOMINANT BACTERIAL GENERA IN THE OSCC AND CONTROL GROUP

OSCC GROUP(n=10)		Number of samples(n)	CONTROL GROUP(n=10)		Number of samples(n)
Bacillus	$ (S) (A)	5	Bacillus	$ (S) (A)	4
Bacterium	$ (S)	3	Enterococcus	$ (S) (A)	4
Buchnera	* (S)	4	Lactobacillus	$ (S) (A)	4
Caulobacter	* (A)	3	Massilia	*	3
Clostridium	* (S) (A)	3	Paenibacillus	*	3
Corynebacterium)	* (S) (A)	3	Streptococcus	$ (S) (A)	4
Desulfutomaculum	# (S) (A)	3			
Enterococcus	$ (S) (A)	3			
Flavobacterium	$ (S)	4			
Gemmata	* (S)	4			
Hymenobacter	* (S)	3			
Lactobacillus	$ (S) (A)	4			
Listeria	$ (S) (A)	5			
Lysinibacillus	* (A)	3			
Marinifilum	$	4			
Ruminococcus	# (S) (A)	3			
Streptococcus	$ (S) (A)	6			
Streptomyces	* (S) (A)	5			
Thermoanaerobacter	# (S) (A)	3			

S- Saccharolytic *** - Aerobe, # - Anaerobe, $ - Facultative anaerobe**

A- Aciduric **Red font – Common to both groups.**

TABLE 6A: DISTRIBUTION OF PREDOMINANT BACTERIAL GENERA IN THE OSCC GROUP WITH RESPECT TO SITE (n=10)

Predominant bacteria in OSCC group	Tongue	Buccal mucosa	Alveolus	Hard palate+Oropharynx
Streptococcus	**1(10%)**	**2(20%)**	**1(10%)**	**2(20%)**
Streptomyces	**2(20%)**	**-**	**2(20%)**	**-**
Bacillus	**-**	**1(10%)**	**3(30%)**	**1(10%)**
Listeria	**1(10%)**	**1(10%)**	**3(30%)**	**-**

Discussion

DISCUSSION

Metagenomics is the analysis of microbial DNA from all genomes within a sample[71] .In this study twenty samples of Oral Squamous Cell Carcinoma (OSCC) patients and healthy controls were studied using Metagenomic Sequencing (16S rRNA gene sequencing). Other methods which are commonly used are whole genomic checkerboard DNA-DNA hybridization, reverse capture oligonucleotide hybridization, Fluorescent In situ Hybridization (FISH) technique and DNA microarray. We used the 16S rRNA sequencing technique as it is useful in identifying unusual bacteria that are difficult to identify by conventional methods, provides genus identification in >90% of cases, and identifies 65–83% of these at the species level. The advantage of the 16S rRNA gene-based analysis is that it bypasses culturing of bacteria as PCR detection is done on DNA extracted from crude samples. The direct amplification of the 16S rRNA gene from DNA samples helps to detect unculturable bacteria which are estimated to exceed 99% of microorganisms observable in nature. Many novel species can be identified by this process of bacterial identification, when there is a significant difference between the phenotypic characteristics and/or 16S rRNA sequences of the unknown bacterium and those of the most closely related ones. As no single test or gene sequence is ideal for the definition of new species in all groups of bacteria, a polyphasic approach is usually used when a novel species is defined[70].

Although 16S rRNA gene sequencing is highly useful in regards to bacterial classification, it has relatively low phylogenetic power at the species level and poor discriminatory power for some genera, as there is no known universal definition for species identification. However, 16S rRNA gene sequencing technique is widely used for establishing a "species" match. Issues to be considered in Small Sub Unit gene sequencing include the number of position ambiguities, sequence gaps, and use of gap and/or nongapped programs with regard to sequence evaluation and analysis. The difficulties that can affect final identification include isolate purity, problems with DNA extraction protocols, and possible chimeric molecule formation[71].

The use of 16S rRNA gene sequencing for definitive microbial identifications requires a harmonious set of guidelines for interpretation of sequence data. The automation of 16S rRNA sequencing is not available yet and interpretation of results often needs significant expertise. In this study, we used the BLAST (Basic Local Alignment Search Tool) analysis with NCBI (National Centre for Biotechnology Information) database, which is a widely used database.

The 16S rRNA gene is conserved and therefore allows design of universal primers. In our study, we have used universal primer for amplifying the 16S rRNA gene. A single pair of the 16S rRNA gene universal primers is capable of amplifying the 16S rRNA gene from diverse bacterial taxa[72].

The universal primer used in our study were forward primer: AGTTTGATC [A/C]TGGCTCAG and reverse primer: GGACTAC[C/T/A]AGGGTATCTAAT.

The oral microbial diversity assessed in OSCC patients by Pushalkar *et al* showed members of eight phyla (divisions) of bacteria. The majority of sequences in combined libraries belonged to Firmicutes *(*45%) and Bacteroidetes (25%). The phylum Firmicutes was the most abundant in the OSCC library as compared with the control library. The other phyla represented in both libraries are Actinobacteria, Proteobacteria, Fusobacteria, SR1, Spirochaete and uncultured TM7 [64].

In our study, a total of 19 phyla were identified of which Proteobacteria(39%), Firmicutes(22%), Actinobacteria(15%) and Bacteroidetes(12%) were the major ones. Other phyla were Euryarchaeota, Spirochaetes, Tenericutes, Cyanobacteria, Verrucomicrobia, Planctomycetes, Nitrospirae, Fusobacteria, Chloroflexi, Vulcanisaeta, Desulfurobacterium, Deinococcus, Flexistipes, Caldithrix, Solemya. The major phyla present in both OSCC patients and healthy individuals was Proteobacteria.

The high prevalence of Firmicutes as reported by Pushalkar *et al*,was not seen in our study. This difference could be due to the use of both saliva and tumor samples by Pushalkar *et al*[63]. In our study, saliva samples were only analysed with the aim of assessing saliva as a diagnostic tool for OSCC.

Similarly, in another study using saliva samples, high levels of colonization of OSCC by facultative oral streptococci and by species of

anaerobic bacteria (Prevotella, Veillonella, Porphyromonas, Streptococcus anginosus, and Capnocytophaga) were demonstrated relative to uninvolved mucosa[64]. The most predominant bacteria present in OSCC patients in our study were Bacillus, Bacterium, Buchnera, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Flavobacterium, Gemmata, Hymenobacter, Lactobacillus, Listeria, Lysinibacillus, Marinifilum, Ruminococcus, Streptococcus, Streptomyces, Thermoanaerobacter. In our controls the predominant bacteria were Bacillus, Enterococcus, Lactobacillus, Massilia, Paenibacillus, Streptococcus. Bacterial genera uniquely found in control group were Massilia and Paenibacillus. It is known that absence of certain bacteria can be responsible for shift in the microbial homeostasis, with alteration leading to the pathogenic bacterial overgrowth in OSCC patients.

In our study, the most common bacteria in OSCC and the control group were Bacillus, Enterococcus, Lactobacillus and Streptococcus. Streptococcus species such as S. salivarius, S. intermedius, S. mitis and non-pathogenic Neisseria species are known to convert ethanol to acetaldehyde which is a Class I Carcinogen, with the capability to induce sister chromatid exchanges, point mutations, DNA adducts and hyperproliferation of epithelium[4].

In our study Streptococcus species were present in 60% of the OSCC subjects. Based on the site of the lesion, Streptococcus was the predominant bacteria present in all the sites [tongue(10%), buccal mucosa(20%), alveolus(10%), palate(20%)]. In OSCC patients, Streptomyces was seen both in

alveolus(20%) and tongue(20%) whereas Bacillus and Listeria were seen only in the alveolar lesions(30%). No correlation was present in relation to the habit history of the patients as the habits were diversely varied among the subjects.

In a review by Chocolatewala *et al* in 2012, majority of the isolates from OSCC patients were saccharolytic and acid tolerant, such as yeasts, Actinomycetes, Bifidobacteria, Lactobacilli, Streptococci and Veillonella. The microenvironment of solid tumors is typically hypoxic with low pH, thus favoring the survival of only acid tolerant bacteria[5]. In our study, the OSCC patients had the saccharolytic bacteriae: Bacillus(50%), Bacterium(30%), Clostridium(30%), Corynebacterium(30%), Desulfutomaculum(30%), Enterococcus(30%), Gemmata(40%), Hymenobacter(30%), Lactobacillus(40%), Listeria(50%), Ruminococcus(30%), Streptococcus(60%), Streptomyces(50%) and Thermoanaerobacter(30%). Flavobacterium(40%) contains both saccharolytic as well as non-saccharolytic species of which Flavobacterium myroides, is non-saccharolytic. The bacteria with aciduric properties present in the OSCC cohort were Bacillus, Buchnera Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Lactobacillus, Listeria, Lysinibacillus, Ruminococcus, Streptococcus, Streptomyces and Thermoanaerobacter.

Bolt *et al* in 2014, found that the prominent pathogens of the normal healthy oral mucosa were aerobes whereas anaerobes were predominant in the OSCC group[3]. In our study 58% of the prominent bacteria in the OSCC group

fall under anaerobes while 42% were aerobes. Within the 58% anaerobes present in the OSCC group, 37% were facultative anaerobes and 21% obligate anaerobes. In the control group 67% of the prominent bacteria were anaerobes and 33% were aerobes. Interestingly all the anaerobes (67%) in the control group were facultative anaerobic bacteria, with no obligate anaerobes, as opposed to 21% obligate anaerobes in the OSCC group.

In the present study a large number of uncultured bacteria were identified. The unculturable bacteria are bacterial sequences that have not been identified and uploaded into the NCBI database, and our study involved comparing the sequences with that available in the NCBI database. These uncultured and sometimes dormant bacteria occupy different ecological microniches, and they maybe involved in latent infections.

The results of our study on the salivary microbiome are of interest as it provides an insight into the diversity present in the salivary microbial populations in OSCC and non-OSCC individuals. Our findings show that though the microbiome is diverse there is a shift towards different species in OSCC compared to controls. These findings need to be validated in larger samples. The species that are unique to OSCC need to be further studied to assess their role and importance, if any, in the etiological and clinical context.

Summary and conclusion

SUMMARY AND CONCLUSION

16S rRNA gene sequencing was done for ten cases of Oral Squamous Cell Carcinoma patients (OSCC group) and ten cases of healthy individuals (control group) and the sequences were identified using the BLAST (Basic Local Alignment Search Tool) analysis in the NCBI (National Centre for Biotechnology Information) database.

- Of the ten cases within control group nine were analysed and one sample could not be sequenced because the quality of the DNA was not optimal.
- All the ten cases of OSCC group were analysed and sequencing was done.
- A total of nineteen phyla were identified in OSCC and control group of which Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were the major phyla.
- The predominant bacteria seen in control group were of the genera Bacillus, Enterococcus, Lactobacillus, Massilia, Paenibacillus and Streptococcus.
- The predominant bacteria seen in OSCC group were of the genera Bacillus, Bacterium, Buchnera, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Flavobacterium, Gemmata, Hymenobacter, Lactobacillus, Listeria, Lysinibacillus,

Marinifilum, Ruminococcus, Streptococcus, Streptomyces, and Thermoanaerobacter..

- The bacteria which were common to both OSCC patients and healthy controls were of the genera Bacillus, Enterococcus, Lactobacillus and Streptococcus.
- The bacteria that were unique to the OSCC group but not seen in the control group were Bacterium, Buchnera, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Flavobacterium, Gemmata, Hymenobacter, Listeria, Lysinibacillus, Marinifilum, Ruminococcus, Streptomyces, and Thermoanaerobacter.
- The bacteria that were unique to the control group but not seen in the OSCC group were Massilia and Paenibacillus.
- The saccharolytic bacteria seen in the OSCC group were Bacillus, Bacterium, Buchnera, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Flavobacterium, Gemmata, Hymenobacter, Lactobacillus, Listeria, Ruminococcus, Streptococcus, Streptomyces and Thermoanaerobacter.
- The aciduric bacteria in the OSCC group were Bacillus, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Lactobacillus, Listeria, Lysinibacillus, Ruminococcus, Streptococcus, Streptomyces and Thermoanaerobacter.

- The aerobic bacteria in the OSCC group were: Buchnera, Caulobacter, Clostridium, Corynebacterium, Gemmata, Hymenobacter, Lysinibacillus and Streptomyces.
- The anaerobic bacteria in the OSCC group were Bacillus, Desulfutomaculum, Enterococcus, Flavobacterium, Lactobacillus, Listeria, Marinifilum, Ruminococcus, Streptococcus, and Thermoanaerobacter. In the OSCC group 22% were obligate anaerobes whereas in the control group only facultative anaerobes were present.

16S rRNA sequencing using Metagenomic Sequencing is a viable and powerful tool to study the oral microbiome. There are variations in the microbiome in OSCC group compared to the control group. The present study was able to identify the bacterial species that further need to be studied.

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Annexures

ANNEXURE – II

DISSERTATION PROTOCOL

1. Title:

Comparative analysis of salivary microbiome in Oral Squamous Cell Carcinoma patients and healthy individuals using 16S rRNA gene sequencing.

2. Name and designation of the principal investigator:

Dr. Ishwarya.S

I year post graduate student

Department of Oral and Maxillofacial Pathology

3. Name of HOD & staff in charge:

Dr. K. Ranganathan, MDS, MS (Ohio), PhD

Dr. K. Uma Devi, MDS

Dr. Elizabeth Joshua, MDS

Dr. T. Rooban, MDS

4. Department where project is to be carried out:

Department of Oral and Maxillofacial Pathology,

Ragas Dental College,

Chennai

5. Duration of the project:

I year

6. Background:

Bacteria abundance profile in saliva can serve as useful biomarkers for carcinoma.Inflammation has been observed in various stages of squamous cell carcinoma. An increasing evidence of the involvement of oral bacteria in inflammation warrant further studies on the association of bacteria in the progression of squamous cell carcinoma.

7. Hypothesis:

There is no difference in the salivary microbiome of Oral Squamous Cell Carcinoma patients and healthy individuals.

8.Aim and Objective:

- To assess the difference in the salivary microbiome of Oral Squamous Cell Carcinoma patients and healthy individuals.
- To ascertain the oral microbiome in saliva samples of Oral squamous cell carcinoma patients and healthy individuals using 16S rRNA sequencing of bacteria.

9. Materials& Method:

Study design:

Cross sectional study

Study sample:

- 10 – Oral Squamous Cell Carcinoma patients.
- 10 – healthy individuals.(control)

Method: Saliva collection (unstimulated saliva)

- The samples are collected after obtaining informed consent.

Analysis based on 16S rRna gene sequencing. (DNA extraction-PCR-16s rRNA sequencing)

The difference in salivary microbiome species present Oral Squamous Cell Carcinoma patients and healthy individuals is analysed.

.**Inclusion criteria:**

- Oral Squamous Cell Carcinoma patients.
- Individuals of age 18 years and above.

Exclusion criteria:

- Patients under chemotherapy and radiotherapy.
- Patients under antibiotic therapy, antifungals and corticosteroids before 3 months.
- Patients with systemic diseases.(Diabetic mellitus, Cardiovascular disorders, Gastroesophageal reflux disorder)
- Pregnant women.
- Patients who are not willing to participate.

Detailed budget plan:

- Total – Rs. 70,000/-

References:

1. Schmidt, Brian L., et al. "Changes in abundance of oral microbiota associated with oral cancer." *PLoS One* 9.6 (2014): e98741.
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Signature of principal investigator

Signature of Head of Department

Remarks of committee

Permission Granted YES / NO

Modifications / comments

ANNEXURE - III

QIAamp DNA Mini Kit

For isolation of genomic, mitochondrial, bacterial, parasite or viral DNA

- Rapid purification of high-quality, ready-to-use DNA
- Consistent, high yields
- Complete removal of contaminants and inhibitors

The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from tissues, swabs, CSF, blood, body fluids, or washed cells from urine. No mechanical homogenization is necessary as the tissues are lysed enzymatically. The convenient spin-column procedure reduces hands-on preparation time to 20 minutes. Purification of DNA using the QIAamp DNA Mini Kit can be automated on the **QIAcube**.

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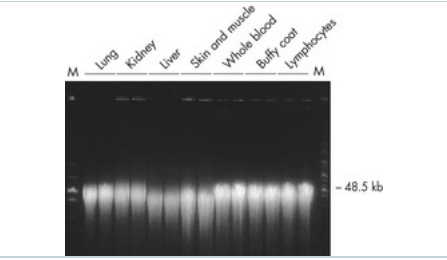


Purification of up to 50 kb genomic DNA.

Size distribution of DNA prepared with QIAamp Kits from the indicated sources (3 µg per lane).

Performance

DNA purified using the QIAamp DNA Mini Kit can be used in a wide range of downstream applications, including PCR and quantitative real-time PCR, Southern blotting, SNP and STR genotyping, and pharmocogenomic research.

The QIAamp DNA Mini Kit yields DNA sized up to 50 kb (see figure "**Purification of up to 50 kb genomic DNA**"). DNA of this length denatures completely and has the highest amplification efficiency. Yields of nucleic acids or DNA depend on the starting material (see table).

QIAamp sample preparation technology is fully licensed, allowing QIAamp purified nucleic acids to be used in any molecular assay or other downstream application without risk of patent infringement.

Typical yields with the QIAamp DNA Mini Kit

Sample	Amount	Total nucleic acid yields (µg)*	DNA yields (µg)†
Blood	200 µl	4–12	4–12
Buffy coat	200 µl	25–50	25–50
Cells	10^6	20–30	15–20

Liver	25 mg	60–115	10–30
Brain	25 mg	35–60	15–30
Lung	25 mg	25–45	5–10
Heart	25 mg	15–40	5–10
Kidney	25 mg	40–85	15–30
Spleen	10 mg	25–45	5–30

* Nucleic acids obtained without RNase treatment.

† Nucleic acids obtained with RNase treatment.

Principle

The QIAamp DNA Mini Kit simplifies isolation of DNA from human tissue samples with fast spin-column or vacuum procedures. No phenol–chloroform extraction is required. DNA binds specifically to the QIAamp silica-gel membrane while contaminants pass through. PCR inhibitors, such as divalent cations and proteins, are completely removed in two efficient wash steps, leaving pure DNA to be eluted in either water or a buffer provided with the kit. QIAamp DNA technology yields genomic, mitochondrial, bacterial, parasite, or viral DNA from human tissue samples ready to use in PCR and blotting procedures.

Procedure

Optimized buffers and enzymes lyse samples, stabilize nucleic acids, and enhance selective DNA adsorption to the QIAamp membrane. Alcohol is added and lysates loaded onto the QIAamp spin column. Wash buffers are used to remove impurities and pure, ready-to-use DNA is then eluted in water or low-salt buffer.

No mechanical homogenization is necessary as the tissues are lysed enzymatically, and the convenient spin-column procedure means that hands-on preparation time is only 20 minutes (lysis times differ according to the sample source). Samples can be processed using either a microcentrifuge or, if blood or other body fluids are being processed, using the QIAvac 24 Plus or QIAvac 6S vacuum manifold. In addition, the rigorous lysis procedure employed makes the QIAamp DNA Mini Kit ideal for purification of genomic DNA from bacteria or parasites. To further reduce hands-on time, genomic DNA purification may be automated on the QIAcube.

Vacuum processing

Blood or other body fluids can be processed by vacuum, instead of centrifugation, for greater speed and convenience in DNA purification. QIAamp Mini spin columns are accommodated on the QIAvac 24 Plus manifold using VacValves and VacConnectors. VacValves should be used if sample flow rates differ significantly, to ensure consistent vacuum. Disposable VacConnectors are used to avoid any cross-contamination. Use of VacConnectors also allows these QIAamp spin procedures to be performed on QIAvac 6S with QIAvac Luer Adapters.

Applications

The QIAamp DNA Mini Kit is ideal for purification of DNA from most commonly used human tissue samples, including muscle, liver, heart, brain, bone marrow, and other tissues, swabs (buccal, eye, nasal, pharyngeal, and others), CSF, blood, body fluids, and washed cells from urine. DNA can be purified from up to 25 mg tissue or from up to 200 µl fluid in 20 minutes, and eluted in 50–200 µl.

Recommended for you

Cat No./ID: 74106

RNeasy Mini Kit (250)

250 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers

$1,342.00

Add To Cart

Cat No./ID: 52906

QIAamp Viral RNA Mini Kit (250)

For 250 RNA preps: 250 QIAamp Mini Spin Columns, carrier RNA, Collection Tubes (2 ml), RNase-free buffers

$1,080.00

Add To Cart

Cat No./ID: 51106

QIAamp DNA Blood Mini Kit (250)

For 250 DNA minipreps: 250 QIAamp Mini Spin Columns, QIAGEN Protease, Reagents, Buffers, Collection Tubes (2 ml)

$659.00

Add To Cart

Enable Biolabs India Private Limited
CIN: U74999TN2015PTC100804
Date: 04.02.2018

WORK COMPLETION LETTER

To whom it may concern

This is to state that Dr Ishwarya Sankaran, a post graduate student from the Department of Oral Pathology at Raagas Dental College and Hospital, Chennai has completed her research investigation pertaining to analysis of bacterial signatures in samples provided, by 16S rRNA gene sequencing analysis.

The research investigation was performed under the supervision of Dr Arvind Ramanathan, and report was submitted after confirming the correctness of the same by Dr Arvind Ramanathan.

Kindest regards,

Dr Arvind Ramanathan, B.D.S., M.Sc., (Genetics), Ph.D (Genetics) (Japan),
Director,
Enable Biolabs India Private Limited, Chennai 603210

Dr ARVIND Ramanathan BDS, MSc (Genetics), PhD (Genetics) (Japan),
Director, Enable Biolabs, URAPAKKAM (next to Vandaloor Zoo), Chennai 603210.
Phone: 96000 24619 | arvind@ebl.org.in

ANNEXURE - V

INDIVIDUAL SAMPLE MICROBIOME LIST

O 1	O 2	O 3	O 4	O 5	O 6	O 7	O 8	O 9	O 10	O 11	O 12	O 13	O 14	O 15	O 16	O 17	O 18	O 19	O 20
Neisseria	Bacillus	Acinetobac	Acidovorax	Actinobaci	Unculturec	Aphanizom	Neisseria	Eikenella	Anaerofusi	Actinokine	no result	Alysiella	Acetobacte	Aerococcu	Acetonem	Acholeplas	Acidamino	Acetonem	Actinocatenispora
Unculturec	Carnobacte	Actinotign	Amantichit	Aggregatibacter		Bacillus	Unculturec	Kingella	Anaerostip	Actinomadura		Aquitalea	Acholeplas	Anaerobac	Acidovorax	Actinomad	Acinetobac	Algoriphag	Actinoplanes
	Catellicocc	Aggregatib	Azoarcus	Aliivibrio		Carnobacterium		Neisseria	Aphanizom	Aerococcus		Bacillus	Acidamino	Aphanizom	Actinoplan	Actinophyt	Alkalitalea	Arsenopho	Algoriphagus
	Enterococc	Aliiglaciecc	Azovibrio	Arsenophonus		Catellicoccus		Vitreoscilla	Bacillus	Aneuriniibacillus		Bosea	Alkaliflexu	Bacillus	Aeromona	Actinoplan	Anaerocoli	Bacillus	Amphibacillus
	Granulicat	Allobaculu	Beta prote	Asticcacaulis		Desulfitibacteria			Clostridiun	Aquimarina		Brachybact	Amycolato	Bavariicoc	Aliiroseova	Actinopoly	Apibacter	Bacterium	Aquitalea
	Kurthia	Alteribacill	Bordetella	Bibersteinia		Enterococcus			Coprococci	Arachidicoccus		Brevundim	Anaerococ	Carnobacte	Amycolato	Alicyclobac	Aquabacte	Blattabacte	Arcobacter
	Lactococcu	Anaerobac	Candidatus	Blochmannia		Fructobacillus			Dolosigran	Arenitalea		Campyloba	Clostridiun	Enterococc	Asanoa	Alloactinos	Archangiur	Borrelia	Arthrobacter
	Listeria	Anaerosali	Castellanie	Buchnera		Granulicatella			Enterococc	Bacterium		Caulobacte	Culturomic	Fictibacillu	Avibacteriu	Altereythro	Bacillus	Borreliella	Bacillus
	Macrococc	Asticcacaul	Chromoba	Buttiauxella		Lactobacillus			Eubacteriu	Blautia		Chloroflexi	Dehalobac	Granulicat	Azoarcus	Amycolato	Bacteroide	Buchnera	Bacterium
	Melissococ	Bacillus	Comamona	Candidatus		Listeria			Exiguobact	Brevinema		Corynebac	Dehalococ	Isobaculun	Bacterium	Arthrobact	Beijerincki	Campyloba	Blautia
	Oceanobac	Bizionia	Cupavidus	Cedecea		Melissococcus			Jeotgalliba	Buchnera		Coxiella-lik	Eubacteriu	Kurthia	Brachymor	Auraticocc	Bizionia	Chryseoba	Brachybacterium
	Paenibacill	Bordetella	Curvibacte	Chelonobacter		Oceanobacillus			Lachnospir	Carboxydocella		Desulfobul	Faecalicoc	Lactobacill	Congregiba	Bosea	Borrelia	Colwellia	Buchnera
	Streprococ	Butyrivibri	Dechlorom	Citrobacter		Streptococcus			Lactobacill	Caulobacter		Desulfoton	Halarsenat	Megaspha	Cronobacte	Bradyrhizo	Caloramati	Corynebac	Cephaloticoccus
	Tetragenoc	Caldithrix	Eikenella	Edwardsiella		Vagococcus			Listeria	Cellulomonas		Desulfovib	Haloglycon	Rummeliib	Crossiella	Brochothri	Campyloba	Desemzia	Chloroflexus
	Vagococcu	Carnobacte	Ferrovum	Gallaecimonas		Weissella			Lysinibacill	Cellulosimicrobium		Devosia	Listeria	Sediminiba	Cryobacter	Cellulomor	Chryseoba	Desulfobac	Colwellia
		Caulobacte	Herbaspiril	Glaciecola					Melissococ	Chryseobacterium		Didymococ	Marinifilun	Staphylocc	Demequina	Couchiopla	Clostridiun	Enterococc	Desulfobulbus
		Cecembia	Herminiim	Haemophilus					Robinsonie	Clostridium		Fervidicola	Muribaculu	Streptococ	Desulfamp	Dermacocc	Coprobacti	Fictibacillu	Desulfomicrobium
		Chitinopha	Kingella	Intrasporangium					Ruminococ	Croceitalea		Fournierell	Odoribacte	Streptomy	Desulfopla	Dokdonella	Desulfovib	Flavobacte	Desulfotomaculum
		Chroogloe	Lamprope	Klebsiella					Streptococ	Cyclobacterium		Gemmata	Porphyrom	Vagococcu	Dokdonella	Fimbriiglol	Dialister	Fusobacter	Didymococcus
		Cloacibact	Massilia	Leclercia					Sulfitobact	Deinococcus		Halorubrur	Propionimi	Viridibacill	Donghicola	Friedmann	Enterococc	Gemella	Flavobacterium
		Clostridiun	Neisseria	Mannheimia					Thermoact	Desulfoluna		Helicobact	Salisaeta	Weissella	Dyella	Geoderma	Erwinia	Gemmata	Gemmata
		Collimonas	Ottowia	Mannheimia						Desulforhopalus		Hymenoba	Sporomusa		Ectothiorh	Glycomyce	Facklamia	Holospora	Gordonibacter
		Corynebac	Pandorea	Marinomonas						Desulfosporosinus		Kitasatosp	Streptococcus		Edwardsiel	Gordona	Flammeovi	Hymenoba	Helicobacter
		Dendrospo	Parabulkho	Marinospirillum						Desulfotomaculum		Kocuria	Succinclasticum		Frateuria	Gordoniba	Flavobacte	Kordia	Hymenobacter
		Desulfuron	Polaromon	Methylococcaceae						Desulfurobacterium		Listeria	Tanerella		Gilliamella	Gryllotalpi	Fluviicola	Leptotrichi	Intestinibacillus
		Dichelobac	Pseudocid	Methylomicrobium						Enterococcus		Malonomo	Williamwhitmania		Halomonas	Halobacter	Geoderma	Listeria	Kordimonas
		Flavobacte	Raistonia	Methylosarcina						Entomoplasma		Mesorhizobium			Hydrocarbo	Halobellus	Halograniu	Lysinibacill	Lactobacillus
		Frateuria	Ramlibacte	Moellerella						Faecalicatena		Microbacterium			Idiomarina	Halohasta	Halolamina	Methanob	Listeria
		Gelidibacte	Rhodofera	Moritella						Flaviramulus		Micromonaspora			Jeotgalicoc	Halolamina	Hydrogenii	Mycoplasm	Lysinibacillus
		Granulicat	Stenoxyba	Necropsobacter						Flavobacterium		Mycobacterium			Legionella	Halomicrol	Lacinutrix	Odoribacte	Marinifilum
		Halococcus	Sutterella	Obesumbacterium						Flexistipes		Paracoccus			Lentzea	Halopiger	Lactobacill	Olivibacter	Massilibacterium
		Halomonas	Thiobacillu	Pantoea						Gemmata		Porphyromonas			Luteibacte	Hamadaea	Legionella	Olsenella	Methanobrevibacter
		Haloterrige	Vitreoscilla	Pasteurella						Geobacillus		Rhodopseudomonas			Mangrovib	Herbiconiu	Lewinella	Paenibacill	Methanotorris
		Izhakiella		Pectobacterium						Gillisia		Ruminococcus			Mannheim	Herbidospo	Lutimariba	Peanut-wit	Microbacterium
		Lactobacillus		Photobacterium						Halogeometricum		Spiribacter			Marinibact	Intrasporai	Massilia	Prevotella	Mycobacterium
		Leptolyngbya		Plesiomonas						Hespellia		Streptococcus			Metallibac	Janibacter	Mesoflavib	Providenci	Nitrobacter
		Leptospira		Proteus						Hungatella		Streptomyces			Methyloco	Janthinoba	Methanob	Salibacteri	Onion yellows phytoplasma
		Lewinella		Pseudoalteromonas						Jannaschia		Tetrasphaera			Microbulbi	Jatrophiha	Microbulbi	Sanguibact	Patulibacter
		Limnothrix		Psychromonas						Jejuia		Thermoanaerobacter			Moraxella	Jiangella	Microtetra	Spiroplasm	Pedobacter
		Lysinibacillus		Rahnella						Kordia		Thermobifida			Mycobacte	Kibdelospo	Moellerella	Streptomy	Photobacterium

Maribacter
Marinifilum
Marinifilum
Methanoculleus
Methanosarcina
Methylomicrobium
Moraxella
Mycobacterium
Myroides
Natronorubrum
Nitrosococcus
Novosphingobium
Paenibacillus
Pelagicola
Polaribacter
Pontibacter
Poseidonocella
Pseudarcicella
Psychrobacter
Psychroflexus
Rhizobium
Roseomonas
Rosevivax
Ruegeria
Ruminococcus
Scardovia
Scytonema
Sedimenticola
Sediminibacillus
Selenomonas
Siansivirga
Silicibacter
Sinomicrobium
Solemya
Spirochaeta
Streptococcus
Rouxiella
Serratia
Skermanella
Vibrio
Xenorhabdus

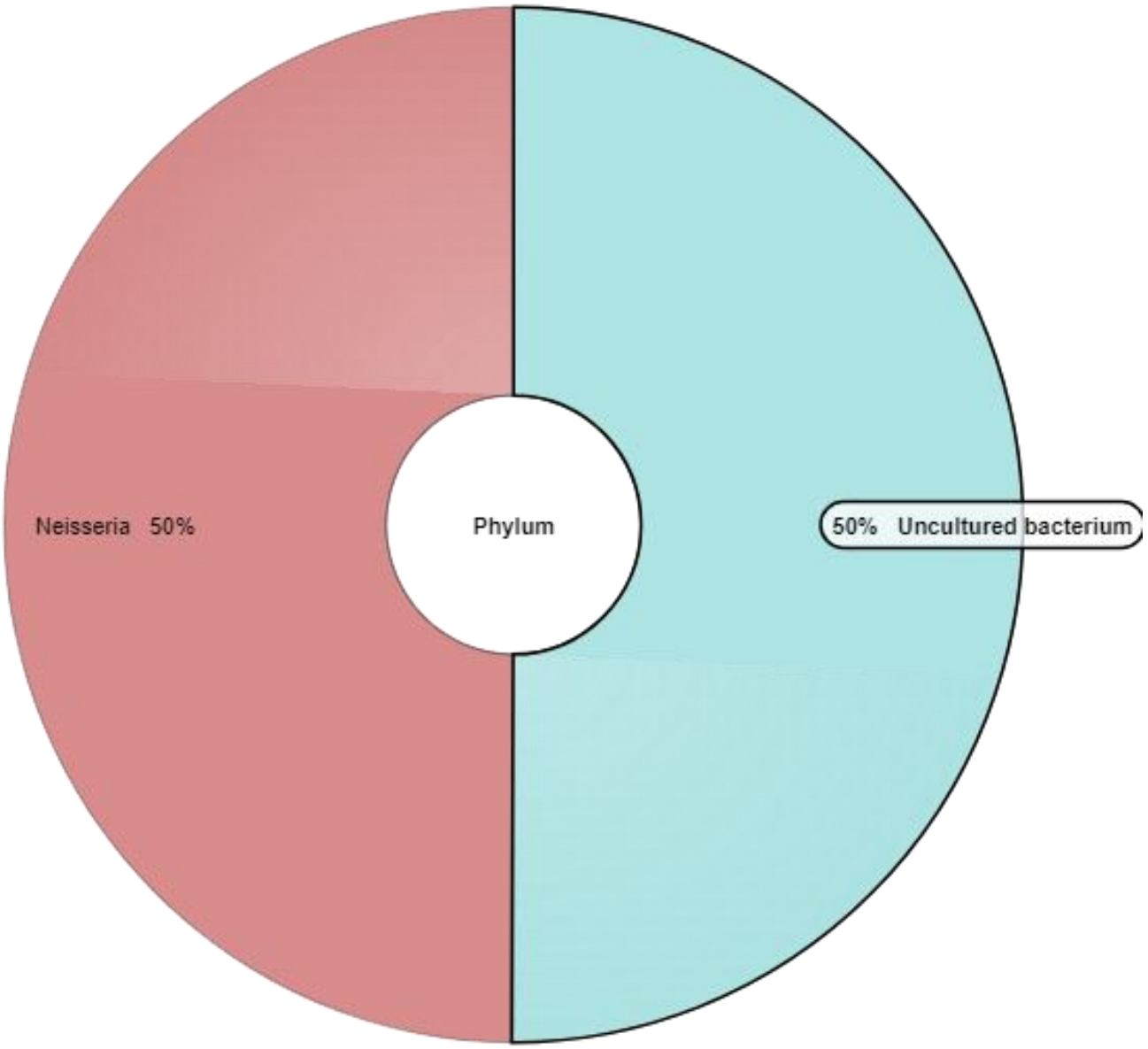
Kordiimonas
Kutzneria
Lacinutrix
Lactobacillus
Litoreibacter
Mariniradius
Mesoplasma
Methanogenium
Micromonospora
Mycobacterium
Mycoplasma
Myroides
Niastella
Nocardia
Nocardiopsis
Nonlabens
Olleya
Onion yellowsphytoplasma
Paracoccus
Prevotella
Pseudonocardia
Pseudorhodobacter
Psychroserpens
Rhodovulum
Runella
Salinimicrobium
Spirochaeta
Stappia
Streptococcus
Streptomyces
Sulfurovum
Tetragenococcus
Vulcanisaeta
Tistlia
Tropicibacter

Paenibacill
Perlucidiba
Photobact
Polycyclov
Porphyrob
Poseidono
Pseudoam
Pseudohali
Pseudomo
Pseudoreu
Psychroba
Rhodanob
Rodentiba
Rudaea
Salipaludib
Schlesneri
Stenotroph
Steroidoba
Streptomy
Sulfurovun
Tepidiphilu
Thermofla
Thiocapsa
Xanthomo
Xenorhabd
Yuhushiell
Kineospha
Kitasatosp
Knoellia fla
Lactobacill
Leifsonia
Lentzea
Lysinibacill
Lysobacter
Marinomo
Marivita
Massilia
Methylmic
Microbacte
Microbacte
Millisia
Mobilicocc
Modestob
Monashia
Mycobacte
Nakamurel
Natronoru
Neorhizob
Nocardia
Paludibact
Peptoniphi
Phycicoccu
Prauserella
Rhizobium Vibrio
Rhodoblas Wolbachia
Rhodococcus
Rufibacter
Saccharopolyspora
Stappia
Streptacidiphilus
Sunxiuqinia
Xanthomonas
Moorea
Moraxella
Niastella
Nitrospira
Nocardiop
Nonlabens
Nostocales
Paenibacillus
Peptoclostridium
Photobacterium
Pontibacter
Prevotella
Prosthecomicrobium
Rhizobium
Rhodococcus
Rosenbergiella
Ruminococcus
Siansivirga
Sneathia
Snodgrassella
Sphingobium
Sporomusa
Streptococcus
Tenacibaculum
Thermoanaerobacter
Thermobifida
Thermonospora
Thermoan
Traorella
Treponem
Ureaplasm
Vagococcu
Wenyingzh
Pseudomonas
Psychroserpens
Ruminococcus
Sphingobacterium
Streptococcus
Streptomyces
Sunxiuqinia
Tetragenococcus
Thermoanaerobacter
Treponema
Wolinella

ANNEXURE VI

MICROBIOME CHARTS OF INDIVIDUAL SAMPLES

SAMPLE O-1



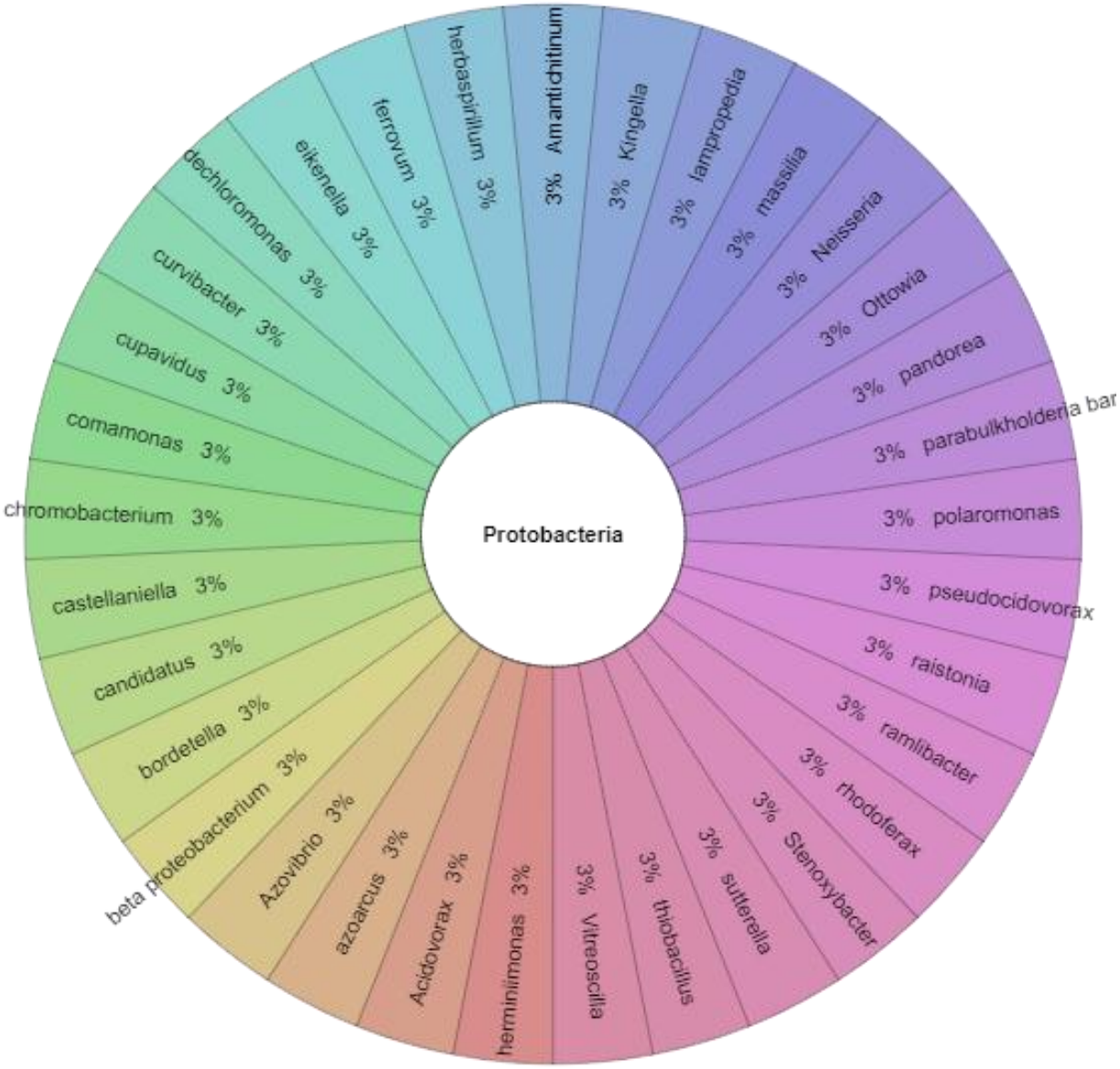
SAMPLE 0-2


Firmicutes
7% Carnobacterium
7% Macrococcus
7% Melissococcus
7% Oceanobacillus
7% Paenibacillus
7% Streprococcus
7% Tetragenococcus
7% Vagococcus
Listeria 7%
Bacillus 7%
Catellicoccus 7%
Enterococcus 7%
Granulicatella 7%
Kurthia 7%
Lactococcus 7%

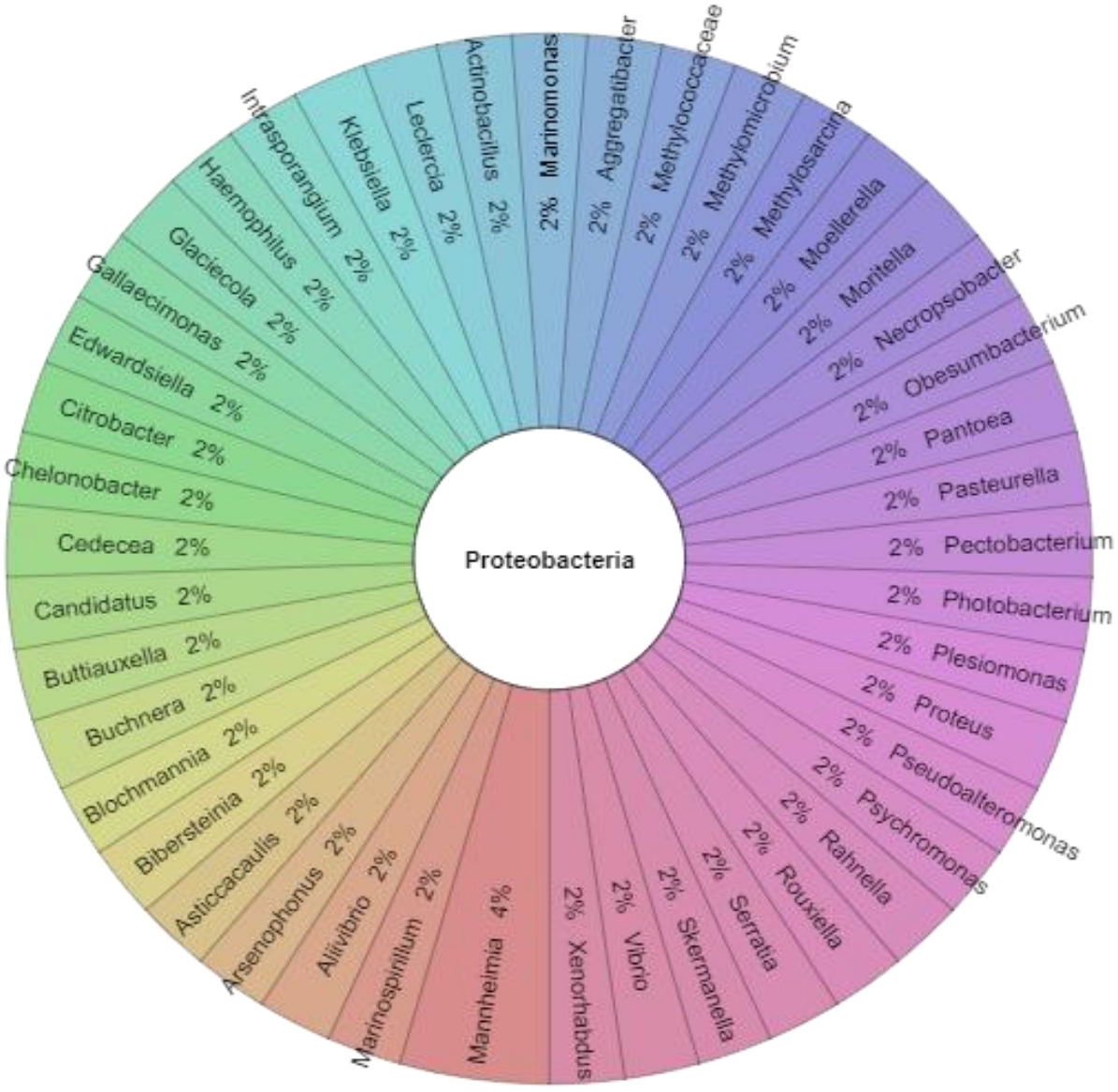
SAMPLE O-3


Microbiome
Firmicutes
Bacteroidetes
Actinobacteria
Euryarchaeota
Cyanobacteria
Spirochaetes
Proteobacteria
Sedimentibacillus 1%
Selenomonas 1%
Streptococcus 1%
Thermincola 1%
Trichococcus 1%
Marinifilum 1%
Blattabia 1%
Chitinophaga 1%
Cloacibacterium 1%
Flavobacterium 1%
Lewinella 1%
Maribacter 1%
Cecembia 1%
Myroides 1%
Polaribacter 1%
Pontibacter 1%
Pseudarcicella 1%
Psychroflexus 1%
Siansivirga 1%
Sinomicrobium 1%
Actinotignum 1%
Corynebacterium 1%
Mycobacterium 1%
Scardovia 1%
Streptomyces 1%
Halococcus 1%
Haloterrigena 1%
Methanoculleus 1%
Methanosarcina 1%
Natronorubrum 1%
Leptolyngbya 1%
Limnothrix 1%
Scytonema 1%
Leptospira 1%
Spirochaeta 1%
Caldithrix 1%
Thermodesulfovibrio 1%
Sclernya 1%
Pelagicola 1%
Izhakiella 1%
Acinetobacter 1%
Alkalitalea 1%
Asticcacaulis 1%
Bordetella 1%
Caulobacter 1%
Colimonas 1%
Desulfuromusa 1%
Dichelobacter 1%
Frateuria 1%
Haliomonas 1%
Aggregatibacter 1%
Methylomicrobium 1%
Moraxella 1%
Nitrosococcus 1%
Novosphingobium 1%
Poseidonocella 1%
Rhizobium 1%
Roseomonas 1%
Ruegeria 1%
Sedimenticola 1%
Silicibacter 1%
Xenorhabdus 1%
Granulicatella 1%
Allobaculum 1%
Anaerobacillus 1%
Anaerosalibacter 1%
Bacillus 1%
Butyrivibrio 1%
Clostridium 1%
Clostridium 1%
Dendrosporobacter 1%
Alteribacillus 1%
Lactobacillus 1%
Lysinibacillus 1%
Paenibacillus 1%
Ruminococcus 1%

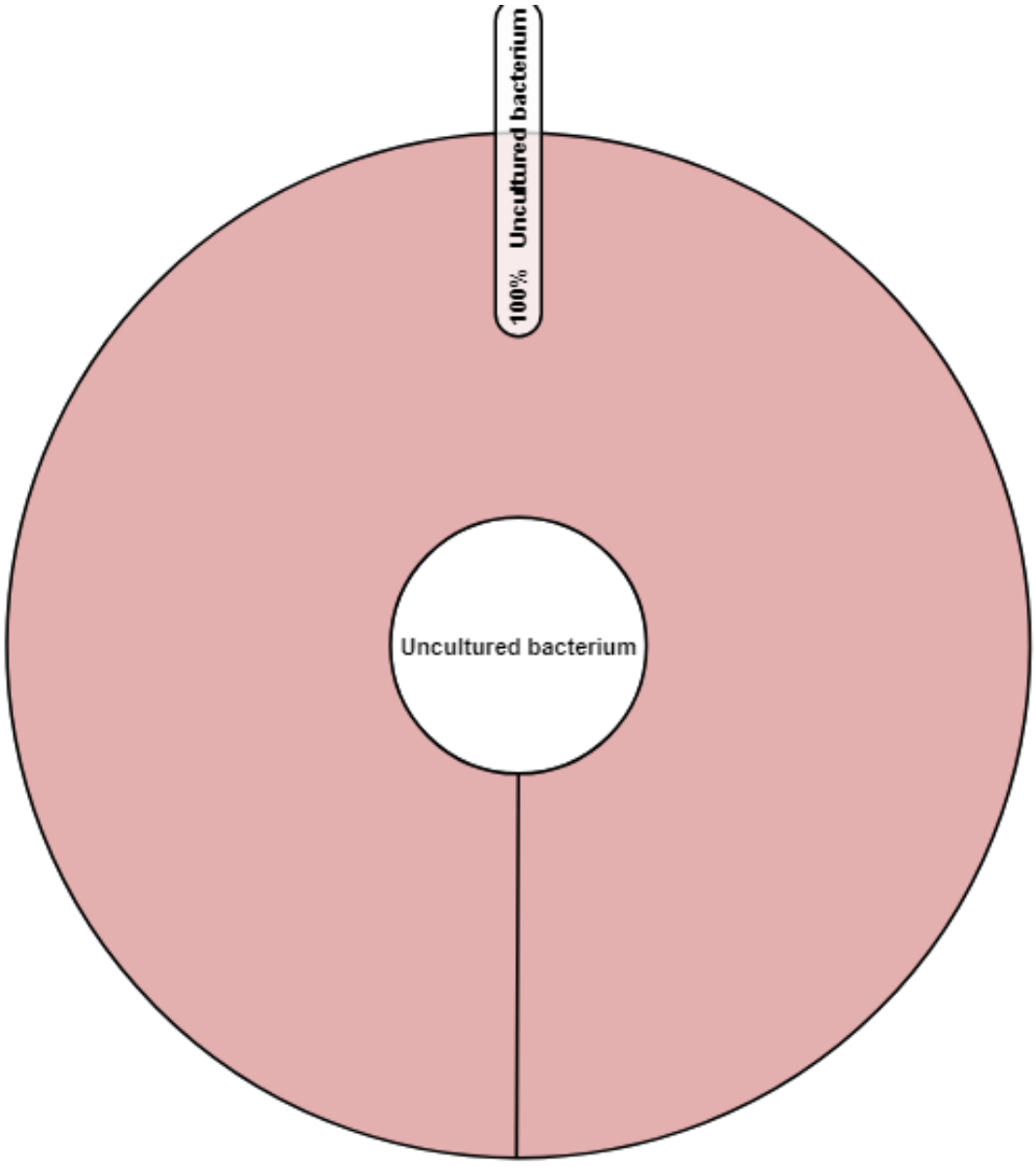
SAMPLE O-4


Protobacteria
3% Amantichitinum
3% Kingella
3% lampropedia
3% massilia
3% Neisseria
3% Ottowia
3% pandorea
3% parabulkholderia bar
3% polaromonas
3% pseudocidovorax
3% raistonia
3% ramlibacter
3% rhodoferax
3% Stenoxybacter
3% sutterella
3% thiobacillus
3% Vitreoscilla
herminiimonas 3%
Acidovorax 3%
azoarcus 3%
Azovibrio 3%
beta proteobacterium 3%
bordetella 3%
candidatus 3%
castellaniella 3%
chromobacterium 3%
comamonas 3%
cupavidus 3%
curvibacter 3%
dechloromonas 3%
eikenella 3%
terrovum 3%
Heliosporillum 3%

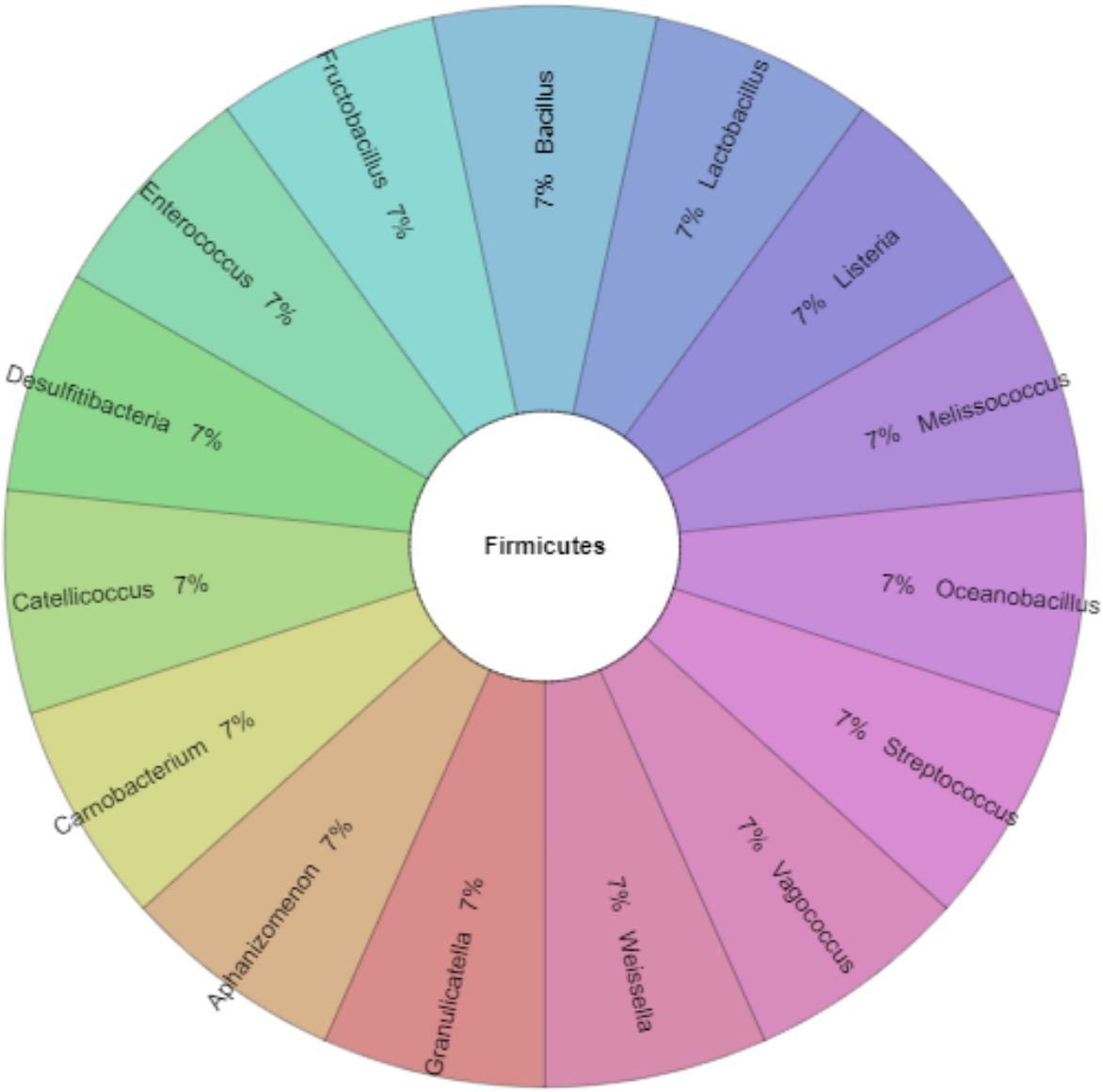
SAMPLE O-5


Proteobacteria
Marinomonas 2%
Aggregatibacter 2%
Methylococcaceae 2%
Methylomicrobium 2%
Methylosarcina 2%
Moellerella 2%
Moritella 2%
Necropsobacter 2%
Obesumbacterium 2%
Pantoea 2%
Pasteurella 2%
Pectobacterium 2%
Photobacterium 2%
Plesiomonas 2%
Proteus 2%
Pseudoalteromonas 2%
Psychromonas 2%
Rahnella 2%
Rouxiella 2%
Serratia 2%
Skermanella 2%
Vibrio 2%
Xenorhabdus 2%
Mannheimia 4%
Marinospirillum 2%
Alivibrio 2%
Arsenophonus 2%
Asticcacaulis 2%
Bibersteinia 2%
Blochmannia 2%
Buchnera 2%
Buttiauxella 2%
Candidatus 2%
Cedecea 2%
Chelonobacter 2%
Citrobacter 2%
Edwardsiella 2%
Gallaecimonas 2%
Glaciecola 2%
Haemophilus 2%
Intrasporangium 2%
Klebsiella 2%
Lecercia 2%
Actinobacillus 2%

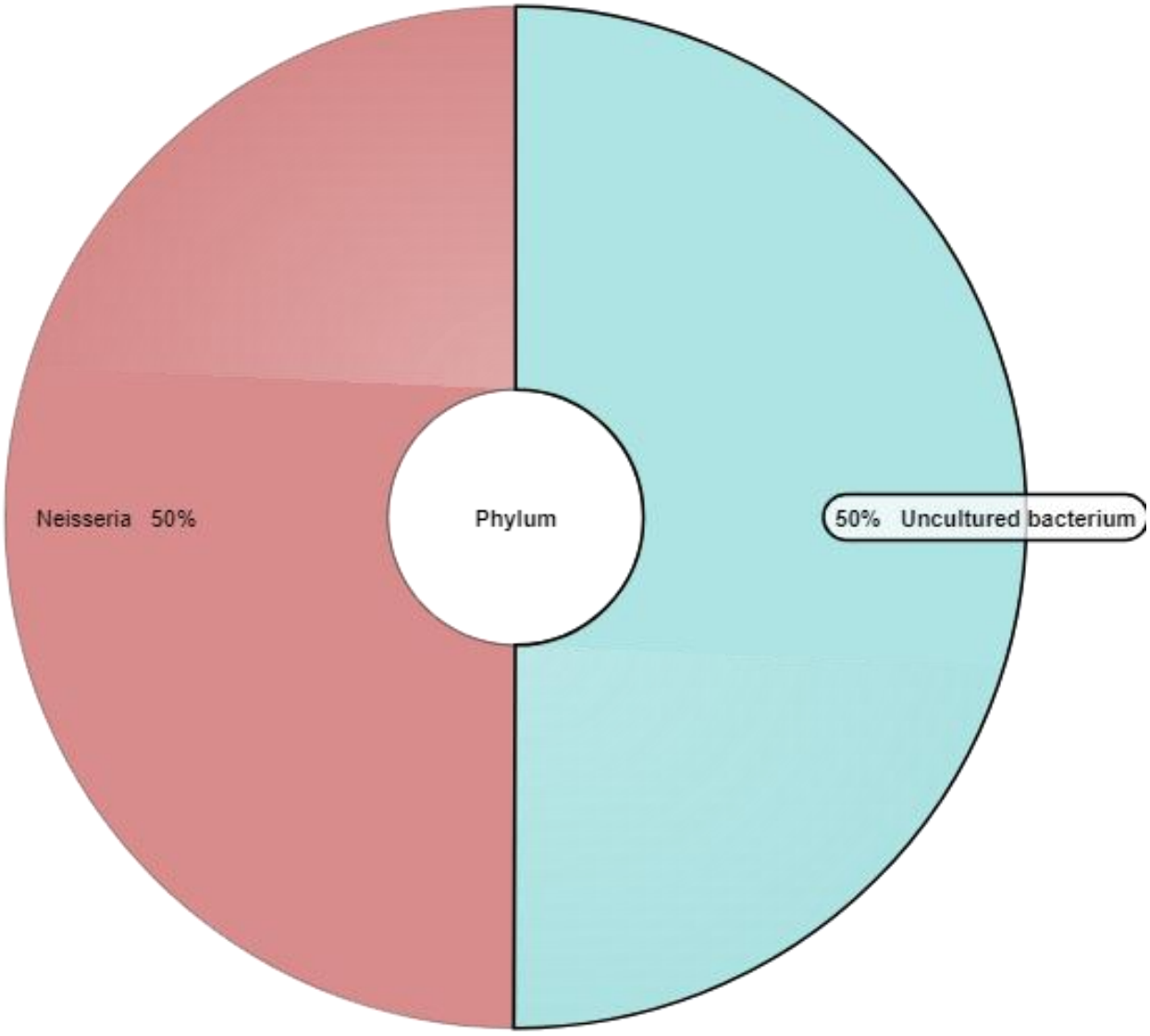
SAMPLE O-6



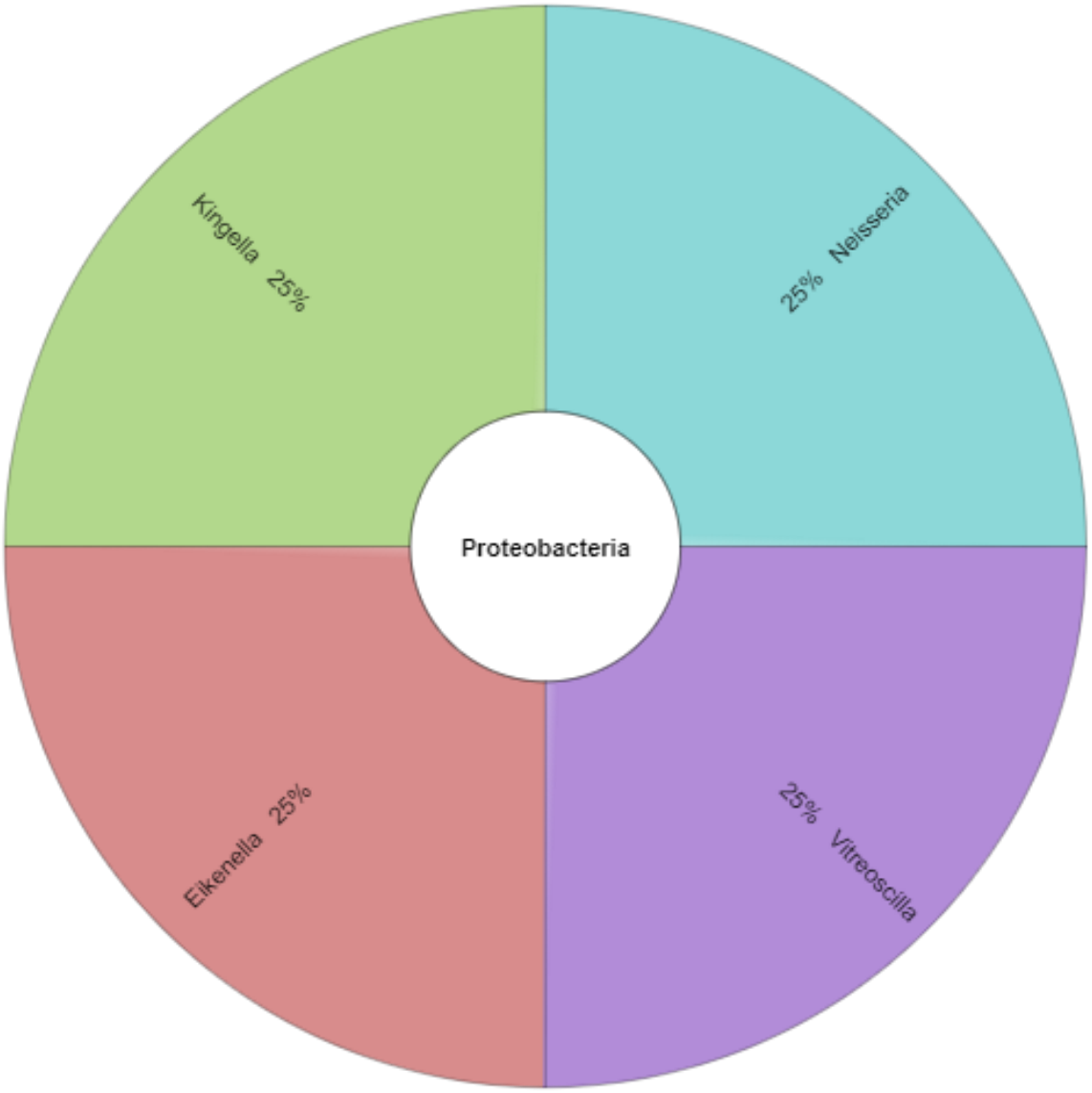
SAMPLE O-7


Firmicutes
7% Bacillus
7% Lactobacillus
7% Listeria
7% Melissococcus
7% Oceanobacillus
7% Streptococcus
7% Vagococcus
7% Weissella
Granulicatella 7%
Aphanizomenon 7%
Carnobacterium 7%
Catellicoccus 7%
Desulfitibacteria 7%
Enterococcus 7%
Fructobacillus 7%

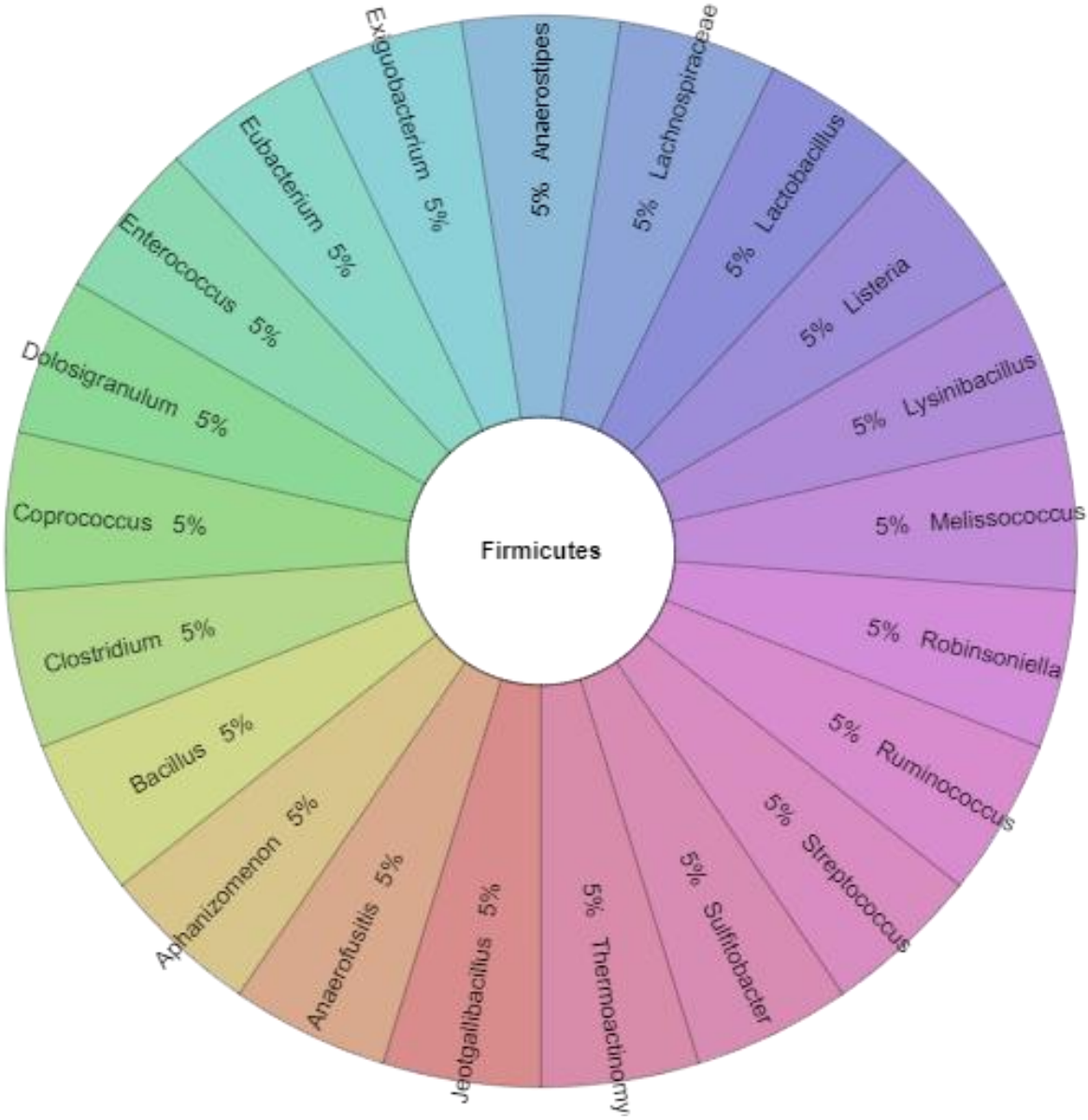
SAMPLE O-8



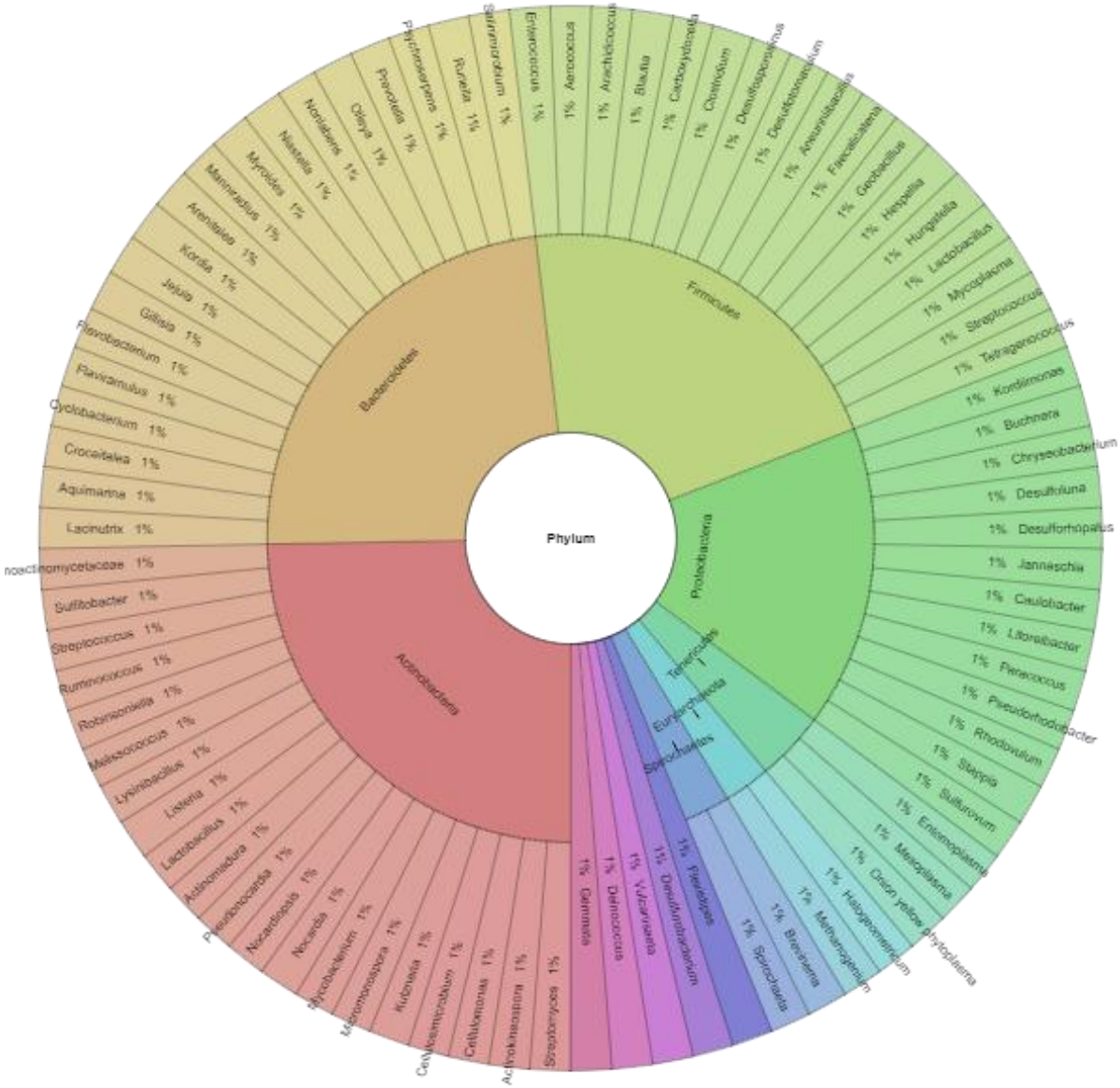
SAMPLE O-9


Kingella 25%
25% Neisseria
Proteobacteria
Eikenella 25%
25% Vitreoscilla

SAMPLE O-10


Firmicutes
5% Anaerostipes
5% Lachnospiraceae
5% Lactobacillus
5% Listeria
5% Lysinibacillus
5% Melissococcus
5% Robinsoniella
5% Ruminococcus
5% Streptococcus
5% Sulfitobacter
5% Thermoactinomy
Jeotgalibacillus 5%
Anaerofusitis 5%
Aphanizomenon 5%
Bacillus 5%
Clostridium 5%
Coprococcus 5%
Dolosigranulum 5%
Enterococcus 5%
Eubacterium 5%
Exiguobacterium 5%

SAMPLE O-11


Phylum
Bacteroidetes
Firmicutes
Proteobacteria
Actinobacteria
Tenericutes
Euryarchaeota
Spirochaetes
Saprospirales 1%
Rubrella 1%
Prevotella 1%
Porphyromonas 1%
Odoribacter 1%
Nonlabens 1%
Nakamurella 1%
Mycoides 1%
Mannheimia 1%
Arenibacter 1%
Kordia 1%
Jejuia 1%
Gillisia 1%
Flavobacterium 1%
Flavicampus 1%
Cyclobacterium 1%
Crocinitalea 1%
Aquimarina 1%
Lacinutrix 1%
Streptococcus 1%
Sulfitobacter 1%
Streptococcus 1%
Ruminococcus 1%
Robinsoniella 1%
Melissococcus 1%
Lysinibacillus 1%
Listeria 1%
Lactobacillus 1%
Actinomadura 1%
Acidothermus 1%
Nocardioides 1%
Nocardia 1%
Mycobacterium 1%
Micromonospora 1%
Kutzneria 1%
Cellulosimicrobium 1%
Cellulomonas 1%
Actinokineospora 1%
Saccharopolyspora 1%
Geminata 1%
Dictyoglomus 1%
Vulcanisaeta 1%
Desulfurobacterium 1%
Pleurocapsa 1%
Spirochaeta 1%
Brevinema 1%
Methanopyrus 1%
Halogeometricum 1%
Cronobacter 1%
phytoplasma
Metaplasma 1%
Enterosplasma 1%
Sulfurovum 1%
Staphia 1%
Rhodovulum 1%
Pseudorhodobacter 1%
Pelagicoccus 1%
Litoribacter 1%
Caulobacter 1%
Janthinobacterium 1%
Desulfohalobium 1%
Desulfofaba 1%
Chryseobacterium 1%
Buchnera 1%
Xanthomonas 1%
Tetragenococcus 1%
Streptococcus 1%
Mycoplasma 1%
Lactobacillus 1%
Hungatella 1%
Herpetila 1%
Geobacillus 1%
Flavobacterium 1%
Desulfotomaculum 1%
Desulfosporosinus 1%
Clostridium 1%
Carboxydocella 1%
Blautia 1%
Arachidicoccus 1%
Aerococcus 1%
Enterococcus 1%

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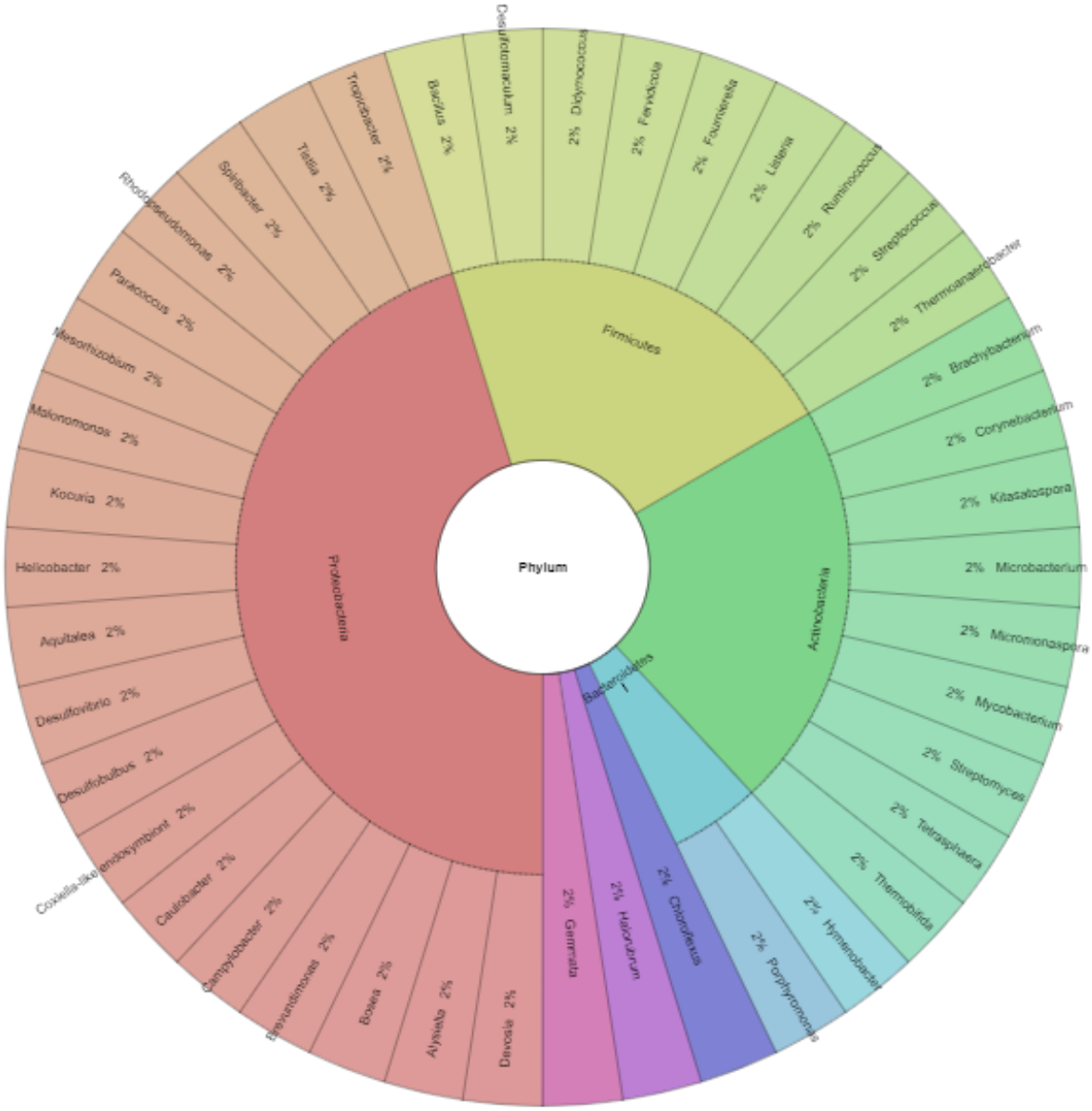
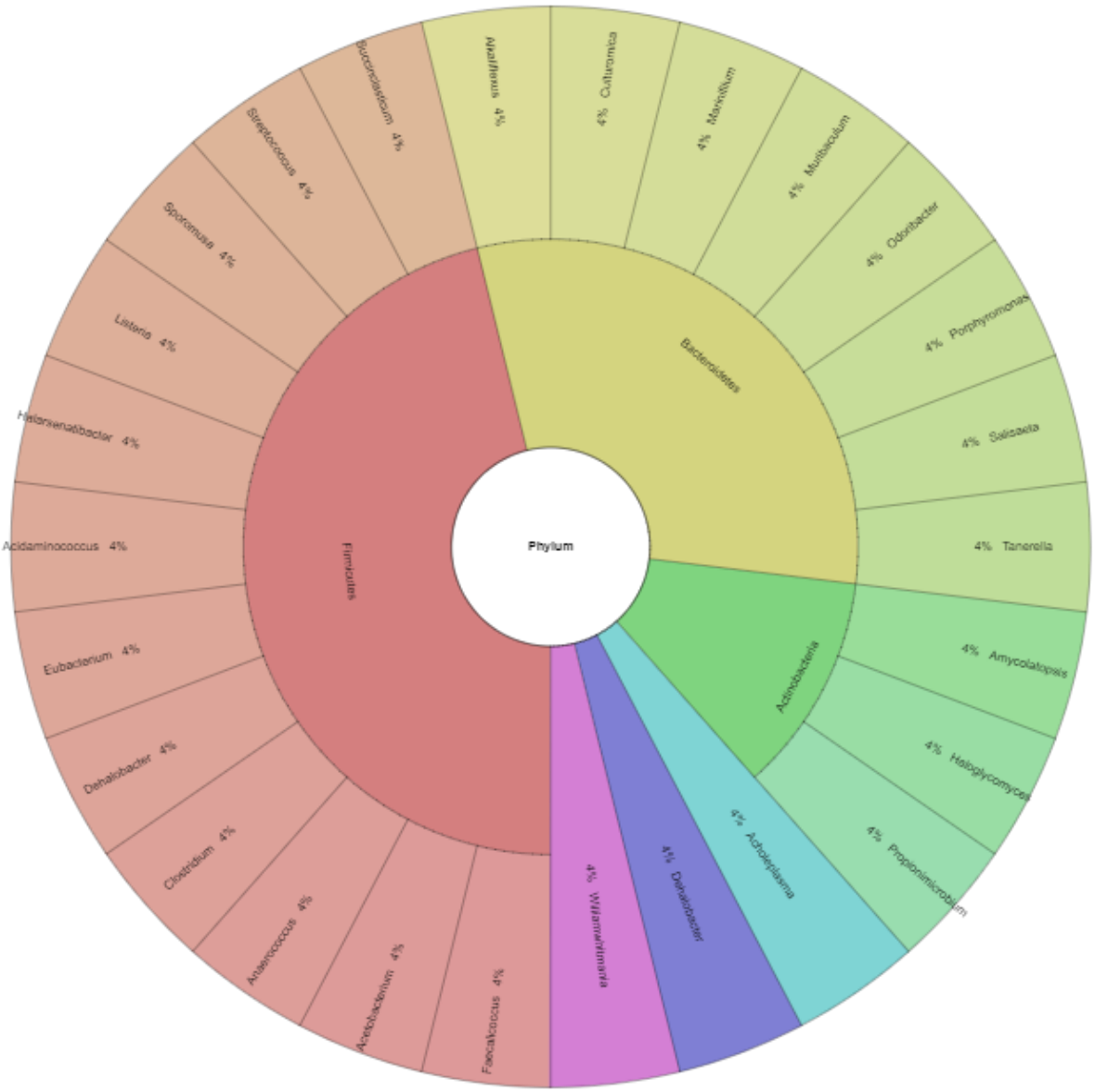
SAMPLE O-12

No results were obtained due to very low DNA concentration in the sample.

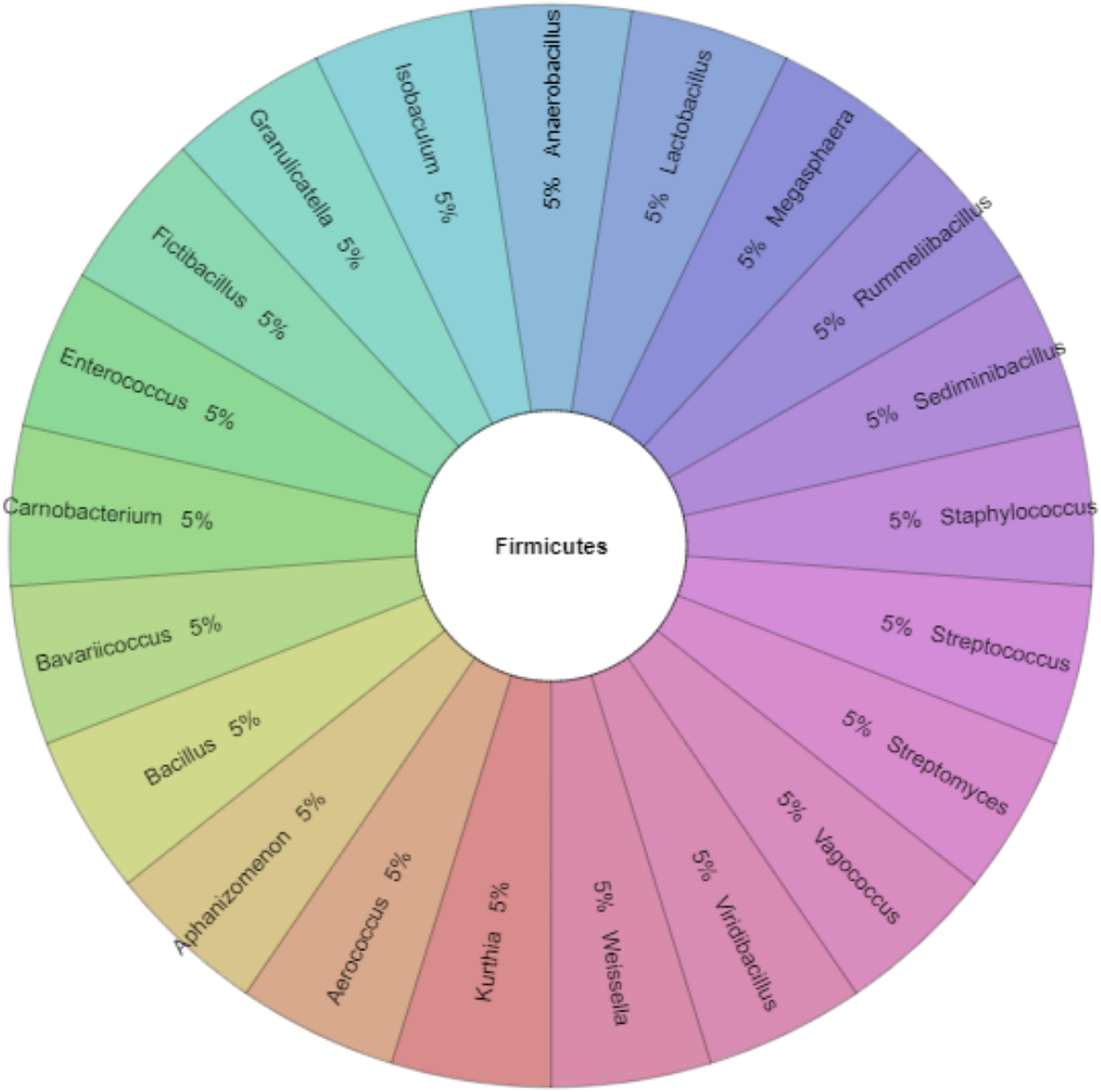
SAMPLE O-13


Phylum
Firmicutes
Actinobacteria
Bacteroidetes
Proteobacteria
Desulfotomaculum 2%
Bacillus 2%
Dialysta 2%
Fervidicola 2%
Faecalitalea 2%
Listeria 2%
Ruminococcus 2%
Streptococcus 2%
Thermoanaerobacter 2%
Brachybacterium 2%
Corynebacterium 2%
Kitasatospora 2%
Microbacterium 2%
Micromonospora 2%
Mycobacterium 2%
Streptomyces 2%
Tetrasphaera 2%
Thermobifida 2%
Hymenobacter 2%
Porphyromonas 2%
Chloroflexus 2%
Haloarcula 2%
Gemella 2%
Devosia 2%
Aliyella 2%
Bosea 2%
Devosiaceae 2%
Campylobacter 2%
Caulobacter 2%
Coxiella-like endosymbiont 2%
Desulfobulbus 2%
Desulfovibrio 2%
Aquitalea 2%
Helicobacter 2%
Kocuria 2%
Malonomonas 2%
Mesorhizobium 2%
Paracoccus 2%
Rhodopseudomonas 2%
Spirilaster 2%
Tetilla 2%
Tropicibacter 2%

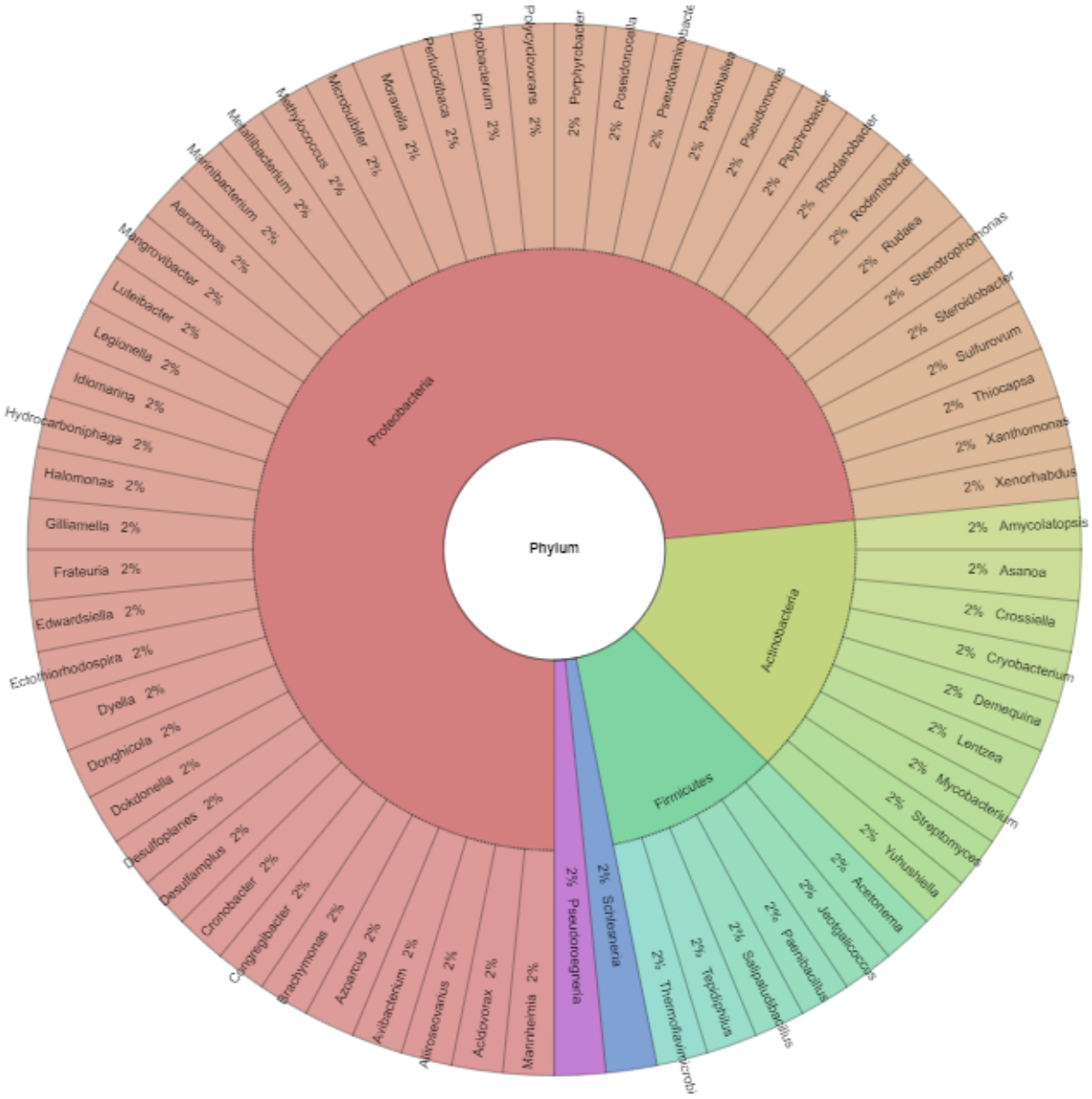
SAMPLE O-14


Phylum
Bacteroidetes
Firmicutes
Actinobacteria
Verrucomicrobia
Deinococcus
Acholeplasma
Propionimicrobium
Halopiyomyces
Amycolatopsis
Tanerella
Salsaeta
Porphyromonas
Odoribacter
Muribaculum
Marinilabilia
Cultramica
Marinilabilia 4%
Butyricicoccus 4%
Streptococcus 4%
Sporomusa 4%
Listeria 4%
Heliorestis 4%
Acidaminococcus 4%
Eubacterium 4%
Dehalobacter 4%
Clostridium 4%
Anaerococcus 4%
Acetobacterium 4%
Faecalicoccus 4%

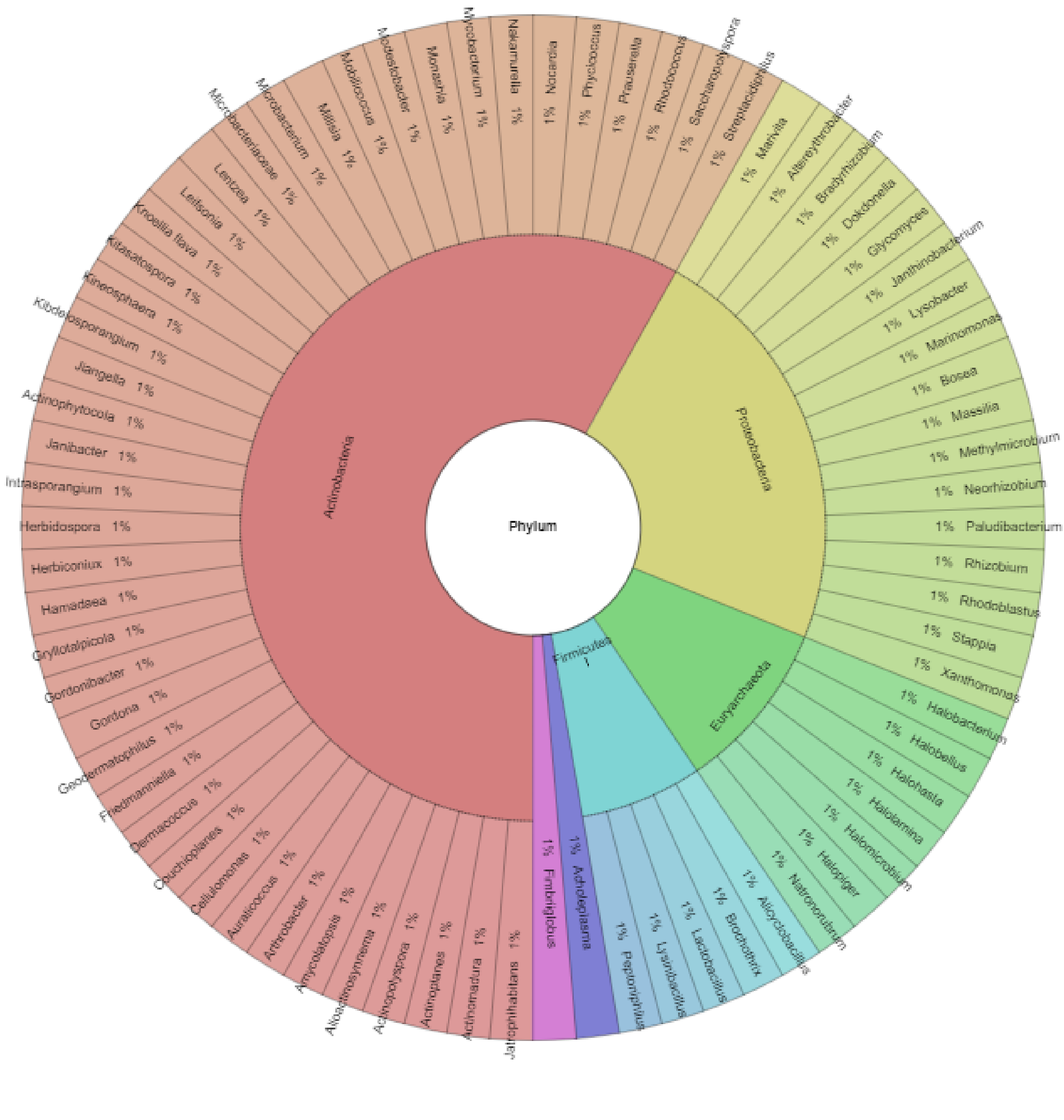
SAMPLE 0-15


Firmicutes
5% Anaerobacillus
5% Lactobacillus
5% Megasphaera
5% Rummeliibacillus
5% Sediminibacillus
5% Staphylococcus
5% Streptococcus
5% Streptomyces
5% Vagococcus
5% Viridibacillus
5% Weissella
Kurthia 5%
Aerococcus 5%
Aphanizomenon 5%
Bacillus 5%
Bavariicoccus 5%
Carnobacterium 5%
Enterococcus 5%
Fictibacillus 5%
Granulicatella 5%
Isobaculum 5%

SAMPLE O-16


Phylum
Proteobacteria
Actinobacteria
Firmicutes
2% Porphyrobacter
2% Poseidoniocella
2% Pseudoalteromonadacte
2% Pseudohaliea
2% Pseudomonas
2% Psychrobacter
2% Rhodanobacter
2% Rodentibacter
2% Rudaea
2% Stenotrophomonas
2% Steroidobacter
2% Sulfurovum
2% Thiocapsa
2% Xanthomonas
2% Xenorhabdus
2% Amycolatopsis
2% Asanoa
2% Crossiella
2% Cryobacterium
2% Demequina
2% Lentzea
2% Mycobacterium
2% Streptomyces
2% Yuhushiella
2% Acetonema
2% Jeotgalicoccus
2% Paenibacillus
2% Salipaludibacillus
2% Tepidibrevis
2% Thermoflavimicrobi
2% Scrofimeria
2% Pseudoruegeria
2% Marinheimia
2% Acidovorax
2% Alloroseovarius
2% Avibacterium
2% Azoarcus
2% Brachymonas
2% Congregibacter
2% Cronobacter
2% Desulfamplus
2% Desulfoplanes
2% Dokdonella
2% Donghicola
2% Dyella
2% Ectothiorhodospira
2% Edwardsiella
2% Frateuria
2% Gilliamella
2% Halomonas
2% Hydrocarboniphaga
2% Idiomarina
2% Legionella
2% Luteibacter
2% Mangrovibacter
2% Aeromonas
2% Marinibacterium
2% Metallibacterium
2% Methylococcus
2% Microbulbifer
2% Moraxella
2% Perlucidibaca
2% Photobacterium
2% Polaromonas

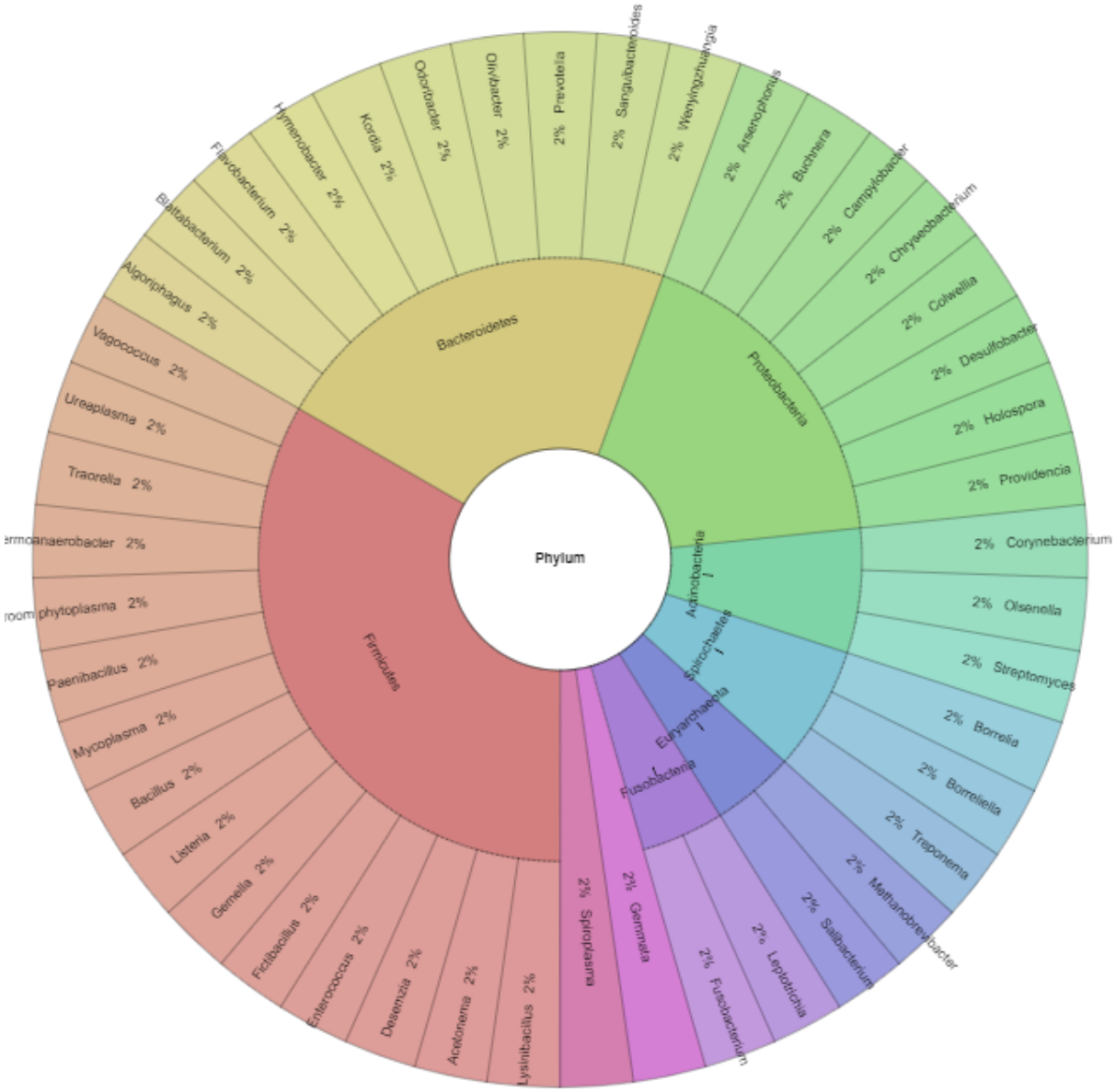
SAMPLE O-17


Phylum
Actinobacteria
Proteobacteria
Firmicutes
Euryarchaeota
Microbacteriaceae 1%
Microbacterium 1%
Millisia 1%
Mobilicoccus 1%
Modestobacter 1%
Monashia 1%
Mycobacterium 1%
Nakamurella 1%
1% Nocardia
1% Phycicoccus
1% Prauserella
1% Rhodococcus
1% Saccharopolyspora
1% Streptacidiphilus
Lentzea 1%
Leifsonia 1%
Knoellia flava 1%
Kitasatospora 1%
Kineosphaera 1%
Kibdelosporangium 1%
Jiangella 1%
Actinophytocola 1%
Janibacter 1%
Intrasporangium 1%
Herbidospora 1%
Herbiconiux 1%
Hamadaea 1%
Gryllotalpicola 1%
Gordonibacter 1%
Gordona 1%
Geodermatophilus 1%
Friedmanniella 1%
Dermacoccus 1%
Couchioplanes 1%
Cellulomonas 1%
Auraticoccus 1%
Arthrobacter 1%
Amycolatopsis 1%
Alloactinosynnema 1%
Actinopolyspora 1%
Actinoplanes 1%
Actinomadura 1%
Jatrophihabitans 1%
1% Fimbriiglobus
1% Acholeplasma
1% Peptoniphilus
1% Lysinibacillus
1% Lactobacillus
1% Brochothrix
1% Alicyclobacillus
1% Natronorubrum
1% Halopiger
1% Halomicrobium
1% Halolamina
1% Halohasta
1% Halobellus
1% Halobacterium
1% Xanthomonas
1% Stappia
1% Rhodoblastus
1% Rhizobium
1% Paludibacterium
1% Neorhizobium
1% Methylmicrobium
1% Massilia
1% Bosea
1% Marinomonas
1% Lysobacter
1% Janthinobacterium
1% Glycomyces
1% Dokdonella
1% Bradyrhizobium
1% Altererythrobacter
1% Marivita

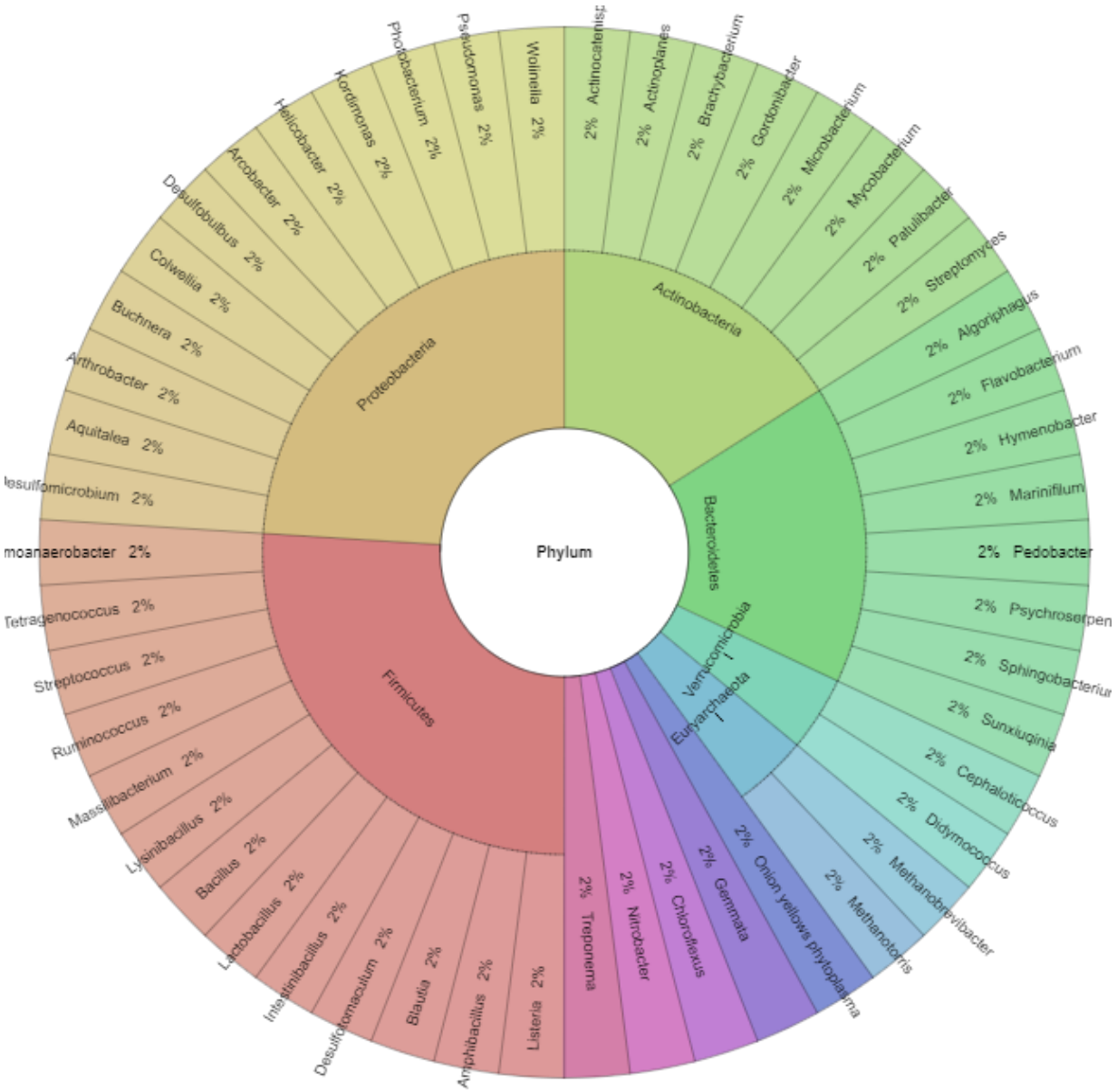
SAMPLE O-18


Phylum
Bacteroidetes
Firmicutes
Actinobacteria
Euryarchaeota
Cyanobacteria
Proteobacteria
Mesoflavibacter 1%
Niastella 1%
Nonlabens 1%
Porticoccus 1%
Prevotella 1%
Siansiviriga 1%
Tenacibaculum 1%
Facklamia 1%
Acidaminobacter 1%
Bacillus 1%
Caloramator 1%
Clostridium 1%
Dialister 1%
Enterococcus 1%
Anaerocolumna 1%
Lactobacillus 1%
Paenibacillus 1%
Peptoclostridium 1%
Ruminococcus 1%
Sporomusa 1%
Streptococcus 1%
Thermoanaerobacter 1%
Geodermatophilus 1%
Microtetraspora 1%
Nocardiopsis 1%
Rhodococcus 1%
Thermobifida 1%
Thermomonospora 1%
Halogranum 1%
Halolamina 1%
Methanobacterium 1%
Moorea 1%
Nostocales 1%
Nitrospira 1%
Sneathia 1%
Borrelia 1%
Massilia 1%
Acinetobacter 1%
Archangium 1%
Beijerinckia 1%
Campylobacter 1%
Chryseobacterium 1%
Desulfovibrio 1%
Erwinia 1%
Hydrogenimonas 1%
Legionella 1%
Lutimaribacter 1%
Aquabacterium 1%
Microbulbifer 1%
Moellerella 1%
Moraxella 1%
Photobacterium 1%
Rhizobium 1%
Rosenbergiella 1%
Shodgrassella 1%
Sphingobium 1%
Vibrio 1%
Wolbachia 1%
Lacinutrix 1%
Alkalitalea 1%
Bacteroides 1%
Bizionia 1%
Coprobacter 1%
Flammeovirga 1%
Flavobacterium 1%
Fluviicola 1%
Aplbacter 1%
Levinella 1%

SAMPLE O-19


Phylum
Bacteroidetes
Proteobacteria
Actinobacteria
Spirochaetes
Euryarchaeota
Fusobacteria
Firmicutes
2% Prevotella
2% Sanguibacteroides
2% Weeyingzhuangia
2% Arsenophonus
2% Buchnera
2% Campylobacter
2% Chryseobacterium
2% Colwellia
2% Desulfobacter
2% Holospora
2% Providencia
2% Corynebacterium
2% Olsenella
2% Streptomyces
2% Borrelia
2% Borreliella
2% Treponema
2% Methanobrevibacter
2% Salibacterium
2% Leptotrichia
2% Fusobacterium
2% Gemmiger
2% Spiroplasma
2% Lysinibacillus
2% Acetonema
2% Desemzia
2% Enterococcus
2% Fictibacillus
2% Gemella
2% Listeria
2% Bacillus
2% Mycoplasma
2% Paenibacillus
2% phytoplasma
2% anaerobacter
2% Traorella
2% Ureaplasma
2% Vagococcus
2% Algoriphagus
2% Blattabacterium
2% Flavobacterium
2% Hymenobacter
2% Kordia
2% Odoribacter
2% Olivibacter

SAMPLE O-20


Phylum
Actinobacteria
Bacteroidetes
Verrucomicrobia
Euryarchaeota
Firmicutes
Proteobacteria
2% Actinocatenispo
2% Actinoplanes
2% Brachybacterium
2% Gordonibacter
2% Microbacterium
2% Mycobacterium
2% Patulibacter
2% Streptomyces
2% Algoriphagus
2% Flavobacterium
2% Hymenobacter
2% Marinifilum
2% Pedobacter
2% Psychroserpen
2% Sphingobacteriu
2% Sunxiuqinia
2% Cephaloticoccus
2% Didymococcus
2% Methanobrevibacter
2% Methanotorris
2% Onion yellows phytoplasma
2% Gemmata
2% Chloroflexus
2% Nitrosacter
2% Treponema
2% Listeria
2% Amphibacillus
2% Blautia
2% Desulfotomaculum
2% Intestinibacillus
2% Lactobacillus
2% Bacillus
2% Lysinibacillus
2% Massilibacterium
2% Ruminococcus
2% Streptococcus
2% Tetragenococcus
moanaerobacter 2%
lesulfomicrobium 2%
2% Aquitalea
2% Arthrobacter
2% Buchnera
2% Colwellia
2% Desulfobulbus
2% Arcobacter
2% Helicobacter
2% Hortimonas
2% Photobacterium
2% Pseudomonas
2% Wolinella

RAGAS DENTAL COLLEGE & HOSPITAL

DEPARTMENT OF
ORAL PATHOLOGY & ORAL DIAGNOSIS

CASE SHEET

OP. No. :

AGE / SEX :

NAME :

DATE :

OCCUPATION :

RELIGION :

ADDRESS :

INCOME :

COMPLAINTS AND DURATION :

HISTORY OF PRESENT ILLNESS :

PAST MEDICAL HISTORY :

HYPERTENSION

ASTHMA

TUBERCULOUS

DRUG ALLERGY

DIABETICS

BLEEDING DISORDERS

CARDIO VASCULAR SYSTEM

ANEMIA

PREVIOUS HISTORY

PRESENTLY ANAEMIC ?

OTHERS

PAST DENTAL HISTORY :

PERSONAL HISTORY :

HABITS..... SMOKING..... BEEDI FILTERED

CIGARETTE

CIGAR NON FILTERED

PIPE

BRUSHING..... No. OF TIMES..... ONCE / TWICE

TYPE..... HARD MEDIUM / SOFT

CHEWING

DRINKING

TONGUE CLEANING..... METAL / PLASTIC SCRAPPER / BRUSHING

FAMILY HISTORY :

CLINICAL EXAMINATION :

GENERAL EXAMINATION :

1. PULSE
2. RESPIRATORY RATE
3. BLOOD PRESSURE
4. WEIGHT
5. HEIGHT
6. TEMPERATURE
7. MENTAL STATUS
8. BUILT
9. SITE OF NUTRITION
10. CYANOSIS
11. JAUNDICE
12. PIGMENTATION
13. CLUBBING
14. SKIN ERUPTION
15. DEFORMITIES

LOCAL EXAMINATION :

INSPECTION :

PALPATION :

PERCUSSION :

LYMPHNODE EXAMINATION :

INTRA ORAL EXAMINATION :

JAW MOVEMENTS :

MOUTH OPENING :

DENTAL CARIES

EROSION

ABRASION

ATTRITION

PLAQUE

CALCULUS

STAINS

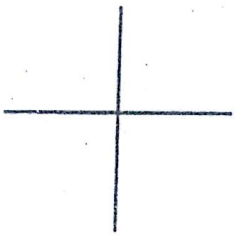
MOBILITY

G / I / II / III

GINGIVAL RECESSION

FURCATION

NUMBER OF TEETH PRESENT :



GINGIVA :

MARGINAL

ATTACHED

ALVEOLAR MUCOSA :

BUCCAL MUCCOSA :

PALATE :

TONGUE :

VENTRAL SURFACE

DORSAL SURFACE

FLOOR OF ORAL CAVITY :

PIGMENTATION :

COLOUR

SIZE

PATCHES

DIFFUSE

ULCERS :

SITE

SHAPE

BORDERS

NATURE OF BASE

CANDIDIAL INFECTIONS

ERYTHEMATOUS

ATROPHIC

PSEUDOMEMBRANOUS

HYPERPLASTIC

MEDIAN RHOMBOID GLOSSITIS

LINEAR GINGIVAL ERYTHEMA

SITE

ANGULAR CHEILITIS

UNILATERAL

BILATERAL

HAIRY LEUKOPLAKIA :

LEFT

RIGHT

BOTH

FISSURED TONGUE :

SUMMARY :

PROVISIONAL DIAGNOSIS :

DIFFERENTIAL DIAGNOSIS :

INVESTIGATIONS :

HISTOPATHOLOGICAL EXAMINATION

CLINICAL DIAGNOSIS :

TREATMENT PLANNING :

CASE ALLOTTED TO :

CONSENT FORM

I, Mr/Ms ..... s/o d/o w/o .....
aged..... hereby agree to participate in the research project titled, **"Comparison of salivary microbiome in healthy and Head and Neck Squamous Cell Carcinoma patients".**

I was informed and explained about the research in the language known to me and I give my consent to provide my saliva for the study. I have given voluntary consent without any individual pressure or duress. I also agree to co-operate with the doctor for the study and I understand the full consequences of the study. I am also aware that I am free to withdraw the consent given at any time during the study in writing.

I hereby give my voluntary consent to participate in the research project titled "Comparison of salivary microbiome in healthy and Head and Neck Squamous Cell Carcinoma patients".

Signature of the participant .....

Date .....

The subject was explained the procedure by me and he/she has understood the same and signed before me.

Name of the researcher .....

Signature of the researcher .....

Date .....

ANNEXURE - VIII

DEPARTMENT DECLARATION FORM

The study titled "COMPARATIVE ANALYSIS OF SALIVARY MICROBIOME IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS AND HEALTHY INDIVIDUALS USING 16S rRNA GENE SEQUENCING" has been done under the guidance of the staff of the Department of Oral Pathology and Microbiology during my post-graduation (2015- 2018). The same has been submitted as a part of the syllabus MDS degree program in Oral Pathology and Microbiology of the Tamil Nadu Dr. M.G.R. Medical University. I shall publish in full or part of this work in any media only with the prior written approval of the head of the department.

Ishwarya.S

Post-graduate 2015-2018

Department of Oral and Maxillofacial Pathology

Urkund Analysis Result

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http://onlinelibrary.wiley.com/doi/10.1111/j.1574-695X.2010.00773.x/full

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5

COMPARATIVE

ANALYSIS OF SALIVARY MICROBIOME IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS AND HEALTHY INDIVIDUALS USING 16S rRNA GENE SEQUENCING. Dr.ISHWARYA.S, POSTGRADUATE STUDENT

INTRODUCTION Microbiome is defined as the collective genome and gene products of the microbiota within an organism. Every human body contains a personalized microbiome that is essential to maintain health but capable of eliciting disease. An imbalance or shift in the microbiota at a given body site results in dysbiosis. Shifts from the core microbiome to dysbiosis has been associated with various diseases1. Oral cancer is a serious and growing problem with more than half a million people affected worldwide of which 90% are Oral Squamous Cell Carcinoma (OSCC).The etiology of oral cancer is multifactorial. Ultraviolet radiation, alcohol, tobacco usage, nutritional deficiencies and viral infections

have been implicated. Around 25% of oral cancer do not have any known risk factors2.

Microorganisms are associated with 20% of fatal cancers in humans.

In the oral cavity chronic inflammation has been observed at various stages of OSCC which could result from persistent epithelial or mucosal cell colonization by microorganism3.

Keeping in mind the

increasing evidence of the involvement of oral bacteria in inflammation,

it has been suggested that the shift in oral microbiome would be a factor in the etiology of chronic inflammation which would influence the pathogenesis of oral cancer. A consortium of microbes rather than one species is usually involved in causing disease. In dental caries, the ecological shift favours growth of acidogenic and aciduric species, namely mutans streptococci, lactobacilli and Bifidobacteria . In periodontal diseases, proteolytic bacteria that challenge the host inflammatory response are in play .The leading bacteria at periodontal destruction sites include members of the so-called red complex, namely P. gingivalis, Tannerella forsythia and Treponema denticola , as well as new taxa such as oral Synergistetes and Saccharibacteria (TM7)4. Bacteria such as Exiguobacterium oxidotolerans, Prevotella melaninogenica, Staphylococcus aureus and Veillonella parvula were isolated from tumours while tumour associated saliva sample showed the presence of Capnocytophagia gingivalis, Prevotella melaninogenica and Streptococcus mitis. Streptococcus mutans, Lactobacilli, and Streptococcus sobrinus were found to be associated with dental caries.

Microbes such as F.nucleatum, Spirochaetes (especially Treponema), Actinomyces, Lactobacillus, Enterococcus faecalis, Dialister species were implicated in the periapical diseases5.

The bacteria that are involved in OSCC need to be identified to establish the role of the microorganism in carcinogenesis. In the background of the importance of microorganisms in

dysbiosis, the present study was done to study the oral microbiota in the saliva of patients with OSCC and normal individuals.

MATERIALS AND METHODS

The study was designed to analyze the oral microbiome in the saliva samples

of Oral Squamous Cell Carcinoma (OSCC) patients and healthy individuals

by 16S rRNA sequencing of bacteria

with BLAST(Basic Local Alignment Search Tool) analysis in NCBI(National Centre for Biotechnology Information) database.

STUDY GROUP

Individuals who are diagnosed with OSCC by biopsy (n =10). Inclusion criteria: • OSCC patients. Exclusion criteria: • Patients under antibiotic therapy, antifungals and corticosteroids 3 months

prior to the study. • Patients with systemic diseases (Diabetic mellitus, Cardiovascular disorders, Gastroesophageal reflux disorder). •

Pregnant women. • Patients who are not willing to participate.

CONTROL GROUP

Individuals who are healthy (n = 10). Exclusion criteria: • Patients under antibiotic therapy, antifungals and corticosteroids 3 months prior to the study. • Patients with systemic diseases (

Diabetic mellitus, Cardiovascular disorders, Gastroesophageal reflux disorder). •

Pregnant women. • Patients who are not willing to participate.

STUDY DESIGN

Ten consecutive OSCC patients and ten healthy individuals satisfying the study criteria were enrolled. The unstimulated saliva samples of ten patients in each group were collected and analysed for

oral microbiome using 16S rRNA sequencing.

STUDY SETTING

After receiving patient’s consent (

Annexure VII),

the study was conducted at Ragas Dental College and Hospital and Madha Trust, a secular charitable institution for poor cancer patients in Chennai, South India. The laboratory techniques were carried out at Enable Biolabs Private Limited, Chennai (Annexure III).

SALIVA SAMPLE COLLECTION

A. Armamentarium

1. Pre-sterilized 50 ml graduated centrifuge tube

2. Saline

3. Gloves

4. Mask

5. Case sheet(Annexure VII)

6. Sharpie permanent marker

7. Consent form

8. Patient apron

B. Patient instruction

1. Do not eat or drink anything but water 1 hour prior to sample collection.

2. Rinse oral cavity with drinking water (room temperature) 1 hour prior to sample collection.

C. Collection

a. 5ml of saline to be swished around oral cavity for 30 seconds.

b. To spit the entire content into the sterile graduated centrifuge tube.

c. The containers with the samples to be labelled. Labels should include the following details:

• Name of the patient:

• Age/Gender:

• Case code:

• Time of collection:

D. Transport

To be carried in ice box. Temperature 3° to 5°C.

E. Storage

Refrigerated between 2°C to 8°C. Methods:

The entire procedure from extraction of bacterial DNA to quantification of DNA and further amplification and sequencing of 16S rRNA was performed by the reagents, (Cat# 51304) from QIAamp TM DNA minikit, Qiagen, Germany (Annexure III).

Bacterial DNA extraction

• Centrifuge 2 ml of oral saline rinse at 3000rpm for 5mins at room temperature to precipitate bacterial cells.

• The precipitated cells were suspended in 100µl cell lysis buffer containing 36% to 50% guanidine hydrochloride(RNA isolation).

• Incubated at 57°C for 2 hours to enable complete lysis of both gram positive and gram negative bacterial cells

• Following lysis, an equal volume of 100% ethanol was added to precipitate the genomic DNA.

• Transfer content to DNA spin columns containing silica membrane

• Centrifuge at 8000 rpm for 1min at room temperature.

• Precipitated DNA gets captured in the silica membrane

• Silica columns were washed twice with wash buffer (supplied by the manufacturer Qiagen)

• Degraded proteins and membrane lipid particles get washed off during the wash steps

• The captured DNA from the silica membrane was eluted with 50µl of elution buffer (supplied by the manufacturer Qiagen).

Quantification of DNA

• The DNA extracted from bacterial cells was quantified by QUBITTM Fluorometer to determine the total DNA concentration.

16S rRNA amplification and sequencing

• 50ng of total genomic DNA was subjected to polymerase chain reaction (PCR) amplification with 16S rRNA gene hypervariable region specific primers.

Forward: AGTTTGATC[A/C]TGGCTCAG

Reverse: GGACTAC[C/T/A]AGGGTATCTAAT.

• The following conditions were be used to amplify the 16S rRNA gene region: After an initial denaturation for 5 min at 95°C

• The DNA was subjected to 40 cycles of 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C, with a final extension for 10 min at 72°C.

• This results in amplification of an 800-bp 16S rDNA fragment, corresponding to Escherichia coli positions 10 to 806.

• The amplified rDNA product was subjected to gel electrophoresis to confirm the size of amplified product, which was then purified and sequenced with forward primer only.

• The sequenced data was then compared with reference bacterial gene sequences deposited in public database (ncbi.nlm.nih.gov) using BlastN program.

•

The microbiome charts were generated using Kronas softwareTM.

• The following values were noted from the results obtained: BLAST (Basic Local Alignment Search Tool) is an algorithm for comparing primary biological sequence information. Query: The input sequence to which all of the entries in a database are to be compared. Score: The score is a numerical value that describes the overall quality of the alignment of base pairs between the query sequence and the database sequence. Higher numbers correspond to higher similarity. Max score: Highest alignment score between query sequence and database sequence. Score is different from max score if several parts of database sequence match different parts of query sequence. Total score: Sum of alignment scores of all segments from the same database sequence that match the query sequence. Query coverage: Percentage of the query length that is included in the aligned segments. E value: Number of alignments expected by chance with a particular score. E is represented as the exponent of 10(eg; 1e-5=1x10-5 =0.00001). Bit score: Log representation of score. Identity: The extent to which two sequences have the same residues at the same positions in an alignment, often expressed as a percentage. Accession number: It is a unique identifier given to a DNA or protein sequence record to allow tracking of different versions of that sequence record and the associated sequence over time in a single data repository. Max Identity: BLAST calculates the percentage identity between the query and the hit in the nucleotide to nucleotide alignment. If there are multiple alignment with a single hit, then only the highest percent identity is shown.

Individual sample results • Top 100 sequences producing significant alignments were taken into consideration for the study. Lower the expected chance value better the significance of identified bacteria. A number close to 0 means that the hit has to be significant and not due to chance. • When there are two or more identical E values, the Max score is then used to sort the hits. The Total score becomes important when BLAST finds multiple, but not joint section of similarity between query and hit.

• If Max score

is equal to the

Total score then only a single alignment is present. If Total score is larger than Max score then multiple alignment is present and their individual scores have contributed to the total score.

•

When a bacteria was present in three or more samples, it was considered as predominant.

Uncultured bacterium

In certain samples(O-1, 0-6, 0-8) presence of uncultured bacterium was noted. The unculturable bacteria are bacterial sequences that have not been uploaded into the NCBI database as the method of identification in 16S rRNA gene sequencing technique involves comparing the sequences in the study sample with that available in the NCBI database. This is because bacteria maybe recalcitrant for culturing. This could be due to lack of necessary symbionts, nutrients or surfaces, excess inhibitory compounds, incorrect combinations of temperature, pressure or atmospheric gas composition, accumulation of toxic waste products from their own metabolism and intrinsically slow growth rate and rapid dispersion from colonies. Low concentration of DNA: DNA concentration can be decreased when extracted by non-commercial protocols. Other components of saliva such as enzymes, hormones, immunoglobulins and other biomolecules can interfere with the quality and quantitiy of the DNA extracted. The concentration of DNA extracted is not affected even when the saliva is frozen or stored for a longer duration.

INDEX

1. Oral Squamous Cell Carcinoma 2. Taxonomic Classification System

3.

Microbiome

• Oral Microbiome

• Clinical Significance

• Microbiome and Cancer

• Oral microbiome and Oral Squamous Cell Carcinoma.

4. Saliva as a Diagnostic Tool

5. Metagenomic Sequencing

ORAL SQUAMOUS CELL CARCINOMA Oral cancers ranks eleventh among the common malignancies globally.

Forty percentage affected are

in developing regions such as South-east Asia. Ninety percent of all oral cancers are squamous cell carcinoma originating from the mucosal epithelium. If detected during its early stages,

the 5

year survival rate of oral cancer is 60-80% 6.The etiology of Oral Squamous Cell Carcinoma (OSCC) is multifactorial and a combination of environmental risk factors and genetic predisposition. The risk factors can be grouped as established, strongly suggestive, possible and speculative factors based on the available global evidence2. Tobacco

along with

alcohol and betel quid usage are the most important etiological factors

in South East Asia.

Risk of oral cancer due to tobacco and alcohol is estimated to be more than 80%7.

Human Papilloma Virus infection is involved in oro-pharyngeal carcinogenesis2 (Annexure IV, Table 1). The average delay time in diagnosing and treating oral cancers is about 2 to 5 months.

Delayed detection may account for high morbidity rate of OSCC patients. Early detection and diagnosis lead to a greater survival rate and play a significant role in successful treatment of the disease8,9. Recently, factors such as the oral microbiome, are being explored for

their role as significant risk factors.

TAXONOMIC CLASSIFICATION SYSTEM A reliable classification system is a prerequisite for scientists and professionals dealing with microorganisms. The ultimate objective of biological classification is the characterization and orderly arrangement of organisms into groups. It is often confused with identification but, as a matter of fact, classification is prerequisite for identification10. The late 19th century saw the beginning of microbial taxonomy. Microbial taxonomy may be defined as the study and classification of the diverse microorganisms with the aim of organizing and prioritizing in an orderly manner. Two kinds of taxonomic and nomenclatural systems are of primary interest- A Linnaean system which is based on the Linnaean hierarchy and a phylogenetic system is a system based on the principle of descent. Linnaen Hierarchy: The Linnaean hierarchy is the series of ranked taxonomic categories based on those adopted by Linnaeus (1758) to which taxa (named groups of organisms) are assigned to seven principal categories-Kingdom, Division/Phylum, Class, Order, Family, Genus, and Species. The Linnaean system of taxonomy has since been complimented with the highest taxonomic rank for prokaryotes, called a “domain”. All prokaryotes are placed within the domains Bacteria or Archaea. Successively lower ranks follow as non-overlapping subsets of the domain: “phylum”; “class”, “order”, “family”, “genus”, “species” and “subspecies”. The “phylum”, “family” and “subspecies” ranks, as well as “suborder” and “subclass”, sometimes used for classification of prokaryotes, were added to the original Linnaean classification scheme. The “species”, assigned to a “genus”, in a binomial combination, is considered to be

the basic unit of microbial taxononomy11. Strain - a “population”, derived from a clonal variant /A group of presumed common ancestry with clear-cut physiological but usually not morphological distinctions. Species –comprising related organisms or populations potentially capable of interbreeding. It is the basic unit of biological classification. Genus - a category of taxonomic classification ranking above the species and below the family comprising structurally or phylogenetically related species. Family - a category of taxonomic classification ranking above the genus and below the order and comprising several related genera. Order - a category of taxonomic classification ranking above the family and below the class comprising several related families. Class - a major category in biological taxonomy ranking above the order and below the phylum comprising several related orders. Phylum - a primary category in biological taxonomy that ranks above the class and below the kingdom comprising related classes. Kingdom - a major category in biological taxonomy that ranks above the phylum and below the domain. Domain - the highest taxonomic category in biological classification ranking above the kingdom.

Phylogenetic classification: The second change involving concepts of taxa was associated with Phylogenetic Systematics or Cladistics. Concepts of higher taxa as groups of similar species were replaced with concepts of higher taxa as clades, that is, monophyletic (holophyletic) groups of species. This new concept of the higher taxon was derived directly from the principle of descent in that it equated higher taxa with units of exclusive common ancestry. Clade - A clade is a group of organisms that includes an ancestor species and all of its descendants Cladogram - A cladogram shows how species may be related by descent from a common ancestor. (Annexure IV, Figure 1) Node-based: The clade stemming from the most recent common ancestor of a and b (Where a and b are organisms, species, or clades). Stem-based: The clade composed of c and all members of x that share a more recent common ancestor with c than with d.b. (Where c and d are organisms, species, or clades, and x is a clade that includes both c and d). Apomorphy-based: The clade stemming from the first ancestor of y to evolve character e.(Where y is an organism, a species, or a clade, and e is a derived character)11. The comparison between the Linnaen and Phylogenetic classification is given in Annexure IV, Table 2.

MICROBIOME Microbiome refers to “the totality of microbes, their genetic information, and the milieu in which they interact”12. ‘Microbiome’ is a terminology

coined by Joshua Lederberg to signify the ecological community of commensal, symbiotic and pathogenic microorganisms that share our body space13.

These microbial organisms that contribute

to

microbiome are termed as ‘Microbiota’12. The human cells are out numbered by the microbes that occupy the body by several folds, thus earning humans the name of ‘supraorganisms’14. The microbiota’s composition can vary according to the environmental sites and the host status8. In health, the microbiome is in a state of homeostasis wherein the majority of the

microorganisms act as commensals or symbiotics15. When this relatively stable state of microbial homeostasis is disrupted, dysbiosis takes place12.

The

anatomical location is a primary determinant for community composition: interpersonal variation is substantial and is higher than the temporal variation seen at most sites in a single individual. Also, there are greater interpersonal similarities than a snap shot view indicates since the microbial system is dynamic in nature12. Diet inventories and 16S rDNA sequencing

characterization of 98 fecal samples have shown that the fecal communities are

clustered into enterotypes distinguished primarily by levels of Bacteroides and Prevotella. Enterotypes are strongly associated with long-term diets, particularly protein and animal fat (Bacteroides) versus carbohydrates (Prevotella).

The

substantial intestinal metagenomic changes is caused by dietary changes and the enterotypes are known to cluster based on dietary abundance of animal protein or carbohydrate16.

Characterization of nasopharyngeal microbiota of 96 healthy children was done in 2011

by barcoded pyrosequencing of the V5–V6 hypervariable region of the 16S-rRNA gene, and compared microbiota composition between children sampled in winter/fall with children sampled in spring. The approximately 1000000 sequences generated represented 13 taxonomic phyla and approximately 250 species-level phyla types (OTUs). Microbiota profiles varied strongly with season, with in fall/winter a predominance of Proteobacteria (relative abundance (% of all sequences): 75% versus 51% in spring) and Fusobacteria (absolute abundance (% of children): 14% versus 2% in spring), and in spring a predominance of Bacteroidetes (relative abundance: 19% versus 3% in fall/winter, absolute abundance: 91% versus 54% in fall/winter), and Firmicutes. This study reveals that there is seasonal variation of nasopharyngeal microbiota in young children which is independent of antibiotic use or viral co-infection17. The vaginal bacterial communities of 396 asymptomatic North American women who represented four ethnic groups (white, black, Hispanic, and Asian) and the species composition was characterized by pyrosequencing of barcoded 16S rRNA genes. The communities were clustered into five groups: four were dominated by Lactobacillus iners, L. crispatus, L. gasseri, or L. jensenii, whereas the fifth had lower proportions of lactic acid bacteria and higher proportions of strictly anaerobic organisms, indicating that a potential key ecological function, the production of lactic acid, seems to be conserved in all communities. The proportions of each community group varied among the four ethnic groups, and these differences were statistically significant [$P > 0.0001$]. Moreover, the vaginal pH of women in different ethnic groups also differed and was higher in Hispanic (pH 5.0 ± 0.59) and black (pH 4.7 ± 1.04) women as compared with Asian (pH 4.4 ± 0.59) and white (pH 4.2 ± 0.3) women18.

A microarray was designed

to detect and quantitate the small subunit ribosomal RNA (SSU rRNA) gene sequences of most currently recognized species and taxonomic groups of bacteria. They used this microarray, along with sequencing of cloned libraries of PCR-amplified SSU rDNA, to profile the microbial communities in an average of 26 stool samples each from 14 healthy, full-term human infants, including a pair of dizygotic twins, beginning with the first stool after birth and continuing at defined intervals throughout the first year of life. To investigate possible origins of the infant microbiota, they also profiled vaginal and milk samples from most of the mothers, and stool samples from all of the mothers, most of the fathers, and two siblings. Most of the breast milk and maternal vaginal samples clustered perfectly by anatomic site of origin. The composition and temporal patterns of the microbial communities varied widely from baby to baby. Despite considerable temporal variation, the distinct features of each baby's microbial community were recognizable for intervals of weeks to months. The strikingly parallel temporal patterns of the twins suggested that incidental environmental exposures play a major role in determining the distinctive characteristics of the microbial community in each baby. By the end of the first year of life, the idiosyncratic microbial ecosystems in each baby, although still distinct, had converged toward a profile characteristic of the adult gastrointestinal tract. The similarity of the microbial community profiles of stool samples from babies 1 year of age and older, to each other and to those of the adult stool samples suggested that the infant gastrointestinal communities converged over time toward a generalized “adult-like” microbiota. The infants' gastrointestinal microbiota was not significantly more similar to that of their parents than to that of other adults. The transition to an “adult-like” profile was found to often follow the introduction of solid foods19. The shift in gut microbial communities

was studied

following antibiotic therapy using a mouse model to control the host genotype, diet, and other possible influences on the microbiota. They employed a tag-sequencing strategy targeting the V6 hypervariable region of the bacterial small-subunit (16S) rRNA combined with massively parallel sequencing to determine the community structure of the gut microbiota. Inbred mice in a controlled environment harbored a reproducible baseline community that was significantly impacted by antibiotic administration. The ability of the gut microbial community to recover to baseline following the cessation of antibiotic administration differed according to the antibiotic regimen administered. Severe antibiotic pressure resulted in reproducible, long-lasting alterations in the gut microbial community, including a decrease in overall diversity 20. Thus, according to the review on microbiota by Cho and Blaser et al, each human over a lifetime develops a densely populated microbiome that is recapitulated in every individual and in every generation12. Microbiome variation and pathology: Cutaneous microbiome: In psoriasis, Firmicutes are over represented and Actinobacteria are significantly under-represented in the psoriatic lesions compared to both the unaffected skin in psoriasis patients and normal controls21. Cutaneous microbiome shifts, such as an increased abundance of Pseudomonaceae in individuals with chronic ulcers treated with antibiotics and an abundance of Streptococcaceae in diabetic ulcers have been reported21. Propionibacterium acnes have been implicated in the dermatological condition, acne23. Gastric microbiome: Gastric microbiota diversity was found to be high in Helicobacter pylori (H.pylori) negative individuals with abundance of prominent gastric phylotypes

(Streptococcus, Actinomyces, Prevotella,Gemella) in the oropharynx which indicates that either many constituents are swallowed from more proximal sites, or that close relatives of the oral microbiota colonize more distally. In contrast, in H.pylori positive persons, H.pylori accounts for < 90% of sequence reads from the gastric microbiota, thus reducing the overall microbial diversity of this microbiota24. H.pylori presence is strongly associated with particular diseases and important age-related differences. Its presence increases the risk for developing peptic ulcer disease, gastric Mucosa Associated Lymphoid Tissue (MALT) tumors, and gastric adenocarcinoma but also is associated with decreased reflux esophagitis and childhood-onset asthma; demonstrating the complex biological interactions with microbiota12. Colon microbiome: Inflammatory Bowel Disease susceptibility is associated with host polymorphisms in bacterial sensor genes such as nucleotide-binding oligomerization domain-containing protein 2 (NOD 2) and toll-like receptor 4 (TLR-4)25. Early childhood antibiotic exposure has been associated with increased risk for Crohn's disease and significantly diminished microbial diversity has been seen. Crohn's disease patients have over-representation of E.faecium and of several Proteobacteria compared to controls26. Gut microbiome associated pathology: Liver: Gut microbiota may be involved in hepatologic conditions, including Non-Alcoholic Fatty Liver Disease (NAFLD), alcoholic steatosis and hepatocellular carcinoma. Patients with cirrhosis have community-wide changes at multiple taxonomic levels, with enrichment of Proteobacteria and Fusobacteria (phyla), and Enterobacteriaceae, Veillonellaceae, and Streptococacceae (family)27. Obesity: In humans, obesity is associated with decreased Bacterioidetes and diminished bacterial diversity (Ley RE et al, 2006). Antibiotic use in human infants, before the age of 6 months was related to obesity development while perinatal administration of a Lactobacillus rhamnosus GG-based probiotic decreased excessive weight gain during childhood28. Rheumatoid arthritis: Dysbiosis within gut lumen can cause dysregulation of host immune responses (local expansion of Th17 cells that activate B cells to produce antibodies) leading to increased antibody production against joints29.

The complexity of dysbiosis and disease is best defined

by Hill's criteria which states that "The criteria include the strength of association, its consistency, specificity, temporality, and biological plausibility, and whether biological gradients are present, experimental support exists, and support can be extrapolated from known causal relationships"30. ORAL MICROBIOME In humans, oral microbiome is

one of

the most complex microbiome31. It is highly diverse, and includes bacteria, virus, fungi, archaea and protozoa15. More than 600 bacterial species have been detected, of which 50% have not been cultivated. A majority of 96% of bacteria belong to the phylum Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes and Fusobacteria; while the remaining 4% belong to Euryarcheota,Chlamydia, Chloroflexi, Synergistetes, Tenericutes and candidate phyla.(divisions SR1 AND TM7).

A candidate phylum is a lineage of prokaryotic organisma for which until recently no cultured representatives have been found32.

Due to the continuum of the oral cavity with the external environment, the oral bacterial flora undergoes dynamic changes in immeasurable rates33. This diversity varies from birth to adulthood due to various external and internal influences. Throughout childhood, the oral microbial load is found to increase but the microbial diversity seems to decrease31. The initial colonizers depend on: 1. Type of delivery: Babies born by vaginal delivery have bacterial communities quite similar to the mother's vagina – predominantly Lactobacillus, Prevotella, and Sneathia spp but babies born by cesarean section have bacteria similar to those present in the mother's skin – predominantly Staphylococcus, Corynebacterium, and Propionibacterium spp34.

2. Personal relationships: The infants show microflora according to the frequency of contact with the surrounding adults and children, domestic animals31.

3. Hygiene habits and diet:

Presence of Streptococcus species in edentulous children

have been demonstrated

thus disproving the fact that these species colonize only during the eruption of teeth. Hence oral hygiene practices become even more important right from birth35.

An increased diet of fermentable carbohydrates can favour the growth of acidogenic and aciduric species.

4. Development of teeth:

Primary dentition: Higher prevalence of bacteria belonging to the class Gammaproteobacteria (Pseudomonaceae, Moraxellaceae, Enterobacteriaceae, Pateurellaceae)

are present.

Permanent dentition: Higher prevalence of bacteria belonging to Veillonellaceae family and Prevotella are seen36. Other factors that can influence oral microbiome composition are genetics, host defences, microbial interactions (Quorum Sensing), receptors for attachment, temperature, atmosphere, pH, and salivary flow37.

Genetics – Genetic polymorphisms associated with interleukin (IL)-1, or other cytokines, can increase the likelihood of detecting certain key periodontal pathogens, and pre-dispose individuals to periodontitis. Host-defences and microbial cross-talk: The host defence system is actively engaged in cross talk with its resident microbiota in order to effectively maintain a constructive relationship. Host cell pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors) are strategically deployed in tissues to sample the extracellular and intra-cellular environments and recognize microbe-associated molecular patterns (MAMPS), such as lipopolysaccharide, lipoteichoic acid, nucleic acid. They activate multiple signalling pathways many of which converge on nuclear factor κB (NF-κB). MAMPs are present on, or are released

from, all microbial cells. The host has evolved systems to enable them to tolerate resident microorganisms without initiating a damaging inflammatory response, while also being able to mount an efficient defence against pathogens. Environmental factors: Nutrients such as amino acids, proteins, and glycoproteins are obtained from endogenous supplies, and mainly from saliva, although gingival crevicular fluid (GCF) is another potential source. Saliva contains amino acids, peptides, proteins, and glycoproteins, vitamins and gases, and it also provides the main buffering capacity for the mouth. The catabolism of the more complex host molecules, such as host glycoproteins, requires the sequential or concerted action of consortia of bacteria, in which their metabolic capabilities are combined. Importantly for the stability of the microbial consortium, the metabolism of these substrates leads to only minor and slow changes to the local pH, which are well tolerated by the normal resident microbiota. In contrast the main impact of diet is the provision of fermentable carbohydrates that leads to ecologically devastating falls in pH, which if repeated frequently enough, lead to the selection of acidogenic and acid-tolerating bacteria and a greater risk of dental caries. Even a small change in pH can alter the growth rate and pattern of gene expression in subgingival bacteria, for example, the expression of proteases by P. gingivalis increases at alkaline pH, and thereby can increase the competitiveness of some of the putative pathogens. This could favour the growth of periodontal pathogens, such as P. intermedia, P. gingivalis, and A. actinomycetemcomitans that have alkaline pH optima for growth. If sustained, the combined selective pressures of the environmental factors will lead to a re-arrangement of community structure and an enrichment of the proportions of the anaerobic and proteolytic component of the microbiota38.

As the child develops into an adult there is a shift in the bacterial population from aerobic or facultative gram positive cocci to anaerobic fastidious gram negative bacteria i.e; from a greater proportion of bacteria from phyla Firmicutes and Actinobacteria to Bacteroidetes, Fusobacteria, Spirochaetes, and Candidatus Saccharibacteria37,38. The set of initial colonizers seems to influence the subsequent colonization, thus setting the base for the complexity and stability of the microbial ecosystem in the adulthood30. Not only postpartum exposure influences the development of microflora but maternal health and hygiene also plays a role. A study has reported that there is 70% intrauterine colonization in amniotic fluid by oral microorganisms.

Pathogenic bacteria such as Fusobacterium nucleatum contributes to the risk of low birth weight and preterm babies38. The oral microbial flora’s complexity depends on oxygen tension, nutrient availability, temperature and host immunological factor exposure39. The proportion of the oral microorganisms may vary according to the colonizing sites. It was found in 2009 that teeth and tongue present a higher microbial load compared to oral mucosa and saliva40. The interplay of all the above mentioned factors is responsible for the development of the oral microbiome and is significant in the determination of health and disease31.

ORAL MICROBIOME – CLINICAL SIGNIFICANCE When microbial homeostasis is disrupted by external or internal factors, oral diseases such as dental caries, pulpal disease, periapical disease, and oral cancer may occur33. Dental caries: When there is an increased dietary

carbohydrate intake, bacteria that ferment the carbohydrates such as Streptococcus mutans, Lactobacilli, and Streptococcus sobrinus adhere to the tooth surface and increase the acidity of the biofilm. This in turn increases the load of these acidogenic bacteria and out-competes the resident flora such as Streptococcus sanguis and Streptococcus gordonii41. Recent studies have shown that Firmicutes, Actinobacteria, and Proteobacteria are the 3 most abundant phyla in patients with caries using Next Generation Sequencing42. The difference in oral microbial diversity between children with severe early-childhood caries (S-ECC) and caries-free (CF) controls

was evaluated in a study

by means of a cultivation-independent approach called denaturing gradient gel electrophoresis (DGGE). Pooled dental plaque samples were collected from 20 children aged 2 to 8 years.

Differences in DGGE profiles were distinguished on the basis of a cluster analysis.

The microbial diversity and complexity of the microbial biota in dental plaque

were found to be

significantly less in S-ECC children than in CF children43.

Periodontitis: A dysbiotic microenvironment has been observed in periodontal inflammation, which is triggered mainly by Porphyromonas gingivalis. This bacteria exerts a keystone effect via host modulation to breakdown homeostasis by remodeling the regular microbiome into a disease-provoking one44.

Endodontic disease: (i) Pulpal disease: P.micra, F.nucleatum and Viellonella species have been implicated in endodontic pulpitits while Atopio genomo species C1, P.alactolyticus, Streptococcus species were found in deep dentinal caries. Rocaset et al noted this shift in microbial population suggesting the change in environment as the cause45. (ii) Periapical disease: Periapical disease includes apical periodontitis and apical abcess. Gram negative saccharolytic rods such as Fusobacterium or Bacteroides are predominantly found in root canal spaces associated with periapical disease. Microbes such as F.nucleatum, Spirochaetes (especially Treponema), Actinomyces, Lactobacillus, Enterococcus faecalis, Dialister species have been implicated in the periapical diseases by recent studies so far which degrade the nitrogenous compounds into short chain fatty acids, ammonia, sulfur compounds, and indole that induce tissue inflammation by modulating immune response and promote apoptosis46.47.48. Halitosis: Actinomyces, Viellonella, and Fusobacterium which are tongue-coating bacteria degrade the nutrients present on the tongue surface to produce short-chain fatty acids, ammonia, sulfur compounds and indole49. These molecules are also present in periodontitis, thus a positive relationship exists between halitosis and periodontitis50. Microbiome and Cancer Even though bacteria were implicated as a potential cause of cancer in the microbial literature in the 19th century, the idea was dismissed51. Various propositions have been put forth recently which have revealed evidence based cancers associated with specific bacterial etiology 5(Annexure IV, Table 3). Khajuria et al in 2017, state that chronic

infections triggered by bacteria can facilitate tumor initiation or progression because, during the course of infection, normal cell functions can undergo the control of factors released by the pathogen. These bacterial factors, namely virulence factors, can directly manipulate the host regulatory pathways and the inflammatory reaction51. In many studies, it has been reported that smoking and alcohol consumption are commonly associated with carcinoma of the palate, while that of chewing tobacco is commonly associated with carcinoma of the alveolus and buccal mucosa. Alcohol is not carcinogenic, but there is increasing evidence that a major part of the tumor promoting action of alcohol might be mediated via its first, toxic and carcinogenic metabolite, acetaldehyde. Acetaldehyde is produced from ethanol in the epithelia by mucosal alcohol dehydrogenases, but much higher levels are derived from microbial oxidation of ethanol by the oral microbial flora. Thus, subjects consuming alcohol are at increased risk of developing cancer because of this synergistic action. Gram positive bacteria and yeasts are associated with higher acetaldehyde production, which could be a biologic explanation for the observed synergistic carcinogenic action of alcohol and smoking on upper gastrointestinal tract cancer. This may open a new microbiologic approach to the pathogenesis of the cancer of the oral cavity and upper gastrointestinal tract. Streptococcus intermedius, Prevotella, Capnocytophaga and Candida albicans

have been

isolated in increased numbers at carcinoma sites51. Paradigms proposed on role of microbiome in carcinogenesis: 1. Several bacteria cause chronic infections or produce toxins which can cause disturbances in the cell cycle and lead to alterations in the cell growth52. 2. Genetic mutation: Chronic infections induce cell proliferation through Mitogen Activated Phosphotidyl Kinase (MAPK) pathways and cyclin D1 that increase the rate of cell transformation and tumor development by increased genetic mutation53.

3. Several infections cause intracellular accumulation of the pathogen, leading to suppression of apoptosis primarily through modulation of the expression of Bcl-2 family proteins or by inactivation of retinoblastoma protein, pRb54. This strategy provides a niche in which the intracellular pathogen can survive in spite of the attempts of the host immune system to destroy the infected cells by apoptosis. Thus, it allows the partially transformed cells to evade the self-destructive process and progress to a higher level of transformation, ultimately becoming tumorogenic51.

4. Many pathogenic bacteria causing chronic infection with intracellular access subvert host cell signaling pathways, enhancing the survival of pathogen54. The regulation of these signaling factors is central to the development or inhibition of tumor formation. The precancerous lesion formed in such infections can regress with antibiotic treatment and clearance of bacteria51.

5. Metabolism of potentially carcinogenic substances by the bacteria. Local microflora may facilitate tumourogenesis by converting ethanol into its carcinogenic derivative, acetaldehyde to levels capable of inducing DNA damage, mutagenesis and secondary hyperproliferation of the epithelium56.

6. Nitrosation - In which microbial cells catalyze the formation of N-nitroso compounds from the precursor's nitrite and amines, amides or other nitrosatable compounds.eg; Escherichia coli51 (Annexure

IV, Figure 2).

Oral microbiome and Oral Squamous Cell Carcinoma:

The bacteria present in the tumor area can be causal, coincidental or potentially protective. They bind to and colonize the mucosal surfaces via a "lock and key" mechanism. Adhesins on bacteria

bind

specifically to complementary receptors on the mucosal surfaces of the host. These adhesins differ from species to species, leading to specificity in attachment to different surfaces. The bacteria that are involved in oral squamous cell carcinoma

need to be identified to establish the role of the microorganism in carcinogenesis. The

specificity of the bacterial species adhering to tumor mucosa could be due to the presence of their complementary receptors or simply due to the irregular

and altered

surface of the lesion favouring microbial retention57.The Table 4 represents the microorganisms isolated from tumor patients

in various studies5.

Table 4 Microorganisms associated with oral cancer Bacteria isolated from tumor specimen Exiguobacterium oxidotolerans, Prevotella melaninogenica, Staphylococcus aureus, Veillonella parvula. Bacteria isolated with the tumor associated saliva sample Capnocytophaga gingivalis, Prevotella melaninogenica, Streptococcus mitis.

Recently a number of studies have been taking place on the microbes involved in OSCC: It was investigated in 2005 whether the salivary counts of 40 common oral bacteria in subjects with an OSCC lesion would differ from those found in cancer-free (OSCC-free) controls. Unstimulated saliva samples were collected from 229 OSCC-free and 45 OSCC subjects

in 2005 by Hooper et al

and evaluated for their content of 40 common oral bacteria using checkerboard DNA–DNA hybridization.

It was

concluded that high salivary counts of Capnocytophaga gingivalis, Prevotella melaninogenica and Streptococcus mitis may be diagnostic indicators of OSCC58. Certain bacterial infections

may evade the immune system or stimulate immune responses that contribute to carcinogenic changes through the stimulatory and mutagenic effects of cytokines released by inflammatory cells. Bacterial toxins can kill cells or, at reduced levels, alter cellular processes that control proliferation, apoptosis and differentiation. These alterations are associated with carcinogenesis and may either stimulate cellular aberrations or inhibit normal cell controls59.

The

microbial populations on the oral mucosa differ between healthy and malignant sites and certain oral bacterial species have been linked with malignancies, but the evidence is still weak in this respect. Nevertheless, oral microorganisms inevitably up-regulate cytokines and other inflammatory mediators that affect the complex metabolic pathways and may thus be involved in carcinogenesis60. With the primary objective to identify any bacterial species within the OSCC tissue a

study was conducted in 2006

using a standard microbiological culture approach. At the time of surgery, a 1 cu.cm portion of tissue was harvested from deep within the tumor mass using a fresh blade for each cut. Diverse bacterial taxa were isolated and identified, including several putatively novel species. Most isolates were found to be saccharolytic and acid-tolerant species. Notably, some species were isolated only from either the tumour or the non-tumor tissue, indicating a degree of restriction. Successful surface decontamination of the specimens indicates that the bacteria detected were from within the tissue. Diverse bacterial groups have been isolated from within the OSCC tissue. The significance of these bacteria within the tumor warrants further study61. In another study the bacterial microbiota present within the oral cancerous lesions, tumorous and non-tumorous mucosal tissue specimens (approximately 1 cm3) were harvested from

ten

OSCC patients at the time of surgery. Bacteria were visualized within sections of the OSCC by performing fluorescent in situ hybridization with the universal oligonucleotide probe, EUB338. DNA was extracted from each aseptically macerated tissue specimen using a commercial kit. This was then used as a template for polymerase chain reaction (PCR) with three sets of primers, targeting the 16S rRNA genes of Spirochaetes, Bacteroidetes and the domain bacteria.

Differences between the composition of the microbiotas within the tumoros and nontumoros mucosae were apparent,

possibly indicating selective growth of bacteria within the carcinoma tissue. Most taxa isolated from within the tumor tissue represented saccharolytic and aciduric species62. The frequency of Streptococcus anginosus infection was assessed in oral cancer tissues and its infection route

was investigated where

Streptococcus anginosus DNA was frequently detected in squamous cell carcinoma (19/42), but not in other types of cancer (lymphoma and rhabdomyosarcoma) or leukoplakia samples63. The most prevalent genera in the OSCC library were

concluded as Streptococcus, Gemella, Rothia, Peptostreptococcus, Porphyromonas and Lactobacillus by Pushalkar et al in 2011.

To understand the role of bacteria in the development of oral cancer, the first step is to identify both cultured and uncultured organisms in the saliva as these organisms have the potential to cause inflammation that may support OSCC progression64.

The microbial flora using cultured saliva and oral swabs from subjects

was assessed with OSCC and healthy controls, wherein Metgud et al concluded

that the median number of colony forming units (CFUs)/mL at the carcinoma site were significantly greater than that at the contralateral healthy mucosa. Similarly, in the saliva of carcinoma subjects, the median number of CFUs/mL were significantly greater than in the saliva of healthy controls65. The bacterial spectra on the surface of OSCC

was identified

in comparison with the oral mucosa of patients with a higher risk to emerge an OSCC and control group to determine their susceptibility to various common antibiotics

by Bolt et al in 2014.

They concluded from their study that the prominent pathogens of the normal healthy oral mucosa were aerobes. The ratio between aerobes and anaerobes was 2:1, balanced in risk patients and inverted in the OSCC group3.

SALIVA AS A DIAGNOSTIC TOOL: Whole saliva is a mixture of fluids produced and secreted by major and minor salivary glands in the mouth and throat. It contains proteins, microorganisms, cellular debris, gingival crevicular fluid, and serum components66. The advantage of using saliva is that: (i) It is non-invasive and many unnecessary biopsies can be avoided. (ii) Decreases the number of hospital visits for the patient. Oral cavity provides a diversity of environments for bacterial communities and consequently microbiome profiles differ for various intraoral surfaces. Given that saliva is in direct contact with the oral mucosa and cancerous lesions, the screening and detection of early OSCC lesions using saliva shows promise67. Also, salivary microbial profiles tend to reflect the prevalence of bacterial pathogens in adherent oral biofilms. A decrease in the salivary count of pathogens can serve as an indicator of therapeutic effectiveness in the treatment of oral disease68. An important advancement in salivary diagnostics is the development of omics-based markers. The term salivaomics was coined to reflect the rapid development of translational and clinical tools based on salivary biomarkers69.

There are several molecular techniques that can be used to identify oral microbiota: Whole genomic checkerboard DNA-DNA hybridization: Hybridization of a selection of labeled whole-genomic DNA probes to sets of sample DNA fixed on a membrane. The specificity of whole-genomic probes is low due to shared genomic sequences with other bacteria. This technique requires cultivable bacteria for constructing the probes and is thus not suitable for studying not-yet cultured- bacteria. Reverse capture oligonucleotide hybridization: Hybridization of a selection of labeled PCR amplified 16S rDNA segments from sets of samples to species-species oligonucleotide probes fixed on a membrane. The probes have low sensitivity when the target bacteria are present at low levels in the sample. Fluorescent In-Situ hybridization(FISH): In-situ hybridization of fluorescent labeled 16S rDNA oligonucleotide probes to bacterial cell rRNA in the sample. The oligonucleotide probes have low sensitivity when the target bacteria are present at low levels in the sample. DNA Microarray: Hybridization of labeled DNA sequences in the sample to target-specific oligonucleotides fixed on a membrane/glass slide. When there are a multitude of unknown bacteria that still lack probes in the arrays it is difficult to obtain, without DNA amplification, enough material from target bacteria found at low levels in a background of other bacteria. 16S rRNA gene sequencing: The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes70.

METAGENOMIC SEQUENCING: Metagenomics is analysis of microorganisms by direct extraction of DNA from all genomes within a sample71. Currently 16S rRNA sequencing has been solely used as a research tool. The ubiquitous and phylogenetically stable bacterial 16S rRNA which is 1500 bp (base pairs) long offers a very useful target for the identification of bacteria down to species level. The 16S ribosomal subunit has highly conserved regions between all bacterial species between which highly variable regions (V1-V9) are present that are used to identify specific bacteria. Universal primers are designed to amplify a specific variable region, of which the most commonly targeted regions are V3, V4 and V672. After sequencing all bacteria are clustered based on their genetic similarity thus representing an Operational Taxonomic Unit (OTU). Grouping at 97% similarity allows identification at species level, while 94% allows for genus level identification of bacteria71,73. This potential to use rapid sequencing in order to understand the impact of bacteria on diseases is huge and

becoming increasingly relevant73.

RESULTS: Ten saliva

samples from Oral Squamous Cell Carcinoma patients and saliva samples from ten healthy individuals (controls) were obtained.

All samples were subjected to 16S rRNA gene sequencing. DISTRIBUTION OF AGE IN THE STUDY GROUPS: (TABLE 1 & GRAPH 1): The distribution of age of the patients was divided into 3 groups: 20-40 years, 41-60 years and those above 61 years of age. OSCC group consisted of

3 (30%) patients in the age group 20-40 years, 5(50%) patients in the age group of 41-60 and 2 (20%) patients in the age group above 61 years. Control group consisted of 4 (40%) cases in 20-40 years, 5 (50%) cases in 41-60 years and 1 (10%) cases above 61 years (p=0.788).

DISTRIBUTION OF GENDER IN THE STUDY GROUPS: (TABLE 2 &GRAPH 2): In OSCC group, 8 (80%) were males and 2 (20%) were females. In Control group, 1(10%) was male and 9(90%) were females.

DISTRIBUTION OF HABITS IN THE STUDY GROUPS (TABLE 3 & GRAPH 3): Based on the prevalence of habits in the study groups, they were categorized in to five groups. They were those without any habits, those with habit of, chewing tobacco alone, chewing tobacco and consuming alcoholic beverages, smoking alone, consuming alcoholic beverages alone. In group I (control group) none of them had any habits. In OSCC group, there were 2(20%) who had no habits, 5 (50%) with habit of chewing tobacco alone, 1 (10%) had the habit of chewing tobacco and consuming alcoholic beverages, 1 (10%) had the habit of smoking & consuming alcoholic beverages and 1(10%) had the habit of consuming alcoholic beverages alone (p=0.010).

DISTRIBUTION OF SITE OF THE LESION IN THE OSCC GROUP (TABLE 4 & GRAPH 4): In the OSCC group, 3(30%) had the lesion in buccal mucosa, 2(20%) had the lesion in tongue, 2(20%) had the lesion in maxillary alveolus, 1(10%) had the lesion in mandibular alveolus, 1(10%) had the lesion in oropharynx and 1(10%) had the lesion in hard palate.

SEQUENCING RESULTS (CHART 1- Master Microbiome chart, CHART 2 – OSCC group microbiome chart, CHART 3 – Control group microbiome chart, TABLE 5, GRAPH 5 (a,b,c) & TABLE 6): All twenty

samples were subjected to 16S rRNA gene sequencing in the variable region V5-V6. A total of 1900 sequences were obtained for all the samples with an average of 100 sequences per sample. One of the sample (control) did not show any sequences due to low DNA concentration. A total of 19 phyla were identified of which Proteobacteria(39%), Firmicutes (22%), Actinobacteria(15%) and Bacteroidetes(12%) were the major phyla.

The most predominant genera present under the four above mentioned major phyla are represented in Table 6.

Other phyla include Euryarchaeota, Spirochaetes, Tenericutes, Cyanobacteria, Verrucomicrobia, Planctomycetes, Nitrospirae, Fusobacteria, Chloroflexi, Vulcanisaeta, Desulfurobacterium, Deinococcus, Flexistipes, Caldithrix, Solemya. Proteobacteria was the major phyla present in both OSCC patients and healthy individuals. The combined groups (OSCC+Healthy individuals) showed 569 bacterial genus with 299 bacterial genus in OSCC group and 270 bacterial genus in healthy individuals (control) group. The most prevalent bacteria present in

OSCC patients were Bacillus, Bacterium, Buchnera, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Flavobacterium, Gemmata, Hymenobacter, Lactobacillus, Listeria, Lysinibacillus, Marinifilum, Ruminococcus,

Streptococcus, Streptomyces, and Thermoanaerobacter. In healthy individuals the prevalent bacteria were Bacillus, Enterococcus, Lactobacillus, Massilia, Paenibacillus, Streptococcus.

The predominant bacteria that are common in OSCC patients and in healthy individuals are Bacillus, Enterococcus, Lactobacillus and Streptococcus. (

Table 6 and Graph 5(a,b,c)). INDIVIDUAL SAMPLE RESULTS (ANNEXURE VI): SAMPLE

O-1 • In the OSCC sample (O-1) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Uncultured bacteria and Neisseria.

• We found that all the bacterial strains present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 1e-82 and ends at a value of 9e-04.

SAMPLE O-2

• In the Control sample (O-2) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Listeria, Bacillus and Streptococcus.

• We found that the bacterial species present in the sample belong to the phyla Firmicutes . The Expectation value (E) starts from 0.004 and ends at a value of 8e-04.

SAMPLE O-3 • In the OSCC sample (O-3) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Listeria, Gemmatta, Colwellia and Mycobacterium.

• We found that the bacterial species present in the sample belong to

the phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Calditrichaeota, Euryarchaeota,

Mollusca, Cyanobacteria, Nitrospirae, Verrucomicrobia .

The Expectation value (E) starts from 0.004 and ends at a value of 8e-04.

SAMPLE O-4

• In the Control sample (O-4) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Vitreoscilla, Neisseria, Acidovorax, Lampropedia, Simonsiella, Herbaspirillum and Parabulkholderia.

• We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 4e-15 and ends at a value of 3e-05.

SAMPLE O-5

• In the OSCC sample (0-5) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Shewanella, Candidatus, Serratia, Buchnera, Marinomonas, Pantoea and Methylomicrobium.

• We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 3e-49 and ends at a value of 9e-43.

SAMPLE O-6 • In the Control sample (0-6) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to uncultured bacteria.

• We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 1e-82 and ends at a value of 2e-80.

SAMPLE O-7

• In the OSCC sample (0-7) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Lactobacillus, Streptococcus, Enterococcus, Weissella, Carnobacterium and Listeria.

• We found that all the bacterial species present in the sample belong to the phylum Firmicutes. The Expectation value (E) starts from 1e-54and ends at a value of 8e-50.

SAMPLE O-8

• In the Control sample (0-8) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to uncultured bacteria and Neisseria.

• We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 1e-95 and ends at a value of 6e-95.

SAMPLE O-9

• In the OSCC sample (0-9) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Neisseria, Kingella and Vitreoscilla.

We found that all the bacterial species present in the sample belong to the phylum Proteobacteria. The Expectation value (E) starts from 1e-91 and ends at a value of 9e-81.

SAMPLE O-10

• In the Control sample (0-10) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Coprococcus, Ruminococcus and Lachnospiraceae.

• We found that all the bacterial species present in the sample belong to the phylum Firmicutes. The Expectation value (E) starts from 0.001 and ends at a value of 6.9.

SAMPLE O-11

• In the OSCC sample (O-11) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Flavobacterium, Clostridium, Enterococcus, Lacinutrix, Streptomyces and Carnobacterium.

• We found that the bacterial species present in the sample belong to 12 phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Tenericutes, Euryarchaeota, Planctomycetes, Defferibacteres, Aquificae, Crenarchaeota, Deinococcus thermus . The Expectation value (E) starts from 0.37 and ends at a value of 16.

SAMPLE O-12

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No results were obtained due to very low DNA concentration in the

sample.

SAMPLE O-13 • In the OSCC sample (O-13) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Flavobacterium, Clostridium, Enterococcus, Lacinutrix, Streptomyces and Carnobacterium.

• We found that the bacterial species present in the sample belong to 12 phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Tenericutes, Euryarchaeota, Planctomycetes, Defferibacteres, Aquificae, Crenarchaeota, Deinococcus thermus . The Expectation value (E) starts from 0.003 and ends at a value of 7e-04.

SAMPLE O-14

• In OSCC sample (O-14) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of

70% and more were selected. • The 100 sequences majorly belonged to Flavobacterium, Clostridium, Enterococcus, Lacinutrix, Streptomyces and Carnobacterium.

• We found that the bacterial species present in the sample belong to 12 phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Tenericutes, Euryarchaeota, Planctomycetes, Defferibacteres, Aquificae, Crenarchaeota, Deinococcus thermus . The Expectation value (E) starts from 0.003 and ends at a value of 9e-08.

SAMPLE O-15

• In the Control (O-15) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Geodermatophilus, Streptomyces and Microbacterium.

• We found that the bacterial species present in the sample belong to the phylum Firmicutes . The Expectation value (E) starts from 0.26 and ends at a value of 7.0.

SAMPLE O-16

• In the Control sample (O-16) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Paenibacillus, Bacillus and Lactobacillus.

• We found that the bacterial species present in the sample belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, Planctomycetes. The Expectation value (E) starts from 0.33 and ends at a value of 33.

SAMPLE O-17

• In the Control sample (O-17) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Actinoplanes, Janibacter, Marinomonas, Kitasatospora.

• We found that the bacterial species present in the sample belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, , Tenericutes, Euryarchaeota, Planctomycetes . The Expectation value (E) starts from 0.098 and ends at a value of 42.

SAMPLE O-18

• In the Control sample (O-18) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Flavobacterium, Clostridium, Enterococcus, Lacinutrix, Streptomyces and Carnobacterium.

• We found that the bacterial species present in the sample belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Euryarchaeota, Cyanobacteria, Nitrospirae, Spermatophyta and Fusobacteria. The Expectation value (E) starts from 1.1 and ends at a value of 25.

SAMPLE O-19

• In the OSCC sample (O-19) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Mycoplasma, Gemmatta and Listeria.

• We found that the bacterial species present in the sample belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Euryarchaeota, Tenericutes, Planctomycetes and Fusobacteria . The Expectation value (E) starts from 0.003 and ends at a value of 6e-04.

SAMPLE O-20

• In the OSCC sample (O-20) top 100 sequences producing significant alignments were taken into consideration for the study in which the bacterial sequences showing minimum identity of 70% and more were selected. • The 100 sequences majorly belonged to Listeria, Gemmatta, Colwellia and Mycobacterium.

• We found that the bacterial species present in the sample belong to 12 phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Spirochaetes, Tenericutes, Euryarchaeota, Planctomycetes, Chloroflexi, Nitrospirae, Verrucomicrobia . The Expectation value (E) starts from 0.004 and ends at a value of 8e-04.

DISCUSSION Metagenomics is analysis of microbial DNA from all genomes within a sample71 .In this study twenty samples of Oral Squamous Cell Carcinoma (OSCC) patients and healthy controls were studied using Metagenomic Sequencing (16S rRNA gene sequencing). Other methods which are commonly used are whole genomic checkerboard DNA-DNA hybridization, reverse capture oligonucleotide hybridization, Fluorescent In situ Hybridization (FISH) technique and DNA microarray. We used the 16S rRNA sequencing technique as it is useful in identifying unusual bacteria that are difficult to identify by conventional methods, providing genus identification in <90% of cases, and identification of 65–83% of these at the species level. The advantage of the 16S rRNA gene-based analysis is that it may bypass culturing of bacteria as PCR detection is done on DNA extracted from crude samples. The direct amplification of the 16S rRNA gene from DNA samples helps to detect unculturable bacteria which are estimated to exceed 99% of microorganisms observable in nature. Many novel species can be identified by this process of bacterial identification, when there is a significant difference between the phenotypic characteristics and/or 16S rRNA sequences of the unknown bacterium and those of the most closely related ones. As no single test or gene sequence is ideal for the definition of new species in all groups of bacteria, a polyphasic approach is usually used when a novel species is defined70. Although 16S rRNA gene

sequencing is highly useful in regards to bacterial classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera, as there is no known universal definition for species identification. However, 16S rRNA gene sequencing technique is widely used for establishing a “species” match. Issues to be considered in Small Sub Unit gene sequencing include: the number of position ambiguities, sequence gaps, and use of gap and/or nongapped programs with regard to sequence evaluation and analysis. The difficulties that can affect final identification include isolate purity, problems with DNA extraction protocols, and possible chimeric molecule formation71. The use of 16S rRNA gene sequencing for definitive microbial identifications requires a harmonious set of guidelines for interpretation of sequence data. The automation of 16S rRNA sequencing is not available yet and interpretation of results often needs significant expertise. In this study, we used the BLAST in NCBI which is a widely used database.

The 16S rRNA gene is conservative and therefore allows design of universal primers. In our study, we have used universal primer for amplifying the 16S rRNA gene. A single pair of the 16S rRNA gene universal primers is capable of amplifying the 16S rRNA gene from diverse bacterial taxa72. The universal primer used in our study were forward primer: AGTTTGATC[A/C]TGGCTCAG and reverse primer: GGACTAC[C/T/A]AGGGTATCTAAT. The oral microbial diversity assessed in OSCC patients by Pushalkar et al showed

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members of eight phyla (divisions) of bacteria. The majority of sequences in combined libraries belonged to Firmicutes (45%) and Bacteroidetes (25%). The phylum Firmicutes was the most abundant in the OSCC library as compared with the control library. The other phyla represented in both libraries are Actinobacteria, Proteobacteria, Fusobacteria , SR1, Spirochaete and uncultured TM7 64.

In our study,

a total of 19 phyla were identified of which Proteobacteria(39%), Firmicutes(22%), Actinobacteria(15%) and Bacteroidetes(12%) were the major ones. Other phyla

were

Euryarchaeota, Spirochaetes, Tenericutes, Cyanobacteria, Verrucomicrobia, Planctomycetes, Nitrospirae, Fusobacteria, Chloroflexi, Vulcanisaeta, Desulfurobacterium, Deinococcus, Flexistipes, Caldithrix, Solemya. The major phyla present in both OSCC patients and healthy individuals

was Proteobacteria. The high prevalence of Firmicutes as reported by Pushalkar et al ,was not seen in our study. This difference would be due to the use of both saliva and tumor samples by Pushalkar et al63. In our study, saliva samples were only analysed

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with the aim of assessing saliva as a diagnostic tool for OSCC. Similarly, in another study using saliva samples, high levels of colonization of OSCC by facultative oral streptococci

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and by species of anaerobic bacteria (Prevotella, Veillonella, Porphyromonas, Streptococcus anginosus, and Capnocytophaga) were demonstrated relative to uninvolved mucosa64.

The most predominant bacteria present in OSCC patients in our study were Bacillus, Bacterium, Buchnera, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Flavobacterium, Gemmata, Hymenobacter, Lactobacillus, Listeria, Lysinibacillus, Marinifilum, Ruminococcus, Streptococcus, Streptomyces, Thermoanaerobacter. In our controls the predominant bacteria were Bacillus, Enterococcus, Lactobacillus, Massilia, Paenibacillus, Streptococcus. Bacterial genera uniquely found in control group were Massilia and Paenibacillus. It is known that absence of certain bacteria can be responsible for shift in the microbial homeostasis, with alteration leading to the pathogenic bacterial overgrowth in OSCC patients. In our study, the most common bacteria that were seen both in OSCC group and the control group were Bacillus, Enterococcus, Lactobacillus and Streptococcus. Streptococcus species such as S. salivarius, S. intermedius, S. mitis and non-pathogenic Neisseria species are known to convert ethanol to acetaldehyde which is a Class I Carcinogen, with the capability to induce sister chromatid exchanges, point mutations, DNA adducts and hyperproliferation of epithelium4. In our study Streptococcus species were present in 60% of the OSCC subjects. Based on the site of the lesion, Streptococcus was the predominant bacteria present in all the sites(tongue(10%), buccal mucosa(20%), alveolus(10%), palate(20%). In OSCC patients, Streptomyces was seen both in alveolus(20%) and tongue(20%) whereas Bacillus and Listeria were seen only in the alveolar lesions(30%). No correlation was present in relation to the habit history of the patients as the habits were diversely varied among the subjects. In a review by Chocolatewala et al in 2012, majority of the isolates from OSCC patients were saccharolytic and acid tolerant, such as yeasts, Actinomycetes, Bifidobacteria, Lactobacilli, Streptococci and Veillonella. The microenvironment of solid tumors is typically hypoxic with low pH, thus favoring the survival of only acid tolerant bacteria5. In our study, the OSCC patients had saccharolytic bacteria such as Bacillus(50%), Bacterium(30%), Clostridium(30%), Corynebacterium(30%), Desulfutomaculum(30%), Enterococcus(30%), Gemmata(40%), Hymenobacter(30%), Lactobacillus(40%), Listeria(50%), Ruminococcus(30%), Streptococcus(60%), Streptomyces(50%) and Thermoanaerobacter(30%). Flavobacterium(40%) contains both saccharolytic as well as non-saccharolytic species of which Flavobacterium myroides, is non-saccharolytic. The bacteria with aciduric properties present in the OSCC cohort were Bacillus, Buchnera Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Lactobacillus, Listeria, Lysinibacillus, Ruminococcus, Streptococcus, Streptomyces and Thermoanaerobacter. Bolt et al in 2014, found

that the prominent pathogens of the normal healthy oral mucosa were aerobes

whereas anaerobes were predominant in the OSCC group3. In our study 58% of the prominent bacteria in the OSCC group fall under anaerobes while 42% were aerobes. Within

the 58% anaerobes present in the OSCC group, 37% were facultative anaerobes and 21% obligate anaerobes. In the control group 67% of the prominent bacteria were anaerobes and 33% were aerobes. Interestingly all the anaerobes (67%) in the control group were facultative anaerobic bacteria, with no obligate anaerobes, as opposed to 21% obligate anaerobes in the OSCC group. In the present study a large number of uncultured bacteria were identified. The unculturable bacteria are bacterial sequences that have not been uploaded into the NCBI database as the method of identification in 16S rRNA gene sequencing technique involves comparing the sequences in the study sample with that available in the NCBI database.

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These uncultured and sometimes dormant bacteria occupy different ecological microniches, and they maybe involved in latent infections.

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The results of our study on the salivary microbiome are of interest as it

provides an insight into the diversity present in the salivary microbial populations between OSCC and non-OSCC individuals. Our findings clearly show that though the microbiome is diverse there is a shift towards different species in OSCC compared to controls. These findings need to be validated in larger samples. The species that are unique to OSCC need to be further studied to assess their role and importance, if any, in the clinical and etiological context.

SUMMARY AND CONCLUSION

16S rRNA gene sequencing was done for ten cases of Oral Squamous Cell Carcinoma patients (OSCC group) and ten cases of healthy individuals (control group) and the sequences were identified using the BLAST (Basic Local Alignment Search Tool) analysis in the NCBI(National Centre for Biotechnology Information) database. • Of the ten cases within control group, one sample could not be sequenced because the quality of the DNA was not optimal. • The predominant bacteria seen in control group were Bacillus, Enterococcus, Lactobacillus, Massilia, Paenibacillus and Streptococcus. • The predominant bacteria seen in OSCC group were Bacillus, Bacterium, Buchnera, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Flavobacterium, Gemmata, Hymenobacter, Lactobacillus, Listeria, Lysinibacillus, Marinifilum, Ruminococcus, Streptococcus, Streptomyces, and Thermoanaerobacter.. • The bacteria which were common to both OSCC patients and healthy controls were Bacillus, Enterococcus, Lactobacillus and Streptococcus.

• The bacteria that were unique to the OSCC group but not seen in the control group were : Bacterium, Buchnera, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Flavobacterium, Gemmata, Hymenobacter, Listeria, Lysinibacillus, Marinifilum, Ruminococcus, Streptomyces, and Thermoanaerobacter. • The bacteria that were unique to the control group but not seen in the OSCC group were Massilia and Paenibacillus. • The saccharolytic bacteria

seen in the OSCC group were: Bacillus, Bacterium, Buchnera, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Flavobacterium, Gemmata, Hymenobacter, Lactobacillus, Listeria, Ruminococcus, Streptococcus, Streptomyces and Thermoanaerobacter. • The aciduric bacteria in the OSCC group were: Bacillus, Caulobacter, Clostridium, Corynebacterium, Desulfutomaculum, Enterococcus, Lactobacillus, Listeria, Lysinibacillus, Ruminococcus, Streptococcus, Streptomyces and Thermoanaerobacter. • The aerobic bacteria in the OSCC group were: Buchnera, Caulobacter, Clostridium, Corynebacterium, Gemmata, Hymenobacter, Lysinibacillus and Streptomyces. • The anaerobic bacteria in the OSCC group were: Bacillus, Desulfutomaculum, Enterococcus, Flavobacterium, Lactobacillus, Listeria, Marinifilum, Ruminococcus, Streptococcus, and Thermoanaerobacter of which 22% were obligate anaerobes whereas in the control group only facultative anaerobes were present. • 16S rRNA sequencing using Metagenomic Sequencing is a viable and powerful tool to study the oral microbiome. There are variations in the microbiome in OSCC group compared to the control group. The present study has narrowed down the bacterial species that further need to be studied.

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members of eight phyla (divisions) of bacteria. The majority of sequences in combined libraries belonged to Firmicutes (45%) and Bacteroidetes (25%). The phylum Firmicutes was the most abundant in the OSCC library as compared with the control library. The other phyla represented in both libraries are Actinobacteria, Proteobacteria, Fusobacteria , SR1, Spirochaete and uncultured TM7 64.

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Members of eight phyla (divisions) of bacteria were detected (Fig. 2a). The majority of sequences in combined libraries belonged to Firmicutes (45% of classified sequences) and Bacteroidetes (25%). The phylum Firmicutes was the most abundant in the OSCC library as compared with the control library. The other phyla represented in both libraries were Actinobacteria (14%); Proteobacteria (10%), Fusobacteria (5%), SR1 (0.6%), Spirochaetes (0.2%), and uncultured TM7 (0.2%).

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with the aim of assessing saliva as a diagnostic tool for OSCC. Similarly, in another study using saliva samples, high levels of colonization of OSCC by facultative oral streptococci

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and by species of anaerobic bacteria (Prevotella, Veillonella, Porphyromonas, Streptococcus anginosus, and Capnocytophaga) were demonstrated relative to uninvolved mucosa64.	and by species of anaerobic bacteria (Prevotella, Veillonella, Porphyromonas, Streptococcus anginosus, and Capnocytophaga) have been demonstrated relative to uninvolved mucosa (
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These uncultured and sometimes dormant bacteria occupy different ecological microniches, and they maybe involved in latent infections.	These uncultured and sometimes dormant bacteria occupy different ecological microniches, and they are involved in latent infections (
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The results of our study on the salivary microbiome are of interest as it	The results of our study on the saliva microbiome are of particular interest as it