CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Characterization of Oral Microbiota in Betal Quid Chewer and Non-Chewer

by

Leeza Khan

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Dedicated to Allah Almighty, Hazrat Muhammad (S.A.W.W) and my father Qamrul Islam Khan. My mother prayers have always enlightened my way throughout my life. It's also dedicated to my brother and all other family members who taught me that the best kind of knowledge to have is that which is learnt for its own sake. They taught me that even the largest task can be accomplished if it is done one step at a time.

CERTIFICATE OF APPROVAL

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My first word of thanks to the one who taught words to Adam, Who blessed man with knowledge, who is the sublime without whose "Kun" nothing is possible. Secondly my humblest thanks from the core of my heart, to our beloved **Prophet Hazrat Mohammad (S.A.W.W)** who is the eternal fountain of knowledge and guidance for the whole mankind.

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(Leeza Khan)

Abstract

There are 700 different types of bacteria, fungi and protozoa found in the oral cavity. Chewing betel quid has been linked to healthy oral microbiota, which can lead to tooth decay, oral leukoplakia, oral submucous fibrosis, and oral disease such as oropharyngeal cancer. Pathogenic bacterial species such as *Streptococcus, Enterococci, and Clostridium* spp. are often associated with betel quid chewers. Samples of non-chewer and betel quid chewer are collected and cultured in nutrient agar. Biochemical analysis of the samples was performed. Blood samples are only 120 out of 200 agar, while the remaining 80 samples show contamination or immaturity. In catalase test, a large numbers of gram-positive bacteria were extracted from non-chewer and a large number of gram-negative bacteria were deducted from betel quid chewers. Mannitol salt agar experiments show a high proportion of phenol red color in chewing and less in chewing while yellow color is found more in chewing and less chewing. In starch hydrolysis tests, a large number of gram-positive bacteria were found in the non-chewer and less in the chewers while a large number of gram-negative bacteria were found in the chewing and small in the non-chewing. In the MacCokney agar test it showed a much reddish color for betel quid chewers than non-chewers while peach color was found more in non-chewers than chewers. A large number of gram-positive bacteria are found in the non-chewers and less ratio in betel quid chewers. while gram-positive bacteria are most commonly found in betel quid chewer and less in non-chewer. In urease tests, a large number of gram-positive bacteria are found in the non-chewer and less number in case for betel quid chewers while gram-negative bacteria are found mainly in the non-chewer and less number in betel quid chewer. In the 16sRNA sequence, a single sequence of chewing and other non-chewing fluids was submitted to the NCBI. The non-chewer entry ID number is OK896990. The following results show a similarity of 99% baseline with bacterial specie '*Streptococci* in non-chewer. While one chewer sequence shows similarity with base pair specie called "*Stenotrophomonas*" "*Stenotrophomonas*" is a gram-negative, aerobic, non-fermentative bacillus closely related to the *Pseudomonas* species. Gram-positive specie named as "*Streptococci*" are facultative anaerobe and catalase negative. It

is usually found in healthy microbiota. *Streptococci* are mainly found in active anaerobes.

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Abbreviations

ADT	Aero-digestive tract
BLAST	Basic Local Alignment Search Tool
HMP	Human Microbiome project
HOMD	Human Oral Microbiome database
LPS	Lipopolysaccharides
MACC	MaCconkey Agar
MMP	Matrix metalloproteinase
MEGAX	Molecular Evolutionary Genetic Analysis
NSPH	Non- Specific plaque hypothesis
OLP	Oral leukoplakia
OPMD	Oral potentially malignant disorder
OSF	Oral sub-mucus fibrosis

Chapter 1

Introduction

1.1 Background

The microbiome is a group of microorganisms that live in our bodies. The term "microbiome" was coined by Nobel laureate Joshua Lederberg to describe a natural collection of symbiotic, commensal, and pathogenic microorganisms. These microorganisms live in the same area of the human body [1]. The number of microorganisms found in our body is equal to or greater than the number of cells [2]. Bacteria that live in human mouth are called the oral microbiome, also known as the oral microflora [3].

A scientist named "Dutchman Antony van Leeuwenhoek" used a built-in microscope to see the oral microbiota for the first time[4] .

The oral microbiota is a collected genome of microorganisms found in the oral cavity. After the intesti nes, it is the second largest network of viruses in humans. Compared to different parts of the body, they have a broad range of protein functions expected. There are two types of microbiota in humans: core and variable microbiota. The primary microbiome is shared by all people, but the flexible microbiome of each individual is different due to lifestyle and differences in body function. The soft and soft tissues of the teeth and oral mucosa, respectively, are two types of areas in the oral cavity where germs can invade [5]. Teeth, tongue,

cheeks, gingival sulcus, tonsils, roughness, and soft palate all help microorganisms grow [6][7]. There is evidence that microorganisms have been performing metabolic activities in humans for at least 500 million years[8]. Co-evolution in microbiome is recorded by various similarities within the composition and organization of human microbiota in many other animals [9]. Throughout the human population, the environment has systematically formed the structure of our microbiota, so it is gradually depleted in the Neolithic, age and modern times[10]. The use of fire, agricultural renaming, increased access to processed foods, and sugar after a commercial revolution therefore the advent of antimicrobial treatment may all contribute to the formation of the human microbiota [11].

Introduction / Introduction of refined sugar in our diet during the early agricultural periods caused the surly bacteria to improve their metabolism to adapt to the 'agricultural' changes after our diet. As a mutant called streptococci it has the ability to fight bacteria by increasing the level of oxidization and showing resistance between the acidic medium through carbohydrate metabolism [12].

The human microbiota is more than just a single-celled organism; it also accompanies them. Microbiomes are highly regulated, systematic and functional groups that form biofilms in the surface, [13] by interactions of interspecies and contraindications that contribute to environmental stability. The bacterium within the biofilm may interact by creating, detecting, and reacting to small differentiated signal molecules, a process known as quorum sensing, which benefits colonization, biofilm formation, rival protection, and adaptability to environmental conditions [14]. Virulence and pathogenic viruses are influenced by quorum sensing functions in biofilms, which play an important role in understanding and controlling bacterial infections, [15].

They form biofilm microorganisms that are highly resistant to host defense and antimicrobial agents [16]. Endogenous human microbial communities are involved in important metabolic, physiological, and immunological activities, such as immune and mucous membranes development, digestion and energy production, energy production, regulation of metabolic processes and fat storage, detoxification and membranes of mucus. natural chemical reactions, the function of the skin and

mucous membranes, and the development and control of the immune response (colonization resistance).

The mouth is one of the places in our bodies where bacteria are most prevalent. The oral microbiome discusses the many communities that are linked to the oral microbiome and the health issue. Mouth bacteria and oral cavity had a symbiotic interaction. Many of them are communalists, meaning they don't hurt other species or allow others to damage them. The only time a bacterium becomes harmful to the oral cavity is when it breaches the commensal barrier and infects the oral microbiota, resulting in infectious disease [17].

There is a strong interaction between the host and the microbiome, which is impacted by various factors of modern-day life, such as nutrition, cigarette use, and stress, and which may cause changes in our microbiome and induce a situation in which this finely tuned ecosystem is no longer balanced. To correct the imbalance. It is necessary to maintain the current condition of harmony.

Many factors are involved in causing an imbalance such as changes in pH and frequency of occurrence, mutations in healthy genes and the formation of new species due to the transfer of horizontal genes [18]. Some such factors as smoking affect healthy microbiome bacteria because commensalism may not occur in the oral microbiota [19].

Heavy metals, antibiotics, biocides, and antibiotics with antimicrobial properties have been introduced to humans as a result of industrial development, leading to a better selection of bacteria that carry antibodies out of the mouth [20]. The publication of Willoughby Miller's book "Microorganisms of the Human Mouth" in 1890, which focused on mixing and beating strings, changed the oral hygiene in developed countries in the late 19th century [21]. This may be one of the main causes of changes in the structure of the oral microbiota [22]. Excessive consumption of acidic beverages and refined sugars or smoked tobacco currently affects the oral ecosystem and spreads diseases such as tooth decay and periodontal disease. The number of people in the oral microbiota also depends on the habit of chewing betel quid, tobacco and alcohol which may cause an imbalance of oral bacteria. Alcohol use may cause infectious diseases to increase population and destroy the

relationship between commensalism and viruses[23]. Chewing betel quid has had a detrimental effect on oral and periodontal health [24].

There is a high rate of chewing worldwide but the highest rate is found in Asian countries e.g. India, Pakistan, china, japan, Taiwan, Pacific islands and migrants from South Asia to countries like the Malay peninsula, east and southern Africa, Europe and North America etc [25]. The population consumes betel quid, and 20-40%of the population in South Asian countries practice this practice [26]. The term "quid" is defined as "an object, or mixture of substances, that is placed in the mouth or chewed and that remains in contact with the mucosa, usually containing one or both basic ingredients, tobacco and / or areca nut, raw or any other form produced or processed". Betel quid, also known as smokeless tobacco, is produced in India. Most Asians use it. The word "paan" is another word for betel quid. Cigarettes, cardamom, saffron, cloves, turmeric, sweeteners, and mustard are some of the ingredients. After caffeine and nicotine, betel quid is the fourth most addictive chemical. The betel quid, placed between the chewing gum and the cheeses, relaxes chewing gum. Chewing betel quid promotes relaxation, relaxation, and fast digestion [27].

Betel quid, a compound betel leaf containing a combination of areca nut and slaked lime, which has no or no tobacco, is known as betel quid [27]. Betel quid with extra tobacco: Betel quid smokers and smokers is at higher risk of oral cancer. Betel quid and Areca nut: Those who chew betel quid and areca nut together have a higher risk of developing oral cancer than those who do not chew [28][29]. In addition, it briefly discusses how betel quid components change depending on a different geographical area[30]. In Taiwan, China, and Papua New Guinea, cigarettes are usually not included in the betel quid mix during any preparation phase, unlike in many other South Asian countries [31][32].

High prevalence of chewing betel quid is found in taxi, truck drivers and agriculture worker due to stress condition. People used betel quid under stress condition and frequently chewing cause burning sensation, less saliva and addiction [33].

The leaf acts as a natural antioxidant and has antibodies and an anti-cancer component the antifungal component acts as an antiseptic while the antibacterial

activity works against germs such as worms. Betel leaf is useful in treating ailments such as colds, coughs, congestion, and other respiratory ailments. Also show medical supplies. Saliva flow increases due to chewing betel quid. It helps protect intestinal parasites, improve pancreatic juice production and digestion. Betel leaf shows antibodies against these diseases e.g. typhoid, cholera and tuberculosis[34]. Chewing betel quid is related to the health of the oral microbiome that causes various oral diseases such as dental caries, oral leukoplakia, oral submucous fibrosis and other types of oral and oropharyngeal cancers. Since chewing betel quid can cause cancer, it is classified as a human carcinogen of Group 1, according to the International Agency for Research on Cancer and the World Health Organization [35]. Chewing betel quid and areca nut can cause other health consequences, including cancer of the mouth, lips and tongue, high blood sugar (type 2 diabetes), birth defects, low birth weight babies, high blood pressure and chronic kidney disease. Chewing betel quid can be addictive and can trigger symptoms such as hunger, insomnia, mood swings, impatience, irritability, and decreased concentration. All of these are some of the major problems related to chewing betel quid[36]. Chewing betel quid alters the oral microbiome by increasing or decreasing oral bacteria including Streptococcus, Actinomycetes, Bacillus Subtilis, and others. These findings suggest that betel quid chewing causes cancer of the mouth by disrupting the body's oxygen supply, as well as other oral and language diseases. Because betel quid chewing is associated with disruption of the oral microbiome, and people around the world chew betel quid for social, cultural, religious, and recreational reasons. The purpose of studying this mutated microbiome in the mouth can lead to a variety of diagnostic purposes and clinical features of these bacteria [37]. The carcinogenicity of betel quid, even when tobacco is not available, is well known[38]. It has been shown that betel quid components of the same type are active in the oral epithelium and cause cellular changes. As a result of these changes, oral carcinogenesis is initiated and developed within the oral mucosa[39]. Oral cancer is the 15th most common cancer in the world and is the leading cause of death from cancer [40]. Oral cancer is the third and fifth leading cause of cancer deaths in South Asian countries, including India, Pakistan,

Sri Lanka, and Bangladesh, respectively[41]. Breast cancer is the third and fifth leading cause of death from cancer [42]. Lungs, abdomen, and cervix. The onset of the disease in various parts of the cervix occurs between the ages of 51 and 55 or older [43]. Men are more likely to have this condition than women, followed by lung and stomach cancer[44]. The growth zone of the disease is determined by the variability of the different epidemiological risk in each region. The cheek (buccal mucosa) and gingiva are the most common areas of infection in South Asian cultures, and the tongue is the most common site of infection in Western societies [45]. The practice of chewing betel quid, which is one of the most important etiological factors in the development of oral cancer and a major threat to public health in many parts of South Asia [46], can be attributed to the strong development of oral cancer in parts of South Asia. Cancer of the mouth is caused by betel quid whether a person chews with or without tobacco [47]. Frequent chewing causes damage to a healthy oral microbiome and rapid growth of oral squamous carcinoma cells known as potentially malignant oral disease (OPMD). Chewing betel quid causes damage to healthy bacteria in the mouth and this practice leads to oral cancer. Betel quid disease OPMD (potentially dangerous oral disease) depends on the habit of chewing it daily for up to 6 months classified as chewing for life. A person who chews up to 12 months is classified as the current chewing gum. Those who did not chew betel quid for 12 months were classified as former chewers. Research reports show that the disorder is more common in those patients who chew betel quid, which is why it depends on their habit of chewing. The high level of OPMD found in a patient chewing betel quid also affects the nervous system [48]. Oral sub mucus fibrosis disorder is caused by chewing betel quid.

1.2 Problem Statement

To better prevent and treat oral disorders caused by chewing betel quid, which causes irreversible damage. It's important to understand and characterize oral microbial diversity.

1.3 Aims and Objectives

The study entails following objectives:

1. To isolate the oral bacterial micro biome from betel quid chewers vs. non-chewers.
2. To biochemically characterized bacteria frequently isolated from the collected samples.
3. To identify the frequently isolated bacterial species.

Chapter 2

Review of Literature

2.1 Oral Microbiome

The development of the microflora begins when the baby meets the uterus and the microflora of the vagina at birth after that microorganism present in the air. The oral cavity of a newborn baby is sterile, and the process of acquiring oral microflora begins from early breastfeeding onwards [49].

The establishment of a bacterial colony after birth is called the pioneer type. Example: *streptococci salavarius*. In 1st year, the oral cavity is attacked by aerobes such as *Streptococcus, Actinomyces, Veillonella, Neisseria and Lactobacillus*. When the tooth decay begins, they settle in the colony in a wasteland. In different birds' habitats, the accumulation of crust is noticeable and the sequence of processes increases as the bacterial population grows over time [50].

2.2 Composition of the Oral Microbiomes

The mouth cavity contains many types of bacteria. It is often in contact and appears to be dangerous to the effects of atmospheric winds [51]. The human microbiota is made up of two parts: the primary microbiome and the flexible microbiome. The primary microbiome is made up of distinct species found in various

parts of the body under healthy conditions. The flexible microbiome is different for each individual and has evolved due to unique lifestyle and genotypic choices[52]. The microbial ecology of the oral cavity is complex, and is a diverse biological setting with different niches that provide a unique habitat for microbial colonization. These sites include gingival sulcus, tongue, cheek, hard and soft palate, under the mouth, throat, saliva, and teeth [53]. Different areas of the mouth are covered by oral bacteria due to their adhesion to their binding receptors in the oral cavity [54]. Bacteria, fungi, viruses, Achaea, and protozoa comprise the common microbiome. Most studies on the common microbiome have focused on viruses, but there are few reports on the microbiome-fungal microbiome [55]. The oral cavity is one of the most widely studied microbiota, with 392 taxa with at least one reference genome and a total genealogy of close to 1500 [56].

About 700 species of prokaryote have been identified. These species are divided into 185 and twelve phyla species, about 54 percent officially identified, 14 percent anonymous (but cultivated), and 32 percent are classified as uncultivated phylotypes. 12 phyla includes *"Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Spirochaetes, SR1, Synergistetes, Saccharibacteria (TM7), and Gracilibacteria (GN02)* [57]. In a healthy mouth, there is an oral microbial preserved at the level of the species. Apart from similarities, the microbiome varies from person to person and site. Because the tongue has many papillae with few anaerobic areas, it has a variety of microbiota that include anaerobes. The buccal and palatal mucosae are an area with a low percentage of bacterial variants [58].

The oral microbiome may undergo major and rapid changes in structure and function, both spatially and temporarily, and progressively develops in the host. These multiplexes, unequal dynamic forces are caused by a variety of factors, including transient capture and food intake, response to pH variation, interactions between bacteria, and, to a large extent, genetic modification and horizontal gene transfer, which provide new features to strain [59]. Bacteria in our oral cavity have a mutualistic relationship based on the mutual benefits. Commensal statistics do not cause damage and keep a check on the various pathogenic species by preventing

them from adhering to the mucosa. Bacteria break the barrier of the commensals and become pathogenic, causing infections and infections [60].

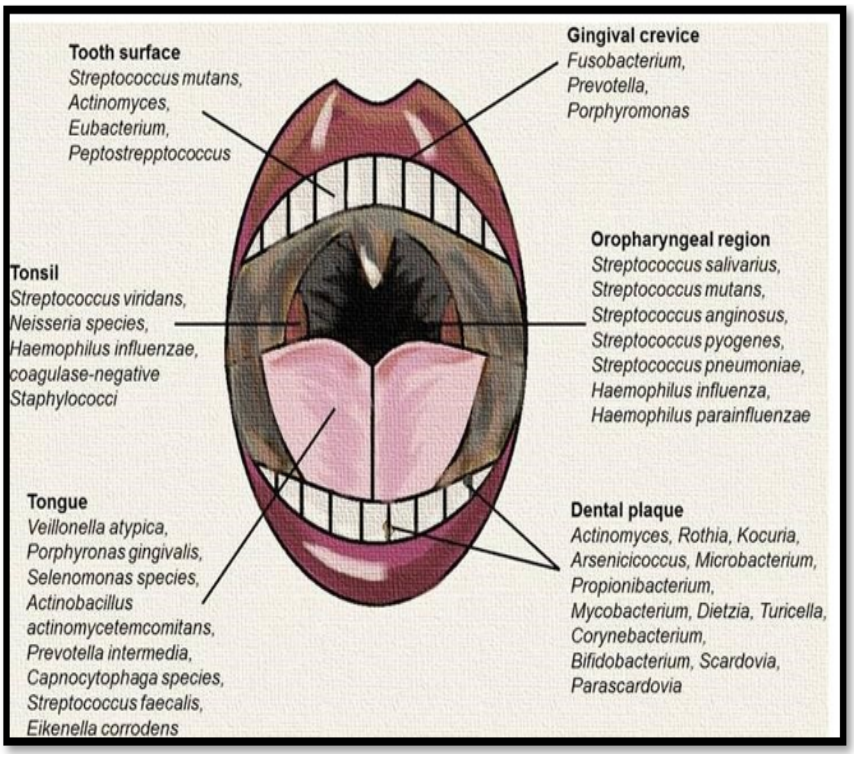

FIGURE 2.1: Predominant microbial communities within different sites of the oral and oropharyngeal region [29].

2.2.1 Types of Bacteria

2.2.1.1 Gram Positive Bacteria

Cocci: *Peptosterptococcus, Abiotrophia, Stomatococcus, Streptococcus.*

Rods: *Rothia, Pseudoramibacter, Prppionibacterium, Lactobacillus, Eubacterium, Corynebacterium, Bifidobacterium and Actinomyces* [61].

2.2.1.2 Gram Negative Bacteria

Cocci: *Neisseria, Veillonella and Moraxella* .

Rods: *Hemophilus, Fusobacterium, Eikenellaa, Desulfovibrio, Desulfobacter, Capnocytophaga, Camphylobacter, Woinella, Treponema, Simonsiella, Selemonas*[61].

2.3 Oral Microbiome Functions

Microbiota has a unique physiology and ecology that is closely associated with host biology[62]. The microbiota has a profound effect on both health promotion and disease progression [63][64]. Biofilm is the most common type of oral microbiota. It helps to maintain oral homeostasis, protect the oral cavity, and prevent infections. Knowing who the microbiome is and the neighbors it communicates with is important to understand the progression of the disease and to understand the mechanisms of key players [65].

Bacteria play a vital role in the human body, including digestion, energy production, differentiation, maturation, and regulation of the intestinal tract and immune system, as well as immune-strengthening functions; regulating fat storage and metabolism; processing of natural chemicals and detoxification; work to prevent skin and mucosa; maintenance of the immune system and balance between pro-inflammatory and anti-inflammatory processes; and promoting germs (resistance to colonization) and preventing the spread of disease [66].

2.4 Host Factor and Oral Microbiome

2.4.1 Intrinsic Host Factors

They are described as biological features of an existing host, which exists naturally and is not modified by the individual. These internal factors play an important role in the oral cavity and have a direct or indirect effect on the oral microbiota [67][68].

2.4.1.1 Saliva

To maintain oral health, saliva is an important component of various function within the oral cavity as it helps to lubricate food, speak properly, clean and protect teeth. Saliva allows the formation of a pellicle in a solid oral cavity that

TABLE 2.1: Role of pathogenic microbes in oral cavity[67].

Sr.No	Pathogenic microorganisms	Role in oral cavity
1	*Streptococcus*	It is the potential initiator of dental carries and play a role in assembly, metabolizing carbohydrate through fermentation and as a byproduct generate acid [68].
2	*Stomatococcus*	It causes sever oral infection in immunocompromised patients. It produces polysaccharide slime.
3	*Neisseria*	It causes infection of oral mucous membrane, but it is not clear that either its role is in periodontal diseases or dental caries.
4	*Veillonella*	By forming biofilm, it starts colonization and facilitate species succession to form dental plaque.
5	*Haemophili*	These bacteria are opportunistic and cause endogenous infection.
6	*Eikenella*	Induce periodontal disease and promote Periodontal pockets
7	*Fusobacteria*	Bacteria play role in oral and extra oral infection and act as bridge by colonizing bacteria of early and late phases and causes dental plaque

helps to colonize and the formation of bacteria that live in the area present in the oral cavity [69][70]. Saliva is a great source of nutrients. It helps to display the molecule in the oral microbiome and shows the symbiotic relationship between the microbe and the host [71].

2.4.1.2 Temperature

The human mouth temperature is maintained between 34-36 °C which provides an ideal environment for the development and growth of a wide variety of oral insects [72]. When a person eats hot or cold liquid or food, it causes a rapid change in the temperature of the mouth but after a while it returns to its original state [73]. Active genes that reflect human competition[74].

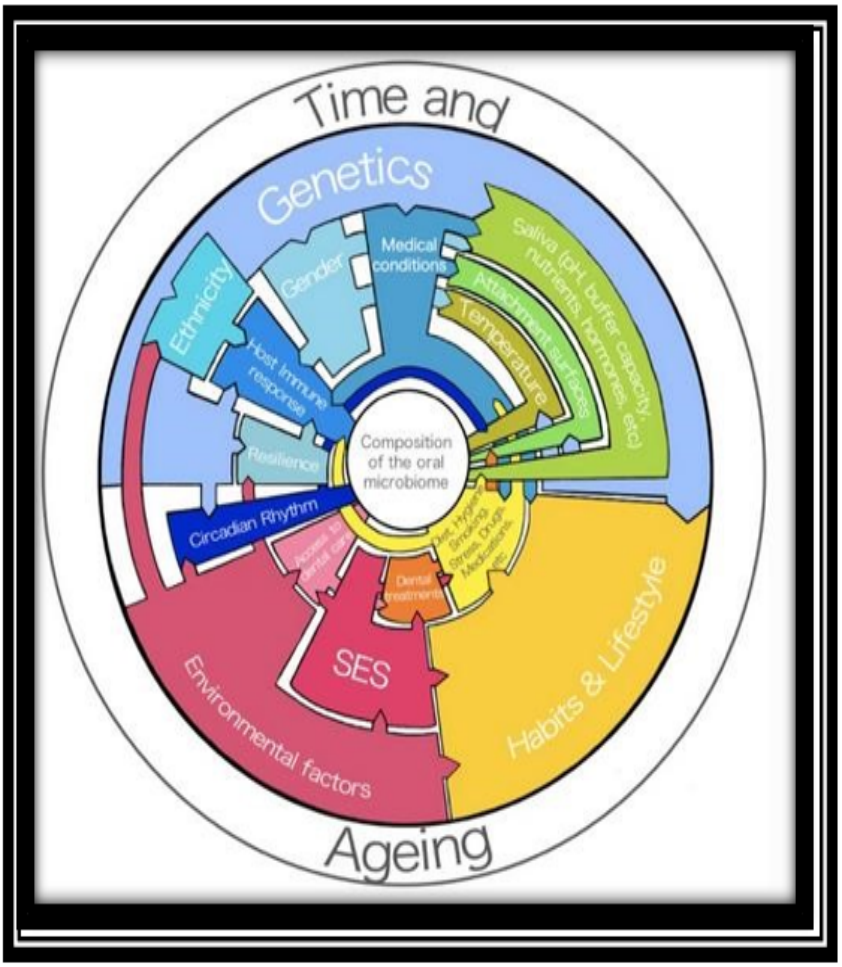

FIGURE 2.2: Host factor that influence composition of oral microbiome [75].

2.4.1.3 Genetics and Immune Response of Host

The composition of oral microbiota also effects ethnicity. Recent studies conclude that person ethnic background play an important role in genetic predisposal for bacterial colonization [76]. The same mechanism of bacterial microbiome composition with genetic background of person is not well defined. The evidence claim that this could be related to alternation in human immune system [77]. Shaping of microbe's community and mechanism of oral cavity colonization is highly affected by prenatal and perinatal processes [78].

Defense mechanism of oral cavity plays a great role in maintain relationship host and resident microbes. Homeostasis between host and microbe is due to innate and adaptive response. These adaptive and innate immune response secretes multiple compound that plays a role in preventing acute inflammation and stay alert

against pathogenic microbes [79].

2.4.2 Extrinsic Host Factor

The extrinsic factors of host microbiota interactions in health are as follow:-

2.4.2.1 Lifestyle

Factors affecting the oral microbiota by lifestyle are diet, tobacco, or drug use. The presence or absence of these features may indicate a disorder of the oral cavity and may result in various diseases or improve the natural balance[80].

2.4.2.2 Diet

A diet high in carbohydrates stimulated in the diet is also a major factor in developing tooth decay and symbiosis as it negatively affects the natural balance of the oral microbiome within the host [81]. The oral microbiome may ferment these carbohydrates into organic acids .
It causes dysbiosis if these organic acids are not reduced and allow for the development of low pH through oral bacterial fermentation [82].

2.4.2.3 Oral Hygiene

Oral hygiene plays vital role in removal of dental plaque by frequently teeth brushing [83]. Fluoride is also a very important part in fast reduction the of the worldwide frequency of dental caries [84].

2.5 Extrinsic Factors Not Modulated by Host

The intrinsic host factors not modulated by host are as follows:

2.5.1 Environmental factors

The environmental factors that can affect the host includes the residential place of host and changes with the place accordingly [85].

2.5.2 Socioeconomic Status

The social status of oral microbial diseases and health is related to social education, status, and social housing[86]. Things are important to the impact of society. The presence of cariogenic bacteria in the saliva of people exposed to stress due to social status. It is noteworthy that high or low levels of salivary cortisol are found in this group of people who cause tooth decay due to the presence of bacteria [88].

2.5.3 Dental Care Approach

Dental care is accessible to the people having availability of oral health professional and good economic status. Both are related to social economic status as well [89].

2.6 Impact of Oral Microbiome on Human Health

The oral microbiome is necessary and is a complex part of the human oral microbiome comprising seven hundred species of microorganisms [89]. Teeth, buccal mucosa, tongue, soft and hard palate are organisms that live in the mouth and make microbial organism rich in the area [90]. In the mouth, a variety of microorganisms are found, including viruses, fungi, and viruses. A healthy person incorporates microbial formation inside the oral cavity. Many other types of bacteria are present in the oral cavity such as Firmicutes, Bacillus, Proteobacteria and Actinomycetes[91]. There are eighty different types of fungi in the mouth and Candida is found to be the most common among them. These live in the oral cavity, but later become infected and make biofilms with Streptococcus due to

imbalance of the oral microbiome [93]. Bacteria found inside the oral cavity and germs are not the first and appear when the human body becomes infected. HIV and Mumps viruses are commonly found [93] Bacterial species are usually found in the oral cavity.

Examples of oral infections are *Streptococcus mutans, Porphyromonas gingivalis, Staphylococcus, and Lactobacillus*[94]. *S. mutans* are a big part of creating plaque and dental caries as well as tooth irritation [95].

P. gingivalis is diagnosed as a periodontal disease, which causes the gums to rot if left untreated.

Lactobacillus produces lactic acid in bacteria and is present in the host body. It also provides health benefits, causing an oral disease called dental caries that is spread by lactic acid fermentation in large quantities[96].

2.7 Oral Hemostasis

When the body is perfectly balanced and healthy, there are various viruses that live like symbiosis. Oral hemostasis can be maintained with the help of oral microbiota in the mouth[97]. It is important for the teeth and other dental tissues. With brushing, oral cavity and immune control, gingival crevicular fluid and saliva containing protein [98].

Substances such as lysozymes, immunoglobin A and lactoferrin retain the microbiota directly or indirectly. The mechanism of action is that the permanent flora has many anti-inflammatory and anti-inflammatory functions that help maintain oral hemostasis [99]. A mouth microbiota with a small number of bacteria reduces the risk of infection.

There is always a balance between commensal bacteria and germs that cause disease in a healthy mouth. In the host's mouth, they produce many antibodies such as immunoglobin and enzymes that maintain symbiosis. Any mutations in the mouth occur as a result of food, then the number of pathogens (streptococcus mutans) increases and causes tooth decay. If the host does not keep food or take preventive measures, then it leads to dysbiosis and causes oral diseases[100].

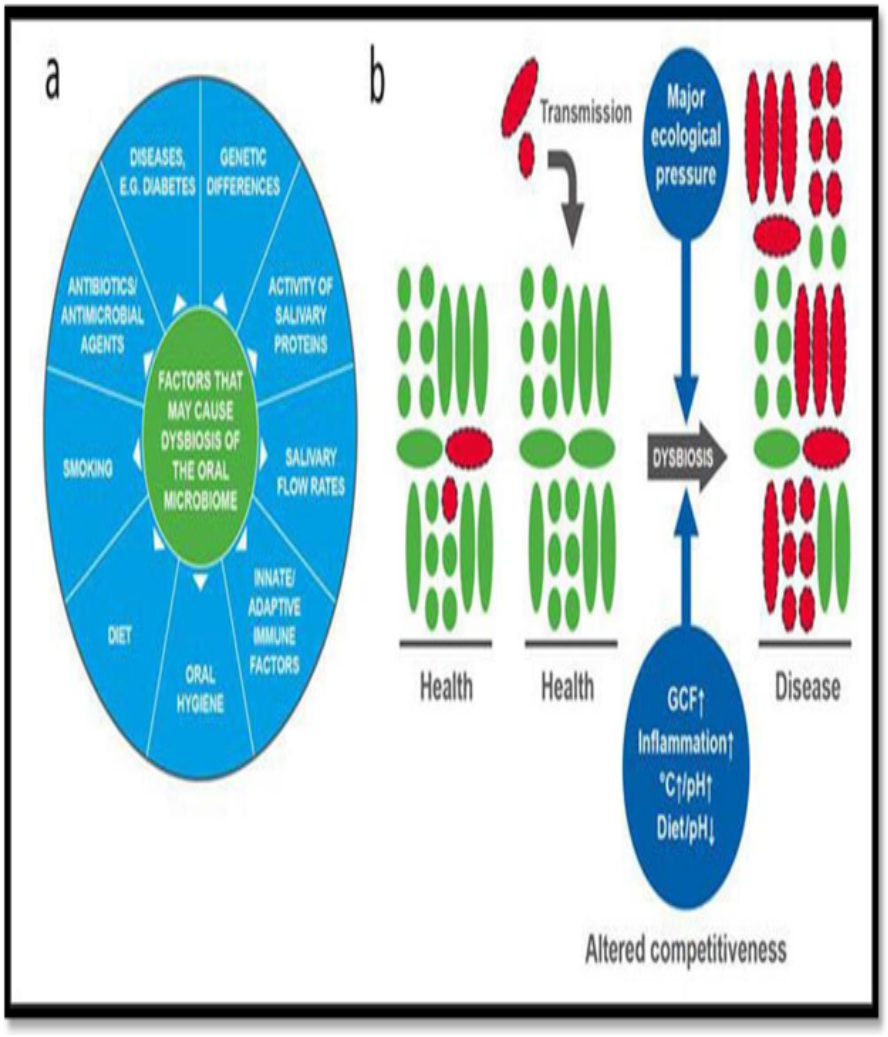

FIGURE 2.3: Shift from Symbiosis to Dysbiosis[101].

2.8 Gut

The oral microbiome is related to the intestines and causes various intestinal diseases. It also causes systemic diseases and inflammation of the intestines, but the cause is not found. Recent studies have shown that the oral microbiota can build up the colon in the gut and cause inflammation of the immune system in the gut. The oral microbiota reaches the gastrointestinal tract through the esophagus, makes microbial changes in the digestive system and affects it. Oral microorganisms of the oral periodontitis are contagious, enter the body through the periodontal blood circulation and release their metabolites in the blood that work throughout the body. This method plays an important role in digestive disorders

caused by the oral microbiome.

P. gingivalis caused by various diseases such as diabetes, and colon cancer through the transmission of the mouth to the intestines. It can destroy microbial and mucosal oral interactions by controlling the expression process and the innate immune system [101].

2.9 Food and Oral Microbiome Interaction

Oral microbiota could affect the body health by consumption of different food types. The factors which are involved are e.g. dietary patterns and food extracts [102].

2.10 Dietary Patterns

It's a very important factor which affects the oral microbiota. There are various dietary patterns in the society for example vegetarians, non- vegetarians, and westerns. Researchers had shown that there is some differences in oral microbiota of vegetarians, non-vegetarians, and western diet relied on oral health and physiology[102].

2.11 Food Extract

Polyphenol is a dietary extract found in apples, grapes, cherries, and red wine. It occurs in the oral cavity, although its effect on the oral microbiota is unknown. according to the study, it was reported that "Alcohol polyphenols" have antimicrobial effect and special properties that reduce the pathogenicity of S. mutans in the mouth. These can be used as a natural remedy against oral diseases. Tobacco, alcohol, and the use of areca nuts can increase the number of *Actinomycetes* and *Streptococcus* while reducing the number of *Parascardovia*. As a result, the oral

microbiota can have a detrimental effect on a person's health when certain foods are digested [103].

2.12 Maintenance of Oral Microbiota

The oral microbiota is maintained by the binding and microbial components, which include the study methods. The bacteria in which they live have anti-inflammatory properties, making it difficult to maintain balance in densely populated areas such as the oral cavity. Despite the prevalence of microbial colonization, malignant oral diseases are rare, similar to the interaction of the immune system with its symbiotic pathogens. Reception in patients with weakened immune systems, which can develop potentially harmful bacterial and fungal infections of the mucous membranes, and oral diseases with non-communicable viruses, emphasizes the importance of this host-microbe interaction.

Minerals are present in both saliva and GCF, which are needed for the growth of small microbial particles and antibodies. The importance of saliva in the health workplace is well known. During brushing, swallowing, speaking, and digestion, saliva containing enzymes and proteins contributes to the maintenance of a healthy microbiota. The oral cavity, like the tongue, produces 108 oral germs per milliliter of saliva. Saliva components are an important source of nutrients for bacteria and are essential for the development of a healthy microbiome. Salivary components such as secretory immunoglobulin A, lactoferrin, lactoperoxidase, lysozyme, statherin, and histatins regulate the microbiome both directly and indirectly, keeping it in shape. Lactoperoxidase, for example, induces the reaction of hydrogen peroxide, a byproduct of bacterial metabolism, and saliocyanate secreted to produce hypothiocyanite. Hypothiocyanite inhibits bacterial glycolysis, which has a direct antibacterial effect. Nitrite, which is made up of oral antibodies from dietary nitrates, is another component of saliva containing antibodies. Nitrite is later converted to nitric oxide, which helps prevent caries by preventing the growth of cariogenic bacteria.

Proteins composed of enzymes, lipids, and similar components (carbohydrates, nucleic acids) are found mainly in saliva, but can also be found in GCF, oral mucosa, and bacteria, and form the acquired pellicle, which helps protect dental areas. in acid attacks by altering the adhesion of bacteria in the dental and epithelial area. Enzymes involved in regulating microbiota balance do not move from active coordination to the acquired pellicle. By completing multiple interactions, the acquired pellicle expands and facilitates bacterial contact in non-destructive dental areas. Saliva's role is to regulate the layers of plaque with the help of many proteins, including as enzymes and glycoproteins, and minerals that control biofilm activities, as well as to help maintain an environment in which biofilms may develop. Plaque biofilm is dislodged by the action of the oral muscles of the cheeks and tongue during speech and mastication, as well as the flow of saliva[104].

2.13 Human Microbiome Project

The National Institutes of Health (NIH) highlighted the importance of the human microbiome (HMP) project in 2008. Recent advances in bioinformatics have improved our ability to study the human microbiome. These developments have created genomic and metagenomic series. These developments have created a wide range of genomic and metagenomics studies examining the function of microorganisms in various ecosystems.

HMP is a combination of several initiatives launched simultaneously in various regions of the world, including the United States, the European Union, and Asia, instead of a single project. Microbial ecologists developed methods to test microorganisms in situ, mainly by sequencing the 16S ribosomal RNA gene, after discovering that¿ 99 percent of bacteria in the area could not be easily reproduced (16S). It is used to identify members of microbial communities at the taxonomic and phylogenetic level. The introduction of high-sequence DNA sequences has led to a significant increase in research into what constitutes a healthy oral microbiota[101]. Nine areas from the oral cavity were found in people with HMP health. The tongue, dorsum, hard palate, buccal mucosa, keratinized gingiva or

gums, tons of palatine, throat, supra- and subgingival plaque, and saliva were all examined. K Li Bihan and Methe (2013) investigated the HMP site and found a small but invasive oral microbiome that was present in most of the samples but in low quantities[105] .

2.14 Database of Human Microbiome

The Human Oral Microbiome Database (HOMD) provides an oral bacterial gene tracking site, as well as an in-depth knowledge of oral bacterial taxa and a 16S rRNA diagnostic tool[105]. The National Institute of Dental and Craniofacial Research established in 2010 information on the only areas that can be cultivated or not.

The goal of expanded HOMD (eHOMD) was to provide the scientific community with a wide range of selected information about the types of bacteria found in the human aerodigestive tract (ADT), including the upper digestive tract, pharynx, nasal passages, esophagus, esophagus, and oral cavity. A sequence of ADT bacterium genome identified by various programs such as the Human Microbiome Project, which is part of the HOMD project, and other successive projects were added to eHOMD as it became available[106].

2.15 Microscopy and Culture

Historically, bacterial taxes have been identified based on culture. Among these were microscopy tests, biochemical and other phenotypic tests, sugar use, growth conditions, and antibiotic sensitivity. The actual variability of the oral microbiome cannot be demonstrated using traditional methods. Attempts by many researchers have now isolated, grown, detected, isolated, and isolated about half of the approximately 700 species of bacteria commonly found in the oral cavity. The basic drawback of conventional culture and cultural-based analysis techniques is that

many bacterial species in biological samples cannot be reproduced, making these methods unsuitable for research[107].

2.16 Sequencing of the 16S rRNA

Two common DNA sequencing techniques are widely used to analyze the ecosystem of oral bacteria uncultivated by 16S rRNA analysis sequences and metagenomics. Preserved genes of 16S rRNA sequences 16S rRNA, while metagenomics requires whole-genome gun sequence (WGS). The "gun" method is used to cut all DNA samples anywhere. The series is then completed using the standard Sanger sequence or the next generation sequence (NGS). To determine the type present in the sample, the most recent study uses a 16S rDNA genetic profile, or shotgun metagenomics when a complete genetic profile is required in a particular habitat[107].

2.17 16S rRNA Characteristics

1. What is 16S rRNA? It is a type of RNA that can be found in the human body. It can be found in almost all bacteria such as the multigene family or operon.

2. The genetic activity of 16S rRNA has not changed over time, which means that random sequence changes are the most accurate measure of time (evolution); and

3. The genes of 16S rRNA (1500 bp) are large enough for informatics purposes[108].

Because it is a highly preserved gene, it is more effective to use it as a marker than to use the full genome, because the gene on our site is less likely to differ from the structure of bacteria collected from natural samples[108]. 16S rRNA profile provides taxonomic information, however WGS metagenomics data can provide

not only taxonomic information, but also biological performance profiles in small communities[108].

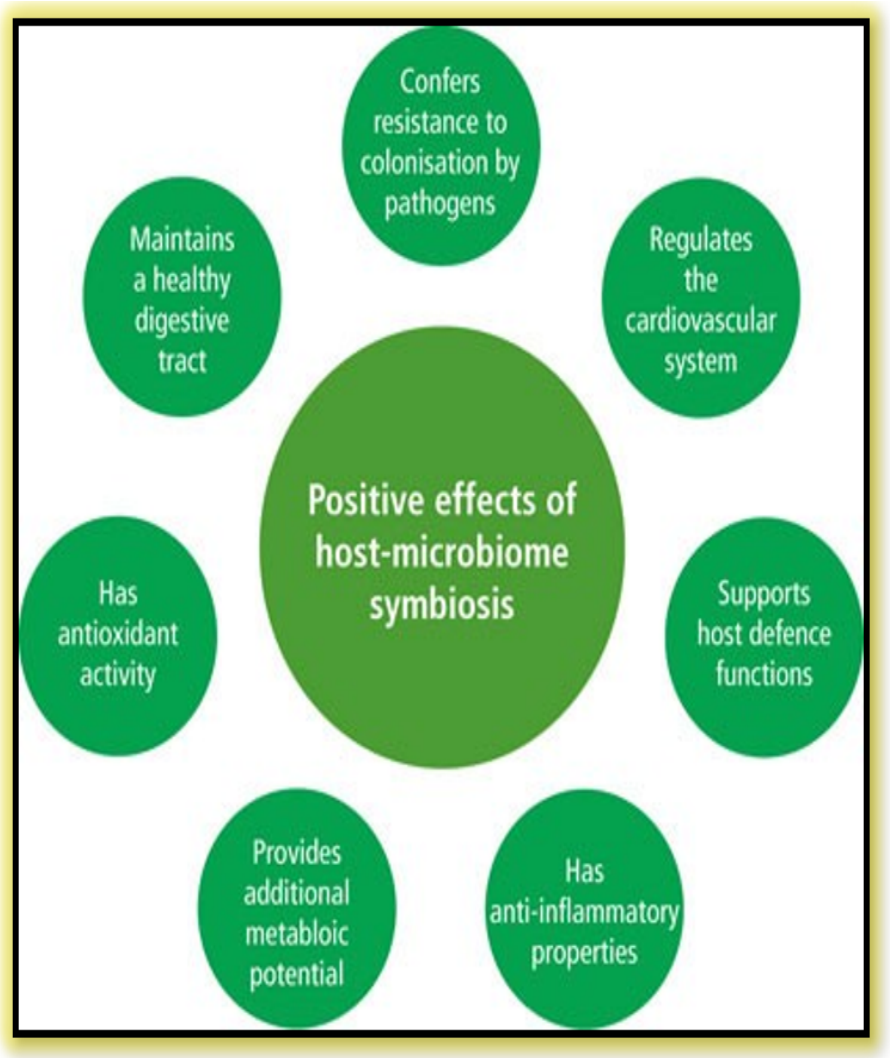

FIGURE 2.4: Positive effect of oral microbiome[108].

2.18 Oral Microbiota Characterization

Many oral germs are sensitive and difficult to grow, requiring complex growth media, specific atmospheric conditions, and long incubation periods. Because most oral antibodies are strong anaerobes, more caution is needed when collecting, transmitting, and storing samples. It is difficult to conduct a full cultural study of building materials, and only allows for the processing of small amounts of samples.

Many oral germs are sensitive and fast growing, requiring complex growth media, specific atmospheric conditions, and long incubation periods. It is difficult to make a full cultural analysis of building materials, and only allows for the processing of small sample values. Many oral germs are sensitive and slow-growing, requiring complex growth patterns, specific environmental conditions, and long incubation periods. Although selected bacteriological media has proven to be accurate in the study of certain species of interest, it may be biased in our understanding of the microbial etiology of oral disease by incorporating a variety of disease factors that thrive in such a culture while others are undetectable[109].

2.19 Methodology of Studying Oral Microbiome

The development of culture-based techniques has greatly improved the identification of microorganisms, many of which can grow culturally. 16S ribosomal RNA (16S rRNA) genetic community analysis is a widely used method based on the study culture of the microbiota. 57 The 16S rRNA gene is found in all prokaryotes and has different components that differ from one bacterium to another, which can be used to differentiate themselves. For comparison, researchers may have access to the Human Mouth Microbiome Database37, a free online resource that includes phenotypic phylogenetic sequencing data, clinical information, and bibliographic information about microorganisms found within the oral cavity. The 16S rRNA genes were isolated, amplified, and classified from a list of samples. If the sequence is detected as similar to a website, a microorganism may be identified; if no similarities were found, the sequence could be sent as a previously unknown phenotypic record.

The main gene sequence for the 16S rRNA gene was laborious, costly, and time consuming. Since the development of NGS technologies such as 454 pyrosequencing (quickly completed) and Illumina MiSeq, the sample output has grown, with up to 27 million sequences being processed simultaneously. Bacterial DNA has been found in a variety of extraction machines, laboratory reagents, and sample

collection methods. Certain extraction kits, laboratory chemicals, and sample collection equipment contain bacterial DNA, which can have a profound effect on research results. In addition to these cases, NGS is a useful tool for conducting extensive genomic research in samples. It has also greatly enhanced our knowledge and understanding of the oral microbiome.

Metagenomics and metaranscripttomics are two of the latest advances in technology. Metatranscriptomics allows researchers to investigate written genes, while metagenomics provides information on the genetic makeup of a microbial group of sample types. 146 Both of these methods have technical challenges, but offer the best opportunities for future research in the genome of oral microbiota gene and metabolic function.

Culturally independent methods add to our understanding of microbiota diversity, but the characteristics of living organisms and energy must be determined by culture. Only 16S rRNA gene sequences are now used to understand one-third of the species of oral microbiota, and researchers are constantly looking for new ways to develop viruses that are now 'unstable. A new method of classifying and cultivating novel types has been developed using siderophores (small computers, closely related to iron-chelating produced by bacteria such as bacteria, fungi, and grass). Its features and power should be culturally promoted. Its features and power must be culturally developed. The genetic sequence of only 16S rRNA is currently being used to understand one-third of the oral microbiota species, and there is a continuous hunt for novel genetic engineering techniques that are currently 'indestructible.' [148]. Using siderophores, a new method has been developed to classify and plant novels (small computers, closely related to iron-chelating produced by bacteria such as bacteria, fungi, and grass).

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New farming methods, combined with emerging biological and bioinformatics techniques and growing compounding powers, will not only help us better understand the oral microbiome, but will also help develop future intervention strategies to maintain health and target disease.

2.20 Oral Microbiota and Systemic Diseases

Oral microbiotas modulate the oral as well as systemic diseases of the body. These diseases are as follow[110] .

2.20.1 Diseases Associated with Oral Microbiome

The oral microbiome is important for oral and general health, and its absence can be detrimental to health. For example, oral bacteria that produce nitrate-reductase help convert food nitrates into nitrite. Salivary nitrite is converted into nitric oxide after ingestion, a potent vasodilator containing antibodies that are essential for cardiovascular health. Nitrite also promotes the production of mucus in the stomach. Nitrate has been shown to lower blood pressure, limit platelet function, and reduce endothelial dysfunction in small amounts. In patients with

hypercholesterolemia, studies have shown that prolonged nitrate intake improves blood vessel function. These benefits were related to changes in the oral microbiome, which prefers organic matter that can reduce nitrites. Although dietary nitrates may increase nitrite production, nitric oxide in tissues can react with superoxide radicals produced by body cells to form peroxynitrite anions, which are associated with cell destruction as DNA damage. The effects of nitrate / nitrite / nitric oxide on health are still being debated; however, many small studies have shown that the use of oral chlorhexidine-containing oral medications can lower nitrite levels in both saliva and plasma while causing a slight increase in blood pressure, it is important to highlight the importance of large-scale research before making any firm conclusions[110] .

2.20.2 Dysbiosis

In the oral cavity, a complex balance between the living species is maintained. It also looks at keeping people healthy (in symbiosis) or managing illness (in dysbiosis). A dysbiosis microbiome is one in which the diversity and dimensions of species or taxa within the microbiota are disturbed. The interaction between the host and the oral bacteria is fluid. In healthy individuals (after childhood microbiome maturation), microbial communities are stable, but biological changes in human health can affect the balance of species within these communities. There are physical changes that a healthy person can respond to without harming his or her dental health, such as aging or hormonal changes during adolescence and pregnancy. The oral environment can be disrupted, leading to dysbiotic changes and loss of social biofilm balance or diversity, by a small or small percentage of animal species and a high risk of disease. Oral dysbiosis is caused by a variety of factors, including dysfunction of the salivary glands (changes in saliva flow and / or formation), tooth decay, gingival inflammation, and lifestyle choices, such as eating and smoking[111] .

Many viruses have a symbiotic relationship with the host; in other words, microorganisms are shown green because many bacteria have symbiotic contact with the

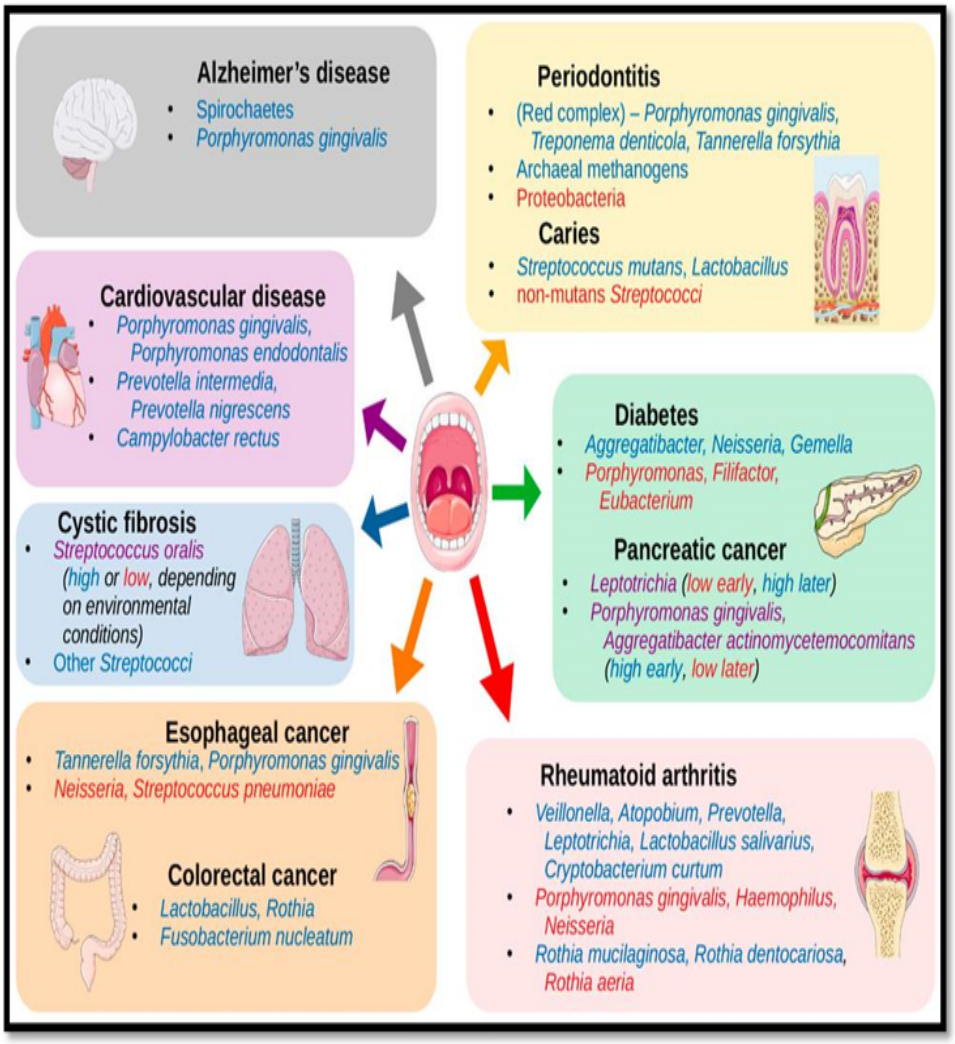


FIGURE 2.5: Oral and systemic diseases associated with the oral microbiome[110] .

host. In healthy domains, cariogenic or periodontopathic viruses (shown in red with dots) are found at low levels that are not clinically consistent; they may also be acquired from close partners (transmission), but their levels will be much lower compared to health-related viruses. Cariogenic or periodontopathic bacteria are a common cause of disease progression. As a result of biomass, they may increase (especially in gingivitis). It is believed that mutations in the natural environment (natural pressure) alter the bacterial competition within biofilm and select new species adapted to the new environment. Factors underpinning this choice should be identified and addressed in order to provide adequate protection against disease[111] .

Oral 'germs' can be found in a lower percentage in healthy areas, and oral disease is caused by a disruption of the natural balance of the microbiome rather than an external 'infection'[165]. Under dysbiosis, these types of disease-related bacteria become more numerous than in healthy settings, where they are usually small and non-toxic parts of biofilm[112] .

Changes in the biofilm development pattern are caused by dysbiotic microenvironments. In addition, there are many other types of mouthwash. A large number of microorganisms can accumulate as a biofilm for dental plaque due to tooth decay (smooth surfaces, holes and cracks, proximal areas, and exposed roots). The plaque biofilm does not naturally disintegrate as it accumulates due to the lack of oral care to disrupt and eliminate it, which may be a major cause of dysbiosis.

2.20.3 Carries and Periodontitis (Dysbiosis)

Several theories have been proposed to explain the link between plaque and dental disease. The nonspecific plaque hypothesis (NSPH) was first proposed in the nineteenth century and developed during the next century. The National Society for Public Health (NSPH) believed that dental diseases were caused by an indirect increase in all microorganisms in tooth plaque. The NSPH was also changed to mean that subgingival colonization was the cause of destructive periodontitis, driven by environmental changes such as plaque accumulation, gingivitis, and gingival exudate. These changes increase the number of oral bacteria and change their dosage, though. These changes increase the number of bacteria in the mouth and alter their proportions, though. There is no single type available on the active and non-functional sites. Because it was thought that any plaque could enter the human tooth, it was decided that the mechanical removal of as much plaque as possible, such as brushing teeth or brushing teeth, would be the best way to prevent infections.

More species can be differentiated and reflected in cultures as laboratory techniques develop. The discovery that kanamycin was very effective in caries-related

strains such as streptococci led to "speculation of a specific plaque," which suggested that only a few species of oral microbiome were involved in the disease process, and that antibiotics directed to these types could cure or prevent disease. (early caries and later periodontitis). Clinical studies that use antibiotics to treat these diseases, on the other hand, have become increasingly frustrating in terms of actual efficacy and the level of long-term therapeutic benefits. Clinical research that uses antibiotics to treat both disorders, on the other hand, is largely unsatisfactory in terms of translation into daily practice and the level of long-term clinical benefit. These findings may be explained by the fact that, as shown earlier, a large number of microorganisms cannot be cultivated, so bias may be introduced by differentiating those that can be grown[113] . The NSPH proposed an ecological plaque hypothesis in the 1980s to explain the link between the habitat they live in, the prevailing ecology, and oral diseases. Changes in the natural environment can contribute to the competition of plaque bacteria, leading to the development of organisms that are better adapted to the new environment. This approach suggests that the disease can be prevented not only by preventing the virus from being blamed directly, but also by interfering with the natural processes that favor the selection and enrichment of these insects. In caries, for example, an increase in sugar or a decrease in saliva flow promotes plaque biofilms to be exposed to low pH values for longer and longer. This is beneficial for organic acids that produce acids and / or are more tolerant of acids than bacteria that thrive in a neutral environment or help lower pH. As figure 2.6 shows the latest model of host – microbe interactions in the etiology of caries.

The theory of ecological plaque was developed with the idea that some small diseases found in small quantities could cause inflammatory disorders by interacting with the human immune system and altering the microbiota, leading to gingivitis and periodontitis. The formation of biofilm causes gingivitis; However, the presence of biofilm is not enough to develop periodontitis. The progression of the disease from gingivitis to periodontitis now requires complex interactions between mediators of the immune response and biofilm. Most tissue damage is produced by

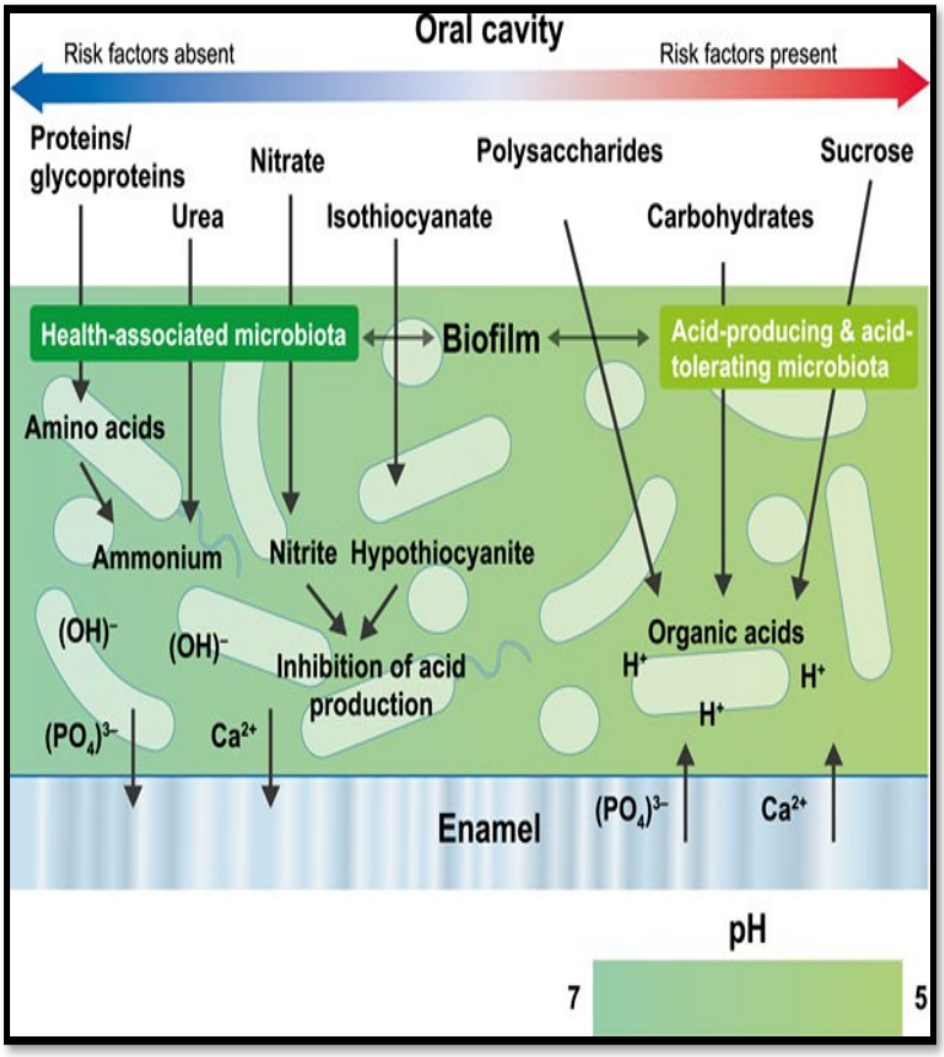


FIGURE 2.6: A contemporary model of host–microbe interactions in the pathogenesis of caries (adapted from de Soet and Zaura and Takahashi) [113] .

a large and uncontrollable amount of inflammation, caused by a number of dysbiotic bacteria. Biofilm formulation causes local inflammation, which increases the flow of nutrient GCF and possibly bleeding, reduces oxygen supply and promotes the growth of anaerobic microbes. Periodontal ecological changes provide an ideal habitat for anaerobic-dependent bacteria and proteins to grow in the gingival crest, leading to a transition from symbiotic to dysbiotic microbiome. Micro-ulceration caused by inflammation of the sulcular epithelium causes blood (hence iron) to flow into the gingival crevice. In this area, the periodontitis-associated bacteria have an easy life span. Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans are two such viruses. By disrupting the immune system's inflammatory

response, which feeds the bacteria on the nutrients produced by the new tissue, dysbiotic microbiota causes periodontal tissue death. Bacteria have evolved to utilize nutrients that are produced from inflammation, leading to increased dysbiosis and tissue disruption, as well as establishing a self-sustaining cycle.

Minor inflammatory changes in clinical life are thought to be equivalent to the presence of a 'health-promoting' periodontal microbiome. The host-microbe relationship is symbiotic, meaning that the host and his microorganisms live together. If biofilm is not disrupted or eliminated regularly, harmful bacteria can form, leading to 'the onset of dysbiosis.' When local inflammation develops, Porphyromonas gingivitis, for example, seeks out iron in the hem and can stabilize and contribute to dysbiosis, with iron supplied by gingival bleeding. . The patient's reaction is accompanied by gingivitis, but due to the growing biofilm, the associated inflammation does not go away quickly and becomes permanent, allowing dysbiosis to develop. Periodontitis is caused by a combination of genetic, environmental, and behavioral factors in certain people. The latter is caused by an overdose and excessive immunological host inflammation, which causes most of the damage to the underlying tissue and ends in prolonged dysbiosis by failing to treat chronic inflammatory inflammation. A vicious cycle maintains inequality, but symbiosis can be reversed by regular biofilm dispersal in a systematic way to aid in the treatment of inflammation. GCF cells represent gingival crevicular fluid, LPS of lipopolysaccharide, MMP of matrix metalloproteinase, and PMN of neutrophilic polymorphonuclear leukocyte.

Biofilm production causes periodontitis to varying degrees, depending on the individual risk profile. In patients not affected by periodontitis, the inflammatory response to gingivitis is almost equal and able to resolve itself, but in affected patients, multiple genetic factors, epigenetic, or patient-modification of the patient (tobacco, alcohol, diet, type 2 diabetes, depression, etc.) can cause exaggerated, yet ineffective and chronic inflammation, which does not resolve in the connective tissue. This is the spread of diabetes, which is related to metabolic disorders, which contribute to the inflammatory response, leading to an inflammatory condition that promotes tissue deterioration and loss of attachment.

Oral microorganisms such as *Filifactor spp., Lactobacillus spp., Prevotella spp., Dialister spp.* It was found to be involved in the cause of detel carrying infectious diseases using "Sequencing technology." Due to its acidic nature, the oral microbiota above dentel has a slight variation and complexity higher than that of a normal healthy person. An increase in *S.acidophilus* in tooth decay can be seen in the oral microbiota of saliva.*"Streptococcus pneumonia", "Micromonas, Eugenia", "Hurdellsa, Tannella", "Porphyromonas'," Clostridium "," Carbachia "*are found in greater numbers in patients with periodontitis than in healthy individuals, while*" Actinomycetes, "Coryneb , "" Neisseria ", and" Carbonophilic "*are found in low numbers[110] .

2.20.4 Diabetes

The most common form of type II diabetes. It is associated with oral disorders such as dental caries, periodontal disease, and mucosal disease. The interaction of the oral microbiota with systemic and oral health and disease is linked, as evidenced by the association. Oral symptoms associated with diabetes include loss of alveolar bone and teeth. Researchers found *Neisseria, Mycobacterium Agrigatibacter, Actinomyces, Fusobacterium, Selenomonas, and Streptococcus* with a higher proportion of oral microbiota of patients with type 2 diabetes compared with non-diabetic individuals. The role of diabetes in tooth loss and changes in the structure of bacteria and oral microbiota in the oral cavity was explained by scientists[111] .

2.20.5 Obesity

Obesity is a serious problem. Studies have shown that it shows interactions with the gut microbiome and the relationship with oral microbiota and obesity is not well defined *Plasmodium, S. genus and S. mutans* raised in the oral microbiota of an obese person while*Haemophillis, Corneobacterium and Staphyloccous* declined. The oral microbiota of obese people shows little environmental variability

and biological decay which is a major cause of the formation of various immune diseases[111] .

2.20.6 Liver Diseases

Liver cancer occurs due to an imbalance of the oral microbiota found in the intestines and is related to liver disease. The scientist studied studies on the intestinal microbiota of cirrhosis patients and compared it to healthy individuals. As a result, a large number of oral microorganisms, such as *Pasteurella genus, Clostridium, Hemophilus, Lactobacillus, and Weirong, Streptococcus* have been found in intestinal microbiota in patients with cirrhosis[110] .

2.20.7 Colon Cancer

Previous reports indicate that there is an association between the oral microbiome and the intestinal microbiota. The bacterium *F. nucleatum* enters all parts of the body through the bloodstream and destroys weakens the immune system. It causes the growth of a tumor on the body[110].

2.20.8 Pancreatic Cancer

The death rate for pancreatic cancer is higher than for any other cancer. Listed fourth is the deadliest cancer-causing cancer. . It is also recognized that the imbalance of the oral microbiota called *P. gingivalis* is the cause of pancreatic cancer[110] .

2.20.9 Rheumatoid Arthritis

Chronic inflammation is the cause of this disease. It is a form of autoimmune disorder. Both rheumatoid arthritis and periodontitis refer to bone loss and inflammation as a pathological process. Patients with rheumatoid arthritis often

have periodontitis. Many species of anaerobic, such as *Cryptobacterium curtum, Leptotrichia, Prevotella, Lactobacillus salavarius, and Atopobium,* are found in rheumatoid arthritis, despite the low levels of *Streptococcus and Corynebacterium.* Patients with rheumatoid arthritis who do not have periodontitis have a high incidence of *Prevotella*, a bacterium associated with periodontitis[110] .

2.20.10 Human Immunodeficiency Virus (HIV) Infection

HIV infection has been linked to oral problems. There is a growing rate of microbial-related disease in HIV-positive patients. Untreated patients have a higher rate of viremia because they have a higher proportion of pathogenic microorganisms *Campylobacter, Prevotella, Veillonella, and Megasphaera* species than healthy patients[110] .

2.20.11 Adverse Pregnancy Outcomes (APO)

Changes in oral microbiota have been found to have a negative effect on pregnant women. It has been reported that high levels of campylobacter rectus bacteria and *Bacteroides forsythus* are found in mothers who have had a negative pregnancy effect (APO). *F. nucleatum* is also a major cause of adverse pregnancy outcomes (APO). It transmits hematogenously to the placenta and has a detrimental effect on pregnancy outcomes[110].

2.20.12 Oral Cancer

Oral cancer caused by the development of a malignant tumor. Different types of oral cancer e.g. tongue cancer, prostate cancer. Jaw cancer, gingival cancer, pharyngeal cancer, prostate cancer, soft and strong sputum cancer. Lip cancer. Maxilla sinus cancer and facial mucosa. Advanced research links the relationship

between oral microbiota and oral cancer. In patients with oral cancer, microorganisms found on the surface of carcinoma tissue called prednisone. S.mutans and gingival carbon dioxide phagocytic increase in patients with oral cancer. These three bacteria are used as diagnostic indicators for oral squamous cell carcinoma[110] .

2.21 Research Analysis

The role of the microbiome of oral bacteria in betel-nut-related oral carcinogenesis, is unclear. There are about 300 bacteria in the oral cavity of healthy people, most of which are compatible organisms that play an important role in maintaining homeostasis by protecting against pathogenic species and reducing inflammation, including the production of proinflammatory cytokines. Nitrogen oxide and other active nitrogen the medium is produced by reducing nitrate and nitrite to nitrogen oxide and other active nitrogen compounds. Those who chew betel nuts generally do not have poor oral hygiene. Both periodontitis and chronic periodontitis have been associated with the exchange of oral antibodies and an increased risk of oral cancer. There have been a few published studies about the limitations associated with betel quid divers, but further research on oral microbiota is needed[111] .

2.22 Questions

1. Is there any difference in oral bacterial microbiome of betel quid chewer and non-chewer?

2. What type of bacterial microbiome exist in oral cavity of betel quid chewer?

Chapter 3

Materials and Methods

3.1 Methodology Flowchart

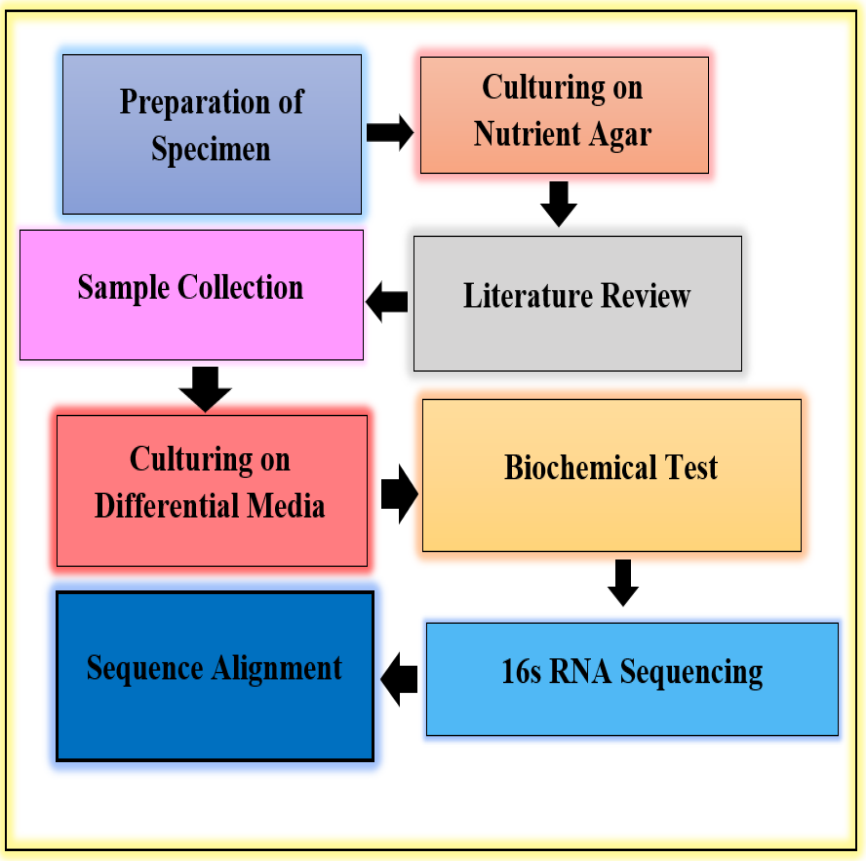


FIGURE 3.1: Methodology of Project

3.2 List of Equipment

1. Autoclave,
2. Magnetic Stirrer,
3. Weighing Balance,
4. Laminar Flow,
5. Incubator,
6. Vortex,
7. Microscope,
8. Shaker,
9. pH Meter,
10. Micro Centrifuge,
11. Centrifuge,
12. Microwave Oven,
13. Refrigerator,
14. Beaker (500ml) ,
15. Spatula,
16. Conical Flasks,
17. Eppendorf Tube,
18. Micropipette,
19. Petri Dishes (10cm),
20. Spirit Lamp ,
21. Plastic bottles,

22. Inoculation Loop,
23. Dropper,
24. Parafilm,
25. Graduated Cylinders (100ml),
26. Glass slides,
27. Falcon tubes,
28. Cotton bud,
29. Aluminum foil.

3.3 List of Chemicals

1. Nutrient Agar(OXOID) MOO3 500g,
2. MacConkeys Agar (OXOID) CM0007 500g ,
3. Mannitol Salt Agar(HIMEDIA) REF M118-500g,
4. Urease Agar (TMAST)500g DM228D,
5. Blood Agar (HIMEDIA) REF MO73 500g,
6. Starch Hydrolysis Test,
7. Simmon Citrate(BIOLAB) REF ECIT20500 500g,
8. EMB (HIMEDIA) REFMO22-500g,
9. Catalase Test,
10. Gram Iodine Solution,
11. Safranin Solution,
12. Crystal Violet Solution and Decolorizing Solution,

13. Phosphate Budder Saline,

14. Distilled Water and Glycerol.

3.4 Sample Collection

Samples are collected by rubbing a cotton swab in the mouth of a chewing and non-chewing gum person, betel quid chewing samples are available at most Pan shop areas, where people usually go to buy betel quid while non-chewing betel quid samples are collected. from various parts of Rawalpindi. In 400ml pure water, 23g NaCl, 62.8g Na2HPO4, and 69.6g KH2PO4 are combined to form PBS (Phosphate Buffer Saline). The pH was then increased to 7.4 with the addition of HCl, and 2ml of PBS was added to each 2ml plastic bottle. Cotton swabs were placed in these bottles containing PBS.

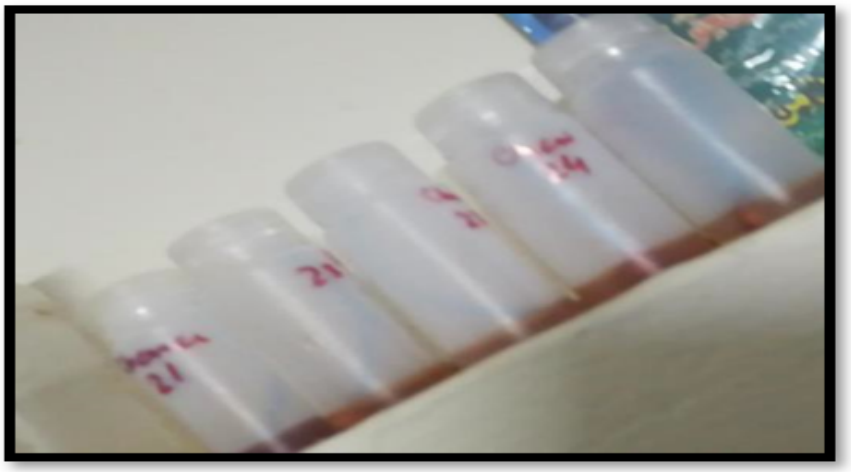


FIGURE 3.2: Sample Collection

3.5 Ethical Clearance

Ethical clearance was received from Ethical committee of" Department of Biosciences and Bioinformatics"

3.6 Sample Exclusion and Inclusion Criteria

Presence of chewing habit for a minimum of 6 months duration and consuming at least 4-5 quid per day.

3.7 Nutrient Agar Preparation

Nutrient agar was used to produce bacteria in collected and chewed samples. A stained-glass cylinder was used to measure 4000ml of distilled water. Nutrient agar is made with a weight of 112.4 g of Nutrient agar and mixed well with 400 ml of distilled water. The prepared medium was autoclaved for 2 hours at 121 ° C. After autoclaved, the media was poured evenly onto the already formed Petri plates within the laminar flow.

3.8 Media Inoculation

Culture medium creates an artificial environment that stimulates natural development conditions for variety of bacteria. Each sample was transferred to a petri plate under laminar flow using a sterile loop, and the plates were labelled as chewers and non-chewers. The samples were then kept in an upside-down position in the incubator for 24 hours at 37°C.

3.9 Growth on Differential Media

3.9.1 Blood Agar

With the help of weighing balance, 96g of Blood Agar was measured and 2400ml of distilled water was added. It was autoclaved at 121℃ for 2 hours. The medium was then poured in a Laminar flow in already autoclaved Petri plates and allowed

to solidify at room temperature. After that, it was properly covered and stored in the refrigerator for later use. Sterile loop was used to pick colonies from nutrient agar and streak on blood agar.

The colour, morphology, and form of the particular bacteria were used to choose the inoculum. After streaking on blood agar, the plates were incubated at 37°C for 24 hours to allow bacteria to proliferate.

3.9.2 Preservation of Purified Strains

A 540ml glycerol stock was produced to maintain the purified strains. 270ml glycerol and 270ml distilled water were used in the preparation. It was autoclave for two hours at 121℃ after thoroughly mixing it in the reagent bottle.

360 blue tips and 360 2.5ml Eppendorf tubes were autoclaved by placing them in a beaker at 121℃ for two hours. A loop full of bacteria was used to make suspension within the Eppendorf tubes. 500ul glycerol tubes were closed carefully. Eppendorf's tubes preserved within the refrigerator at -4℃ .

FIGURE 3.3: Strains are preserved in glycerol at -4℃ .

3.10 Gram Staining

3.10.1 Preparation of Crystal Violet Solution

Crystal violet solution was prepared by dissolving 30g of crystal violet in 100ml of ethanol.

3.10.2 Preparation of Gram Iodine Solution

Gram iodine solution was prepared by dissolving 6.67g potassium iodide in 1g sodium bicarbonate in 100ml of distilled water.

3.10.3 Preparation of Safranin Solution

To make the stock solution, 2.5g of safranin was dissolved in 100ml of 95 percent concentrated ethanol. By diluting a portion of the stock solution with five parts distilled water, the working solution was prepared.

3.10.4 Preparation of Distaining Solution

50 ml of 95% ethanol and 50 ml of acetone are combined to form a decomposition solution. 100ml of the separation solution is made in this way. For grammar, it was stored in falcon tubes.

3.10.5 Gram Staining Procedure

"Hans Christian" Gram received a grammatical procedure in 1844. It is used to distinguish between gram-positive and gram-negative bacteria. To do this, the glass slide was cleaned with 75% ethyl alcohol, and the dilute was prepared in a beaker by mixing a loop full of pure bacterial culture with 2ml of distilled water.

On the slide, a drop of bacterial suspension was placed. The slide was allowed to dry in the air naturally. Next, the slide was heated with an air lamp for 60 seconds to disinfect after it had dried in the air. The heat-resistant bacteria were given a drop of crystal violet and left for 30 seconds before being washed with distilled water. After that, the slide was given 3-4 drops of gram iodine and left for one minute. After one minute, the slide is rinsed with distilled water. The slide is then washed with pure water before being dyed with a decolorizer containing 95 percent ethanol and then run through a sieve to ensure that the color is not washed away. 3-4 drops of safranin are used, and the stain is allowed one minute before it is rinsed off and wiped with a cover slip. Gram-negative bacteria show a pink color and gram-negative bacteria show a purple color.

3.11 16S rRNA Sequencing

The sequence use of ‘16SrRNA, which appears to be highly conserved, was the first method used to investigate microbial ecology. It is the cheapest way to test microorganisms in society[109]. The stosred species were sent to the 16S sequence to test the microbiota associated with the smokers against the non-smokers and the samples were analyzed by Microgen Korea.

3.12 BLAST and Phylogenetic Analysis

The Basic Local Alignment Search Tool [BLAST] is an NCBI tool for aligning sequence with a series of references and measuring similarities based on similarities, differences, and spaces. Based on the BLAST results of type S1–785, which gave a 99.43 percent similarity index and a 50 percent questionnaire, MEGAX was used to establish the evolutionary history of species.

A very similar pressure sequence of S1–785 was selected, which resulted in a total of 10 sequences which was very similar to the S1–785 type. Their sequences were split and stored in their separate file.

3.13 Biochemical Characterization

For biochemical characterization, different biochemical tests were performed by pure bacterial strains obtained.

3.13.1 Tests for Gram-Positive Bacteria

3.13.1.1 Catalase Test

Catalase is a decaying enzyme that breaks down hydrogen peroxide into water and oxygen. Anaerobic carbohydrate metabolism produces hydrogen peroxide as a by-product. When this oxidative product is found in the body of bacteria. It has a negative impact on their ability to live. Catalase testing uses a reagent containing 3%hydrogen peroxide.

The bacterial loop was removed from the clean tissue and placed on a slide. Two drops of 3% hydrogen peroxide are added to the virus to test whether hydrogen peroxide is produced.

Catalase tests were used to detect Gram-positive viruses. For this test, 2 ml of hydrogen peroxide solution was placed in the test tube. The blisters appeared in the samples. The presence of gram-positive bacteria is indicated by blisters. For those who chew and those who do not chew.

3.13.1.2 Mannitol Salt Agar

It can be used as a sorting and sorting media. For the weight balance, 266.4g of Mannitol Salt Agar was measured and 2400ml of pure water was added. Autoclaved for 2 hours at 121 ° C. The contents are poured into the Laminar flow on Petri plates that are already autoclaved and allowed to harden at room temperature.

Pure bacterial species were strands on mannitol salt medium plates. After covering the plates with parafilm, they are placed in an incubator at 37 ° C for 24 hours.

3.13.1.3 Starch Hydrolysis Test

Tests are used to identify microorganisms that are capable of producing hydrolyzing starch. 15g of sugar, 7.5g of peptone, 4.5g of gelatin, 22.5g of Nutrient Agar, 4.5g of beef, and 2.25g of yeast extract in 2400ml of water a-distilled in this test. The pH of the media was then adjusted to 7.3.

The medium was autoclaved for two hours at 121 ° C before being placed on the petrol plates in the laminar flow. Plates containing starch media contained stripes of bacterial strains. The plates are then wrapped in parafilm and stored in an incubator at 37 ° C for 48 hours. Apply 2-3 drops of iodine solution to each Petri plate after incubation.

The clear area around the development line behind the iodine solution shows the beauty of the starch, while the dark blue color of the iodine solution indicates a lack of starch. Plates containing starch media were broken down into a pure tradition using a sterile loop by making one.

3.13.2 Tests for Gram-Negative Bacteria

3.13.2.1 Simmon Citrate Test

Experiments are used to determine the use of citrate in bacterial species. Tests were performed to test the citrate use of bacterial species. Types that use citrate are citrate positive and some are citrate negative. To perform this test, 57.6g of Simmon citrate was extracted and dissolved in 2400ml of distilled water in a conical plant. Simmon test is very important in the identification of the gram negative bacteria. Because they do not changed the color when this test was performed.

The solution is automatically applied for two hours, at 121 ℃. After this, he poured the media on the Petri plate. Bacteria were taken from a germ-laden loop on each plate, and were labeled with citrate media plates.

These plates are then stored in an incubator for 37 ℃ 24 hours after wrapping it with parafilm. Citrate positive bacteria change media color from green to blue, while citrate negative bacteria do not affect local color.

3.13.2.2 Urease Test

The test is based on samples of urea-based bacteria. 55.2g of media and 2400ml of distilled water are added to solid flask. Before being autoclaved for two hours at 121 ℃, conical flasks were neatly integrated and covered to avoid contamination. After that, the contents are poured into 120 autoclaved Petri plates in the laminar flow and allowed to harden at room temperature. Plates containing Urea Agar Base were sterilized using a sterile loop. Plates were stored in the incubator at 37 ℃ after being covered with parafilm for 24 hours. Bacteria that change the yellow color of Urea Agar Base to pink have a positive urease and some that do not change color are urease negative should have a specie.

3.13.2.3 MacConkey Agar

It can be used both as a means of selection and classification. This test determines how lactose is used. 2400ml clear water is placed in 124.8g MacConkey agar. Autoclaved at 121 ° C for 2 hours. The medium was then placed on petrol plates using Laminar Flow. Pure bacterial strains were infested with medium-sized MacConkey agar plates using a sterile loop on each plate. The plates were placed in an incubator at 37 ° C for 42 hours after being covered with parafilm. Bacteria change from a strong pink-red color to a dark red in the middle. Lactose-positive forms are therefore defined as those that are lactose-rich. Lactose-free bacteria are those that do not change color when exposed to lactose.

3.13.2.4 Eosin Methyl Blue (EMU)

EMB is available for commercial use in a mixed form. When commercial powder is added to water, it produces a medium containing 10.0 g / L peptone (Bactopeptone or Gelysate), 5.0 g / L lactose, 2.0 g / L dipostassium phosphate, 13.5 g / L agar, 0.4 g / L eosin, and 0.065 g / L blue methylene. The final pH was less than 0.2. Gram negative bacteria can thrive in the media containing these components, while Gram positive viruses cannot. Blue Methylene and eosin dyes

in EMB suppress Gram positive bacteria, allowing Gram negative bacteria to thrive. Eosin methylene blue medium, on the other hand, helps in distinguishing E. coli from nonpathogenic lactose-fermenting gram negative rod bacteria.

3.14 NCBI Submission

After the removal of low- quality sequences, sequences were submitted in the NCBI.

Chapter 4

Results and Discussions

4.1 Sample Collection

Sample collection was carried out during the month of January. With the help of cotton swab, samples were collected from the buccal mucosa, upper gums, below the tongue and hard plate. Cotton swaps were gently rubbed in above mentioned areas and they transferred in glass bottle of size 2ml containing PBS. Collected samples were transported to the lab in a container and kept in the refrigerator at -2℃. 200 samples were collected, 100 for chewer and 100 for non-chewer. Later only 120 were left due to zero growth of 40 chewer and 40 non-chewers samples. Nutrient agar was used for the initial growth of bacteria. These bacterial colonies were isolated, picked and streaked on blood agar. The purpose of streaking on blood agar was to differentiation of hemolytic bacteria specially streptococcus pneumonia specie. For isolation of bacterial strains multiple culture steps were performed.

4.2 Nutrient Agar Growth

Bacterial growth on cultured media required an artificial environment, which aids in the stimulation of natural conditions for growth. Beef extract, agar, and peptone

help compensate nutrition agar, which provides important nutrients for bacterial growth and genome replication [122]. The goal of nutritional agar is to allow gram positive and gram-negative bacteria to grow together. The culture plates have been shown after 24 hours in both chewers and non-chewers of betel quid.

(a) (b)

FIGURE 4.1: (a) Betel quid Chewer's growth on Nutrient Agar , (b) Betel quid non-chewer's growth on Nutrient Agar.

4.3 Growth on Blood agar

Only 5% of sheep blood is included in blood agar's composition, which aids in the development of bacteria that require particular conditions and nutritional supplies. Blood agar was used as a medium to discriminate and identify hemolytic bacterial strains. Microbes named *Villanelle and Streptococcus* produced large amounts of acid [123]. Only 120 of the 200 samples taken and cultivated show growth, while the remaining 80 samples indicate contamination or no growth. When a person

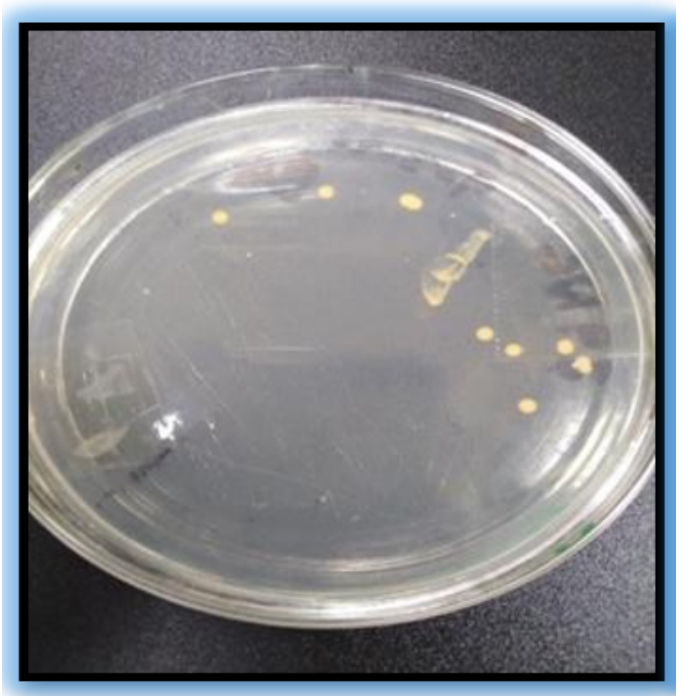
eats betel quid and areca nut orally for a longer length of time, the oral bacterial colonies change and the environment becomes acidic.

FIGURE 4.2: Left side: Bacterial growth on Blood Agar from chewers betel quid chewers and Right side: Bacterial Growth on Blood Agar from non-chewers betel quid chewers.

4.4 Isolation and Purification of Bacterial Strain

After 24-48 hours at 37 ° C, a ritual that indicated colonies or growth was disturbed in the blood agar to be separated and monitored. The most common types of pathogen were selected for the purification and separation of biochemical chemicals

from all pathogen species found in betel quid and non-betel-based divers. The culture was further disturbed until pure colonies were not found.



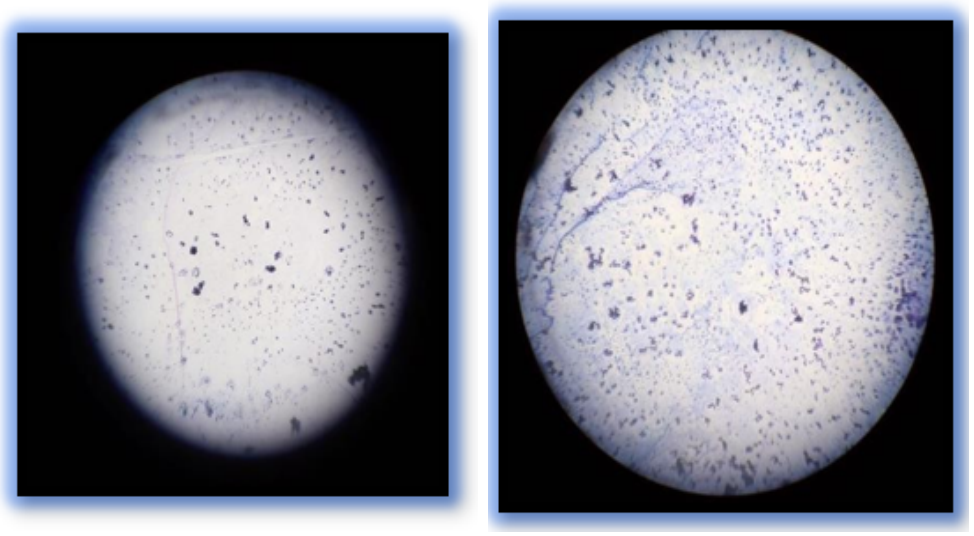
(a)

(b)

FIGURE 4.3: (a) Isolated bacterial growth from betel quid chewers , (b) Isolated bacterial growth from non-chewers betel quid chewers

4.5 Biochemical Characterization

Biochemical characterization tests were done for 60 samples of chewers and 60 samples of non-chewers of betel quid. Results of biochemical tests conducted are given below.



(a) (b)

FIGURE 4.4: (a) Isolated gram positive bacteria from chewers betel quid chewers , (b) Isolated gram positive bacteria from non-chewers betel quid chewers

4.5.1 Isolation of Bacterial Strain

Pure cultures are contaminated using the Gram staining method. In 1884, a Danish physician named "Hans Christian Gram" used the Gram staining method, also known as color separation, to pollute pure cultures. Bacteria are separated by Gram negative and Gram positive bacteria using this method. Gram stain reaction produces two different colors due to changes in the chemical composition of the cell walls of bacteria. Gram positive bacteria have a thicker peptidoglycan layer than Gram negative bacteria, and are surrounded by an outer layer containing lipid. In Gram-negative bacteria, lipid levels are high, leading to large holes that cause crystal violet leakage, leading to bacterial extermination and the need for retention. The peptides are not thick and are very attached to the gramme cell wall

causing dehydration and closing of the pores, allowing the main stain to remain. Gram positive bacteria retain their primary color and do not stain when applied using a Gram method, however Gram negative bacteria lose crystal Crystal violet, iodine solution, alcohol, and safranin are factors used in Gram stain. The results revealed a significant difference in gram contamination between chewers and non-grammar. Of the 60 chewed samples, 30 were gram negative and 41 were gram positive. Of the 60 non-chewing samples, 22 were *Gram positive* and 17 were *Gram negative*. *Peptosterptococcus, Abiotrophia, Stomatococcus*, and *Streptococcus* are among the most common gram *Positive bacteria* in the oral microbiome, according to the study.

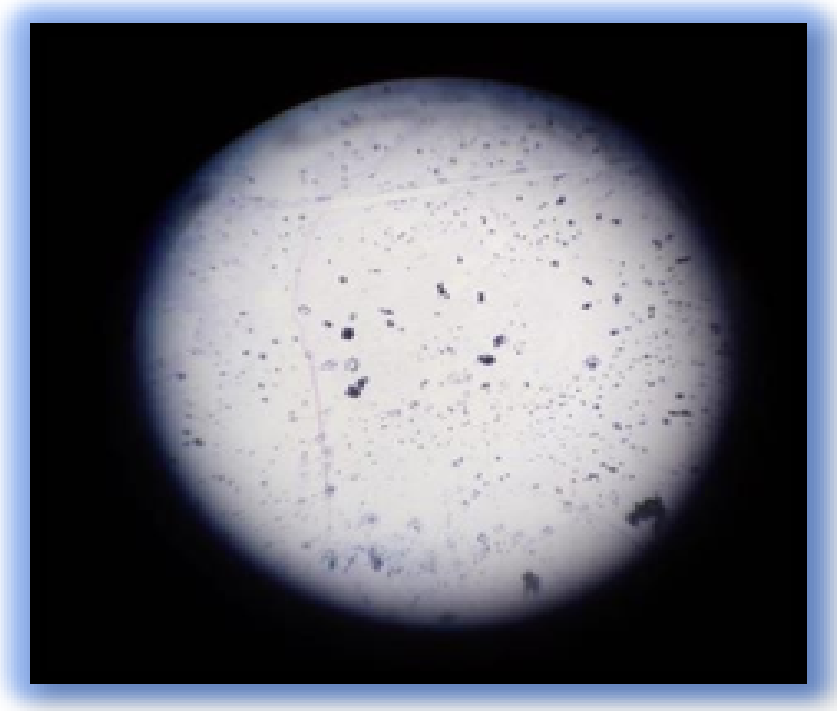


FIGURE 4.5: Staining of pure culture.

Pseudoramibacter, Propionibacterium, Lactobacillus, Eubacterium, Corynebacterium, Bifidobacterium, and Actinomyces , Rothia, Pseudoramibacter, Propionibacterium, Lactobacillus, Eubacterium, Corynebacterium, Bifidobacterium, and Actinomyces. The *Streptococcus* genus was very abundant in a healthy fauna oral microbiome. Extracts of leaf stem from various species have been shown to be effective against human pathogenic microorganisms. All ethanol and ethyl acetate citations have

been shown to be effective, while benzene and hexane quotations have been found to be effective. Leaves and extracts of essential oils have also been shown to be effective against a variety of Gram-negative and Gram-positive bacteria, as well as clinical sites for Pseudomonas aeruginosa and enteropathogenic Escherichia coli.

4.5.2 Catalase Test

Catalase test was used to detect the enzyme catalase in betel quid chewers and non-chewers. The presence of bubbles in the test tube indicates a positive effect, whereas the absence of bubbles indicates a negative effect of catalase.

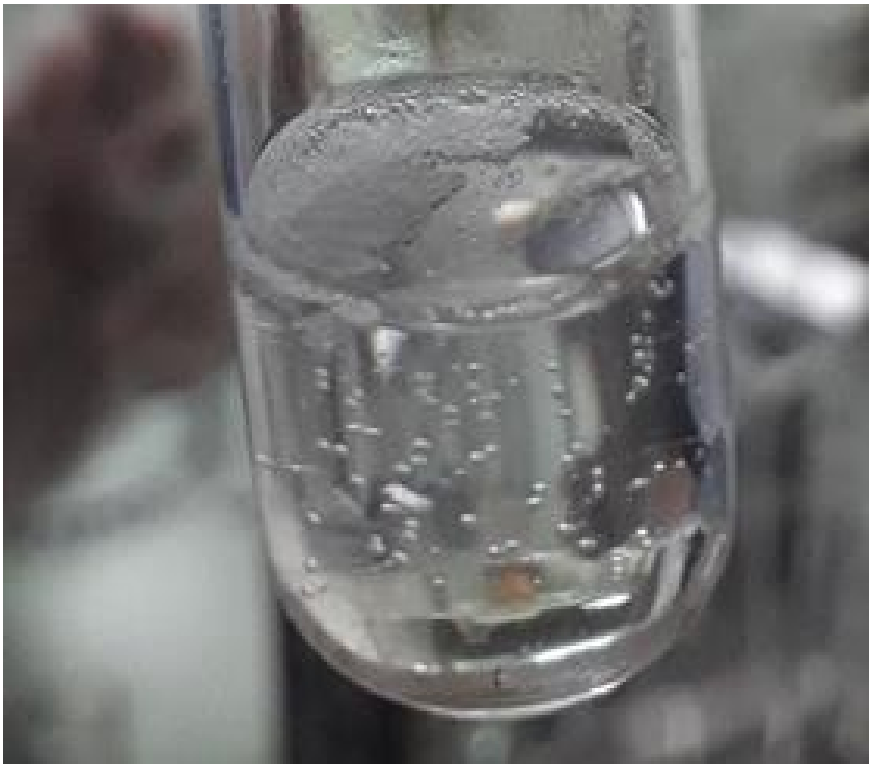


FIGURE 4.6: Staining of pure culture.

Out of 60.40 digesters and 39 non-digesters, there was a negative reaction to catalase testing. Both chewing and non-betel quid cheeses have been tested to have an average of 60 percent. Presence of *Streptococci, Enterococci*, and *Clostridium* spp. The presence of catalase is indicated. *Streptococci* in the oral microbiome are a potential catalyst for dental caries and play a role in binding, metabolizing

carbohydrates by fermentation, and producing acid as a byproduct, while Enterococcus saliva. Microbiota show small differences in oral lesion and epithelial precursors lesion in betel quid chewers. Comparison of the microbiota of patients with cirrhosis with healthy groups reveals the greater number of oral infections, such as Pasteurella genus, *Clostridium,Hemophilus, and Lactobacillus.*

4.5.3 Starch Hydrolysis

These experiments were used to identify the animal's ability to extract starch from hydrolyze and to distinguish animal species based on their amylase activity. It is also used to test the microorganism's ability to extract starch by hydrolyze by producing exoenzymes such as oligo-1,6-glucosidase and a-amylase. Because starch molecules are too large to fit into a bacterial cell, certain bacteria that produce exoenzymes help to divide the starch into smaller units that might be used by microorganisms. When the organisms were hydrolyzed with starch, there was no discoloration in the center, thus iodine solution was added to the plate as a reference after incubation.

FIGURE 4.7: In the presence of Iodine starch produces a dark blue color of the medium, a clear zone near a colony into the blue medium tells amylolytic activity.

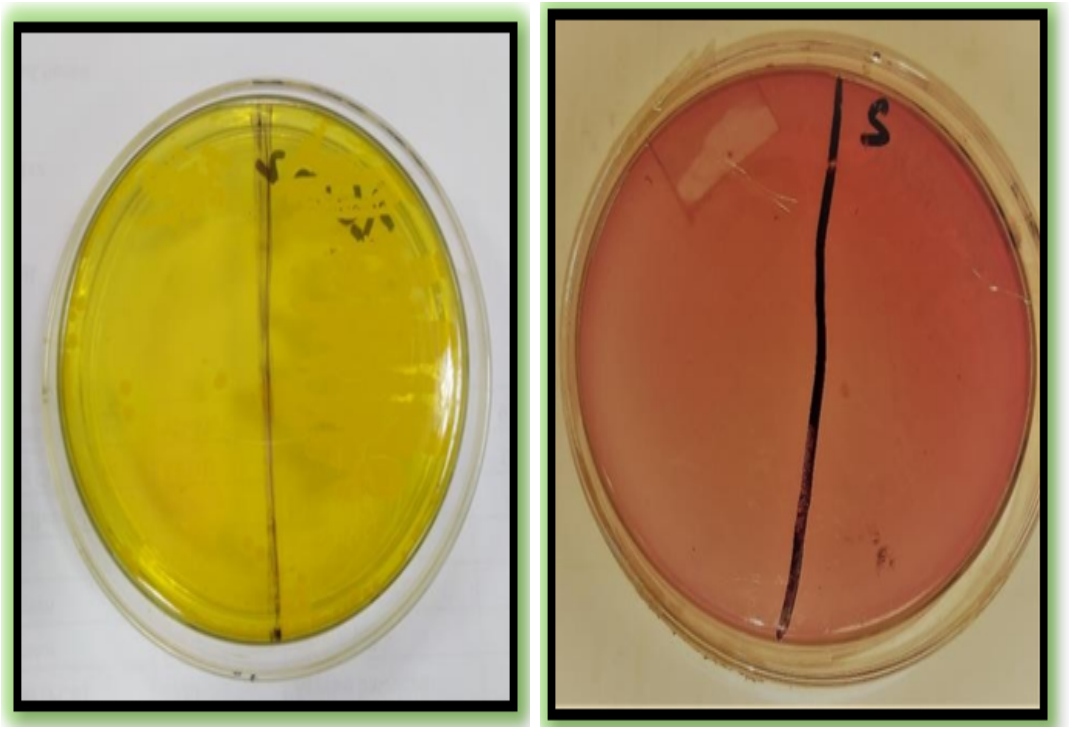
When iodine is added to non-hydrolyzed starch, it turns a dark blue tint. Its

final hydrolyzed products do not get a dark blue color with iodine. In all colonies producing starch by hydrolyze, the most obvious areas [136]. The remaining plate has a dark blue color caused by iodine which forms a mixture of color and starch. The medium may be blue, purple, or dark in color (depending on the amount of iodine). These experiments were performed on both (chewed and non-chewed) test to determine the ability to produce exozy enzymes. The addition of iodine to the samples indicates a clear indication of the presence of bacillus subtilis type of bacteria in both (chewing and non-chewing gum). It has been reported that chewing betel quid alters the oral microbiota by increasing or decreasing commensal microorganisms, such as *Streptococcus, Actinomycetes, Bacillus subtilis,* etc. bacterial type. Bacillus subtilis is closely related to the spread of oral diseases. There were 41 positive results and 19 negative results for non-smokers. While chewing 31 positive and 29 negative for starch hydrolysis.

4.5.4 Mannitol Salt Agar

In microbiology, it is often used as the preferred growth and dividing area. It promotes the development of one type of virus while undermining the growth of others. MSA selects against many Gram-negative bacteria and selects other Gram-positive bacteria (*Staphylococcus, Enterococcus, and Micrococcaceae*) that tolerate high salt content. It contains a high concentration (about 7.5–10 percent) of salt (NaCl), which prevents many germs, making it selective in fighting Gram-negative bacteria and choosing other Gram-positive bacteria (*Staphylococcus, Enterococcus, and Micrococca*).

It also acts as a differentiator of mannitol-fermenting staphylococci, as it contains carbohydrate mannitol and a red phenol index, an indicator of the acid pH to be produced by mannitol-fermenting staphylococci. Some *Stagylococci-negative staphylococci* form small pink or red colonies that do not change color in the center, but *Staphylococcus aureus* develops yellow colonies with yellow spots. When an organism fertilizes mannitol, it produces an acidic substance, which causes the red phenol in agar to turn yellow.



(a) (b)

FIGURE 4.8: (a) *Staphylococcus aureus* on MSA with yellow color , (b) *Staphylococcus epidermidis* on MSA with phenol red color

The change in color from red to yellow indicates the presence of the S-bacterial type. aureus while red-colored samples indicate the type of *S.epidermis* bacterium [138]. Of the betel quid chewers, 37 samples showed color stagnation but there was bacterial growth and 23 samples showed color change from red to yellow. by chewing betel quid. It was found that 41 non-smokers were *S. aures* bacterial strain while 19 are *S.epidermis* indicating that under pathogenic conditions *S.aures* bacteria are commonly found inside the mouth causing oral cancer and diabetes.

4.5.5 Eosin Methyl Blue (EMU)

The sudden decrease in the pH of EMB agar is a key factor in the development of the raw metallic sheen characterized by rapid E.coli for lactose fermentation and high acid production. Bacteria that can tolerate lactose are colorless or light lavender. Gelatin enzymatic digest, lactose sugar (which helps identify lactose

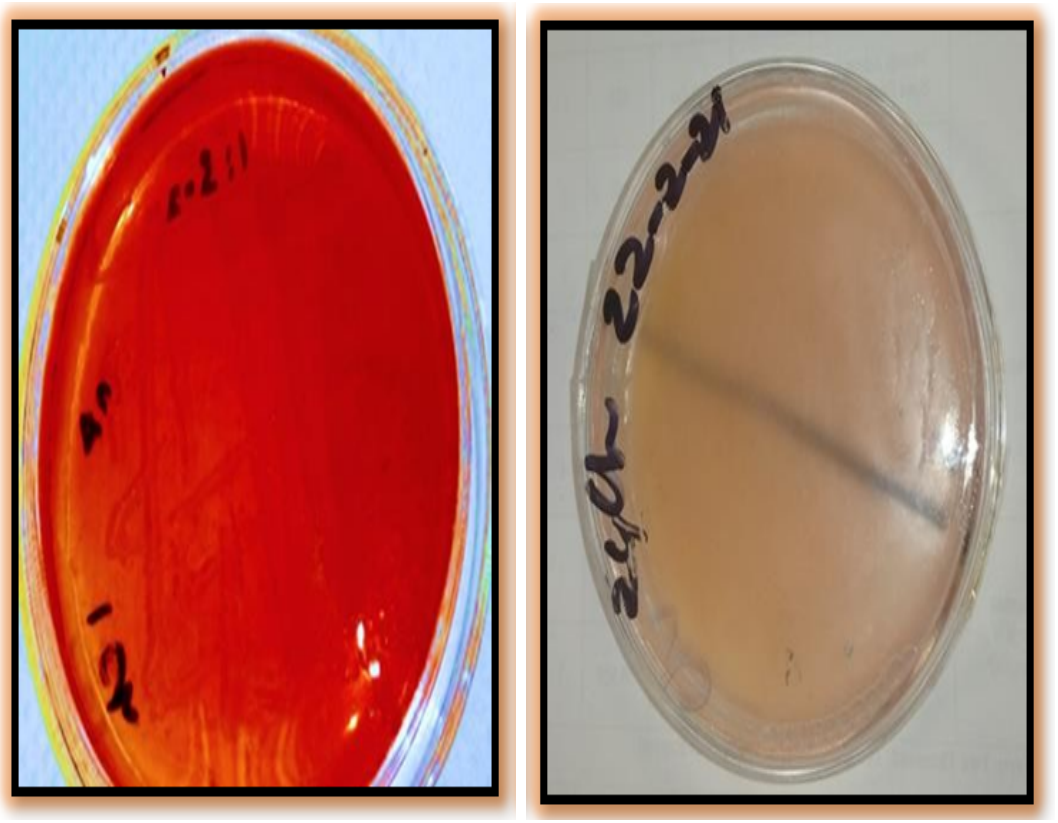
fermenter from non-lactose fermenter), dipotassium phosphate, eosin Y-indicator, agar, and methylene blue are the main components of EMB. These media outlets are mainly used to differentiate lactose fermenter bacteria into non-lactose fermenter bacteria. EMB provides important results. The appearance of a green sheen in the middle indicates the presence of *E.coli*.

EMB inhibits the growth of gram-positive bacteria because the colors contained may be harmful to its growth. Colors of various colors could be found. Microorganisms are killed by blue methylene and other dyes that have a reversible ability to reduce oxidation. Methylene blue, a gram-positive oxidation-reducing agent, is at risk of bacterial growth. Gram-positive bacteria cannot grow in the EMB area. Gram negative viruses are shown by EMB to spread. Microorganisms are killed by blue methylene and other dyes that have a reversible ability to reduce oxidation. Gram positive bacteria show immaturity in the presence of eosin methyl blue. There were 30 chewing samples, and 22 of them showed viral growth in EMB media.

FIGURE 4.9: Sample showing results for gram negative bacteria.

4.5.6 MacConkey Agar

MacConkey agar is used to separate gram-negative bacteria and to separate lactose fermenting gram-negative bacteria into lactose-free, gram-negative bacteria. It is used to differentiate coliform and intestinal bacteria in water, dairy products, and sensible bio-samples. The osmotic equilibrium medium is maintained by sodium chloride. Medium red is an indicator of pH that turns red to a pH below pH of 6.8 and above a pH of 6.8, it is colorless.



(a) (b)

FIGURE 4.10: (a) Lactose fermenting bacteria, (b) Non-lactose fermenting bacteria.

A change in the color of the media was noted, 14 red for chewing gum and 11 for the non-chewing gum. Although pale-colored samples showed the presence of *Staphylococcus* spp they were 46 in chewers and 49 in non-smokers. The red color indicates the presence of E. aerogenes while a pale pink color indicates the presence of *Staphylococcus* spp. It has been reported that the percentage of malignant

bacteria is small in the oral microbiota of betel quid chewers. *E. aerogenes* is a type of bacterium found in vegetables.

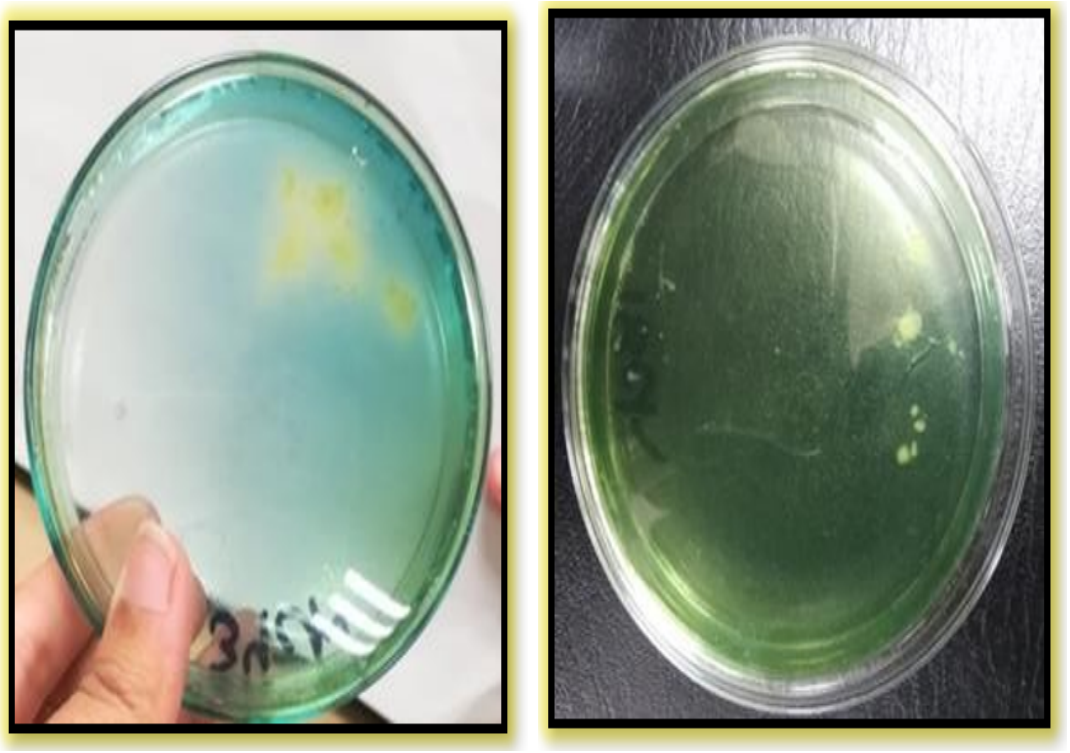
4.5.7 Urease Tests

The potential for hydrolyze urea with the enzyme urease was used to differentiate the animal species in this experiment. It can also be used to identify a variety of *Enterobacteriace* species, such as *Proteus, Klebsiella,*and a few species of *Yersinia and Citrobacter*, as well as certain species of *Corynebacterium.* This test is used to determine the presence of *H. pylori* in abdominal biopsy samples. The pH change is reflected in the change of red phenol color from light orange to pH 6.8 to magenta (pink) at pH 8.1, as ammonia production produces alkaline medium. Within 24 hours, the urease-positive bacteria turn all over pink. The presence of H.pylori in the mouth is caused by the introduction of betel quid and tobacco, and is the most common cause of oral infection (cancer). Urease positive samples were obtained from 13 of 60 chewing samples and 15 non-chewing samples.

FIGURE 4.11: Left side: Positive Urease Test and Right Side: Negative Urease Test.

4.5.8 Simmon Citrate Agar Test

Based on the use of citrate, this test is used to distinguish between gram-negative and gram-positive bacteria. The main purpose of Simmon citrate agar is used for identification. Enterobacteriaceae. Sodium chloride, sodium citrate, ammonium dehydrogen phosphate, dipotassium phosphate, magnesium sulphate, bromomethyl blue, and agar are some of the ingredients in simmon citrate. The use of citrate induces an alkaline reaction, changing the color of the medium from green to blue, indicating the presence of Enterobacter, Citrobacter, Klebsiella, and other bacteria. Enterobacter is a bacterium that causes periodontitis and plaque. In the case of a negative effect, there is no change in the color of the media.



(a) (b)

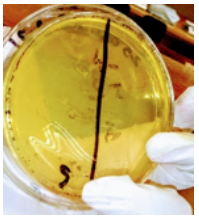
FIGURE 4.12: (a) Positive Citrate Test , (b) Negative Citrate Test

Only 18 out of 60 chewers had a positive response on the simmon citrate test, whereas 42 out of 60 have a negative result. For non-chewers, 31 of the samples are negative, whereas 29 are positive. The high proportion of citrate consumption in non-chewers is due to their high carbohydrate intake.

4.6 Preservation of Prevalent Strains

The bacterial plate that seems to be more prevalent were further purified by streaking and culturing them repeatedly hence, the purified strains are obtained that was shown below 4.1.

TABLE 4.1: Preserved strains from Gut Microbiota of *Culex quinquefasciatus*

S#	Reference Code	Media	Colony Color	Pigmentation	Figures
1	EMB Plate 1	EMB	Purple	Purple	
2	EMB Plate 1	EMB	Purple	Purple	
3	MSA Plate 1	MACC	Pink	Pink	
4	MSAPlate 2	MACC	Yellow	Yellow	
5	MACC Plate 1	MACC	Red	Red	
6	MACC Plate 2	MACC	Violet	Violet	

4.7 Biochemical Analysis

Biochemical analysis of the samples was performed.

4.7.1 Blood Agar

Blood samples are only 120 out of 200 agar, while the remaining 80 samples show contamination or immaturity.

TABLE 4.2: Results of Biochemical Characterizations of bacterial pure strains obtained from non- chewer and chewers of betel quid .

For Non-Chewer		**For Chewer**	
NC1	+ ve	CH01	+ ve
NC2	+ ve	CH02	+ ve
NC3	+ ve	CH03	+ ve
NC4	+ ve	CH04	+ ve
NC5	+ ve	CH05	+ ve
NC6	- ve	CH06	+ ve
NC7	+ ve	CH07	+ ve
NC8	+ ve	CH08	+ ve
NC9	+ ve	CH09	+ ve
NC10	- ve	CH10	- ve
NC11	+ ve	CH11	- ve
NC12	+ ve	CH12	- ve
NC13	+ ve	CH13	- ve
NC14	- ve	CH14	- ve
NC15	- ve	CH15	- ve
NC16	+ ve	CH16	- ve
NC17	- ve	CH17	- ve
NC18	- ve	CH18	- ve
NC19	- ve	CH19	+ ve
NC20	- ve	CH20	- ve

4.7.2 Mannitol Salt Agar Test

Mannitol salt agar was also know as the MSA. Mannitol salt agar was experiments show a high proportion of phenol red color in chewing and less in chewing while yellow color is found more in chewing and less chewing.

TABLE 4.3: The biochemical test of the Mannitol salt agar (MSA) in the chewer and non-chewer.

Non- chewers	MSA	Chewers	MSA
NC1	Phenol Red	CH01	Yellow
NC2	Phenol Red	CH02	Yellow
NC3	Yellow	CH03	Phenol Red
NC4	Yellow	CH04	Yellow
NC5	Yellow	CH05	Yellow
NC6	Phenol Red	CH06	Yellow
NC7	Yellow	CH07	Yellow
NC8	Yellow	CH08	Yellow
NC9	Phenol Red	CH09	Phenol Red
NC10	Yellow	CH10	Phenol Red
NC11	Yellow	CH11	Phenol Red
NC12	Phenol Red	CH12	Phenol Red
NC13	Phenol Red	CH13	Phenol Red
NC14	Yellow	CH14	Phenol Red
NC15	Yellow	CH15	Phenol Red
NC16	Yellow	CH16	Yellow
NC17	Yellow	CH17	Yellow
NC18	Yellow	CH18	Yellow
NC19	Yellow	CH19	Yellow
NC20	Yellow	CH20	Yellow

4.7.3 Starch Hydrolysis Test

In starch hydrolysis tests, a large number of gram-positive bacteria were found in the non-chewer and less in the chewers while a large number of gram-negative bacteria were found in the chewing gum and small in the non-chewing gum.

TABLE 4.4: The biochemical test of the Starch hydrolysis in the chewer and non-chewer.

Non-chewers	**Starch Hydrolysis Test**	**Chewers**	**Starch Hydrolysis Test**
NC1	- ve	CH01	- ve
NC2	-ve	CH02	+ve
NC3	+ve	CH03	- ve
NC4	+ ve	CH04	+ve
NC5	+ve	CH05	- ve
NC6	+ ve	CH06	+ve
NC7	+ ve	CH07	- ve
NC8	+ ve	CH08	+ve
NC9	+ ve	CH09	- ve
NC10	- ve	CH10	- ve
NC11	- ve	CH11	+ ve
NC12	+ ve	CH12	+ve
NC13	+ ve	CH13	-ve
NC14	+ ve	CH14	-ve
NC15	+ ve	CH15	+ve
NC16	+ ve	CH16	+ve
NC17	+ ve	CH17	+ve
NC18	- ve	CH18	+ve
NC19	- ve	CH19	+ve
NC20	- ve	CH20	-ve

4.7.4 Maccokney Agar Test

In the MacCokney agar test it showed a much reddish color for betel quid chewers than non-chewers while peach color was found more in non-chewers than chewers. A large number of gram-positive bacteria are found in the non-chewers and less ratio in betel quid chewers. while gram-positive bacteria are most commonly found in betel quid chewer and less in non-chewer.

TABLE 4.5: The biochemical test of the MacCokney in the chewer and non-chewer.

Non- chewers	**MacCokney Agar**	**Chewers**	**MacCokney Agar**
NC1	Red	CH01	Peach
NC2	Peach	CH02	Peach
NC3	Peach	CH03	Peach
NC4	Red	CH04	Peach

Table 4.5 continued from previous page

Non- chewers	MacCokney Agar Test	Chewers	MacCokney Agar Test
NC5	Peach	CH05	Peach
NC6	Peach	CH06	Peach
NC7	Red	CH07	Peach
NC8	Red	CH08	Peach
NC9	Red	CH09	Peach
NC10	Red	CH10	Peach
NC11	Peach	CH11	Peach
NC12	Red	CH12	Peach
NC13	Peach	CH13	Red
NC14	Peach	CH14	Peach
NC15	Peach	CH15	Red
NC16	Peach	CH16	Peach
NC17	Peach	CH17	Peach
NC18	Peach	CH18	Red
NC19	Peach	CH19	Red
NC20	Peach	CH20	Red

4.7.5 Simon Citrate Agar

In Simon Citrate Agar, a large numbers of gram-positive bacteria were extracted from non-chewer and a large number of gram-negative bacteria were deducted from betel quid chewers.

Table 4.6

Non- chewers	Simon Citrate Agar	Chewers	Simon Citrate Agar
NC1	+ ve	CH01	-ve
NC2	+ ve	CH02	- ve
NC3	+ ve	CH03	- ve
NC4	+ ve	CH04	- ve

Table 4.6 continued from previous page

Non- chewers	Simon Citrate Agar	Chewers	Simon Citrate Agar
NC5	- ve	CH05	- ve
NC6	- ve	CH06	- ve
NC7	- ve	CH07	- ve
NC8	- ve	CH08	- ve
NC9	- ve	CH09	- ve
NC10	- ve	CH10	- ve
NC11	- ve	CH11	+ ve
NC12	+ ve	CH12	+ ve
NC13	+ ve	CH13	- ve
NC14	+ ve	CH14	- ve
NC15	+ ve	CH15	- ve
NC16	+ ve	CH16	- ve
NC17	- ve	CH17	- ve
NC18	+ ve	CH18	+ ve
NC19	- ve	CH19	- ve
NC20	-	CH20	-

4.7.6 Urease Test

In urease tests, a greater number of gram-positive bacteria are found in the non-chewer and less number in case for betel quid chewers while gram-negative bacteria are found mainly in the non-chewer and less number in betel quid chewer.

TABLE 4.7: The biochemical test of the Urease Agar in the chewer and non-chewer.

Non- chewers	Urease Agar	For chewers	Urease Agar
NC1	- ve	CH01	- ve
NC2	- ve	CH02	- ve
NC3	- ve	CH03	- ve

Table 4.7 continued from previous page

Non- chewers	Urease Agar	For chewers	Urease Agar
NC4	- ve	CH04	- ve
NC5	+ ve	CH05	- ve
NC6	+ ve	CH06	-
NC7	- ve	CH07	- ve
NC8	- ve	CH08	- ve
NC9	- ve	CH09	- ve
NC10	- ve	CH10	- ve
NC11	- ve	CH11	- ve
NC12	- ve	CH12	- ve
NC13	+ ve	CH13	- ve
NC14	+ ve	CH14	- ve
NC15	- ve	CH15	- ve
NC16	- ve	CH16	- v
NC17	- ve	CH17	- ve
NC18	+ ve	CH18	- ve
NC19	- ve	CH19	- ve
NC20	- ve	CH20	-

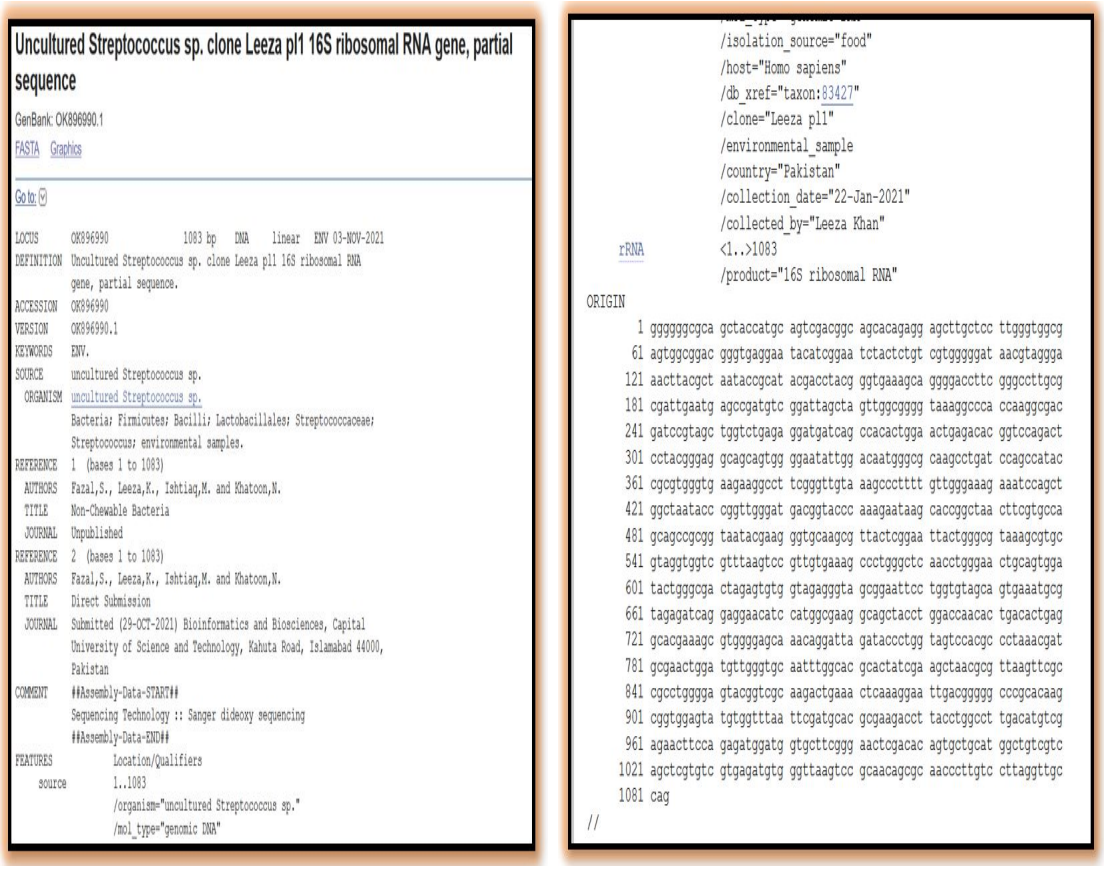
4.8 16s RNA Sequencing

In the 16sRNA sequence, a single sequence of chewing and other non-chewing fluids was submitted to the NCBI. The non-chewer entry ID number is OK896990.The following results show a similarity of 99%baseline with bacterial specie "*Strep- tococci* " in non-chewer while betel quid chewers indicates the similarity of the base pair bacterial strain *Stenotrophomonas spp. "Stenotrophomonas "*is a gram- negative, aerobic, non-fermentative *Bacillus* closely related to *Pseudomonas* species. Gram-positive, non-motile, non-spore-forming, catalase-negative from in pairs or chains known as "*Streptococci* ". The throat, nasopharynx, and mouth are the

most common places to be found. Gram-positive coccus also a facultative anaerobe and catalase negative. For future recommendations, metagenomics of chewer and non-chewer metage- nomics were performed using a large number of samples to identify differences in the chewer and non-chewer oral microbiome to determine the association of healthy and pathogenic microbiome with post health. The chemical analysis of betel quid leaves performed and their biological impact on the microbiome in the laboratory should be evaluated.

4.9 NCBI Submission

After the removal of low quality sequences, the strain sequence was submitted in NCBI and the accession number given from the GenBank is OK896990.



Uncultured Streptococcus sp. clone Leeza pl1 16S ribosomal RNA gene, partial sequence

GenBank: OK896990.1

FASTA Graphics

Go to:

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  ORGANISM  uncultured Streptococcus sp.
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            Streptococcus; environmental samples.
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  AUTHORS   Fazal,S., Leeza,K., Ishtiaq,M. and Khatoon,N.
  TITLE     Non-Chewable Bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1083)
  AUTHORS   Fazal,S., Leeza,K., Ishtiaq,M. and Khatoon,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-OCT-2021) Bioinformatics and Biosciences, Capital
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      241 gatccgtagc tggtctgaga ggatgatcag ccacactgga actgagacac ggtccagact
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      421 ggctaatacc cggttgggat gacggtaccc aaagaataag caccggctaa cttcgtgcca
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     1081 cag
//
```

(a) (b)

FIGURE 4.13: (a) Submission on NCBI , (b) Sequence on NCBI

4.10 Multiple Sequence Alignment of Sequence

When the sequences of the bacteria isolated from the betel quid chewers and betel quid non-chewers, were aligned through multiple sequence alignment, it was noticed that there was a difference of 36 nucleotides among their sequences and some gaps were also noticed among these sequences.

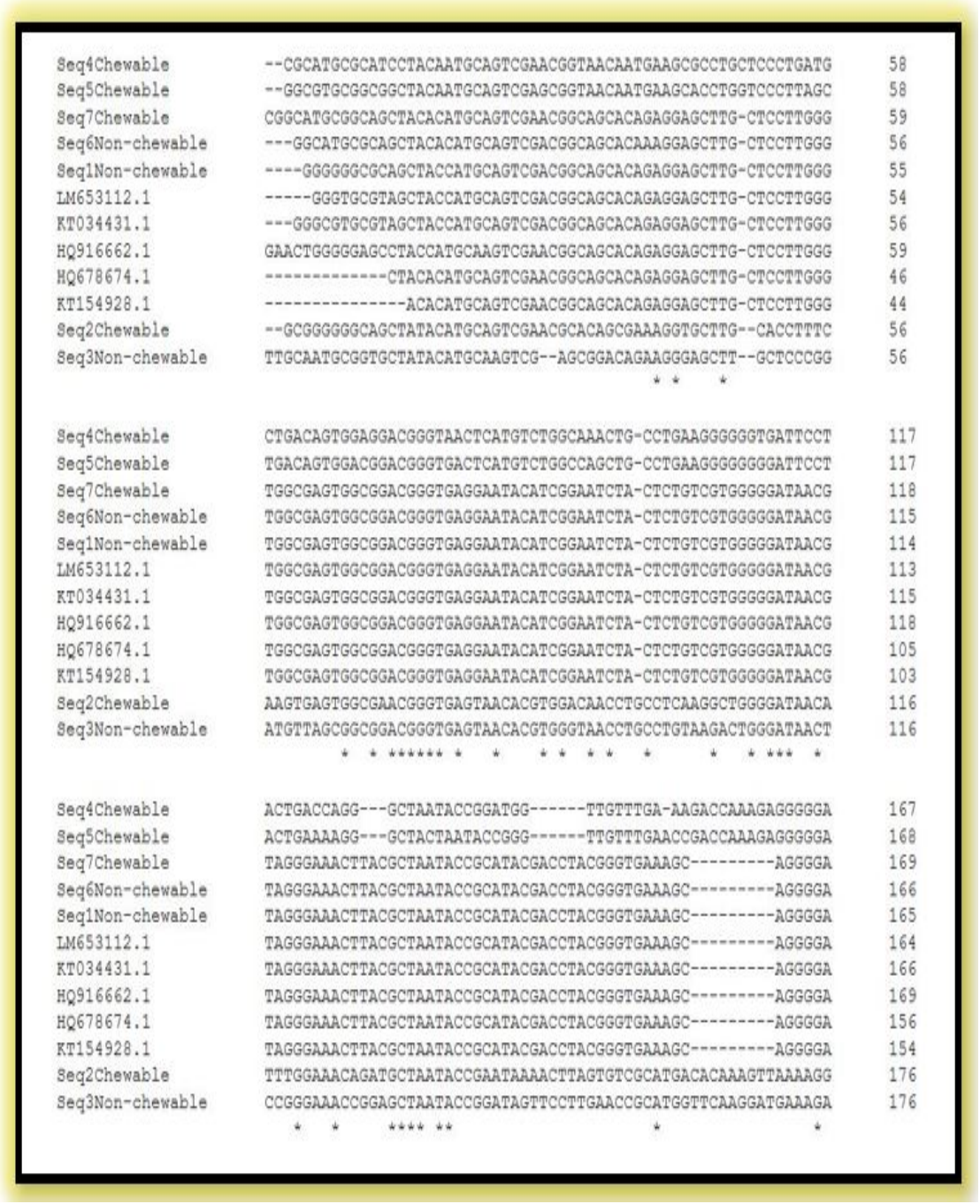

FIGURE 4.14: Multiple Sequence Alignment of the Bacterial Sequences Isolated from Beta Quid Chewers and Beta Quid Non-Chewers.

4.11 Discussion

Both beneficial and harmful bacteria live in the human mouth. Betel quid-associated oral disorders, such as oral cancer and periodontitis, may worsen the imbalance in the formation of bacterial flora[111] .The oral microbiota of betel quid chewers is variable, which has an effect on betel quid-related oral diseases[112]. Some of the most common types of bacteria found in the Betel quid chewing gum group may be linked to oral cancer, depending on our results. Bacterial problems in the oral cavity have been linked to the development of cancer. Oral squamous cell cancer (OSCC) can be detected using high amounts of saliva in *Capnocytophaga gingivalis, Prevotella melaninogenica, and Streptococcus mitis*[113]. By interacting directly with oral epithelial cells with Toll-like receptors, *Porphyromonas gingivalis and Fusobacterium nucleatum* promoted the growth of oral cancer. Bacteroides fragiles and *F. nucleatum* is associated with colorectal cancer in addition to oral cancer[114], while both *P. gingivalis and F. nucleatum*is linked to pancreatic cancer and cancer[115]. In cancerous lesions, several types of Treponema have been found in large numbers. These cancer-causing oral cancers may contribute to the development of oral cancer and ulcers caused by betel quid chewing. Although the exact cause of this increase in oral carcinogenesis is unknown, other theories have been proposed. Bacterial particles can cause cancer exchange by reactivating inflammatory mediators produced by the oral mucosa. Bacterial fimbria cause cancer by increasing intercellular interactions, and bacterial products such as synthetic sulfur compounds, organic acids, active oxygen species, and active nitrogen species stimulate carcinogenesis[116]. Confirmation of this speculation will require further investigation. According to Chao1 and Observed Species, there is no significant difference in the number of viral species between the two groups.

The level of homogeneity and the number of bacterial species may be significantly different between Betel quid groups and non-chewing groups, or they may be similar, according to the data. These data confirmed the variability of each type of level in our study. Carcinogenesis is caused by active nitrogen species. Confirmation of these findings will require further investigation[117]. Previous findings of a Guam

island survey revealed significant differences in the number of bacterial species and homogeneity. The explanation for the differences in data on the number of species of bacteria found in the Sri Lankan and Guam samples is unknown. Additionally, betel quid chewing habits, such as the components and timing of chewing, may vary from country to country. As a result, the observed differences may be related to the common differences between Sri Lankan quid chefs and Guam Betel. More research is needed to truly understand this situation[119]. In healthy gingival sulcus, gram-positive *Cocci*, especially *Streptococcus* sp. and *Actinomycetes* sp., and yeasts, protozoa, etc., exist in equal proportions. A few bacteria have been linked to oral diseases such as periodontitis and dental caries, which are related to a variety of complex and complex sub-gingival microbiota including Gram-negative and Gram-positive bacteria, facultative and anaerobic organisms, and species of about 500 bacteria. separated by a subgingival crack. , a microbial niche that has found great interest. Eating habits, environmental factors, and lifestyle are all associated with the onset of other diseases. According to reports, worldwide some 600 million people chew betel nut. In South and Southeast Asia, betel quid, sometimes known as areca nut, has long been popular. According to the Guptas and Warnakulasuria, small numbers of people in the world chew arecanut, and the practice is widespread in the Indian subcontinent, including the provinces of Asia and Melanesia[120].

Types found inside the oral cavity include viruses, fungi, protozoa, and viruses. Oral commensal bacteria, commonly referred to as the recipe flora, are among the organisms. The most common type of microorganism found in the mouth is bacteria, which includes both anaerobic and aerobic strains. The anaerobic flora is characterized by *Lactobacilli, Leptotrichiabuccalis, and Veillonella.* Aerobic flora includes *Streptococcus viridans, Cogulase Negative Stapylococci (CONS), Diphtheroids, and Neisseria catarrhalis.* Mutations (oral hygiene, gingival syndrome, periodontal disease, oral mucosa condition, and bacterial manifestations) have been shown to be important relationships, and Choudhur nevertheless claimed that chewing betel quid leads to poor health of periodontal. A scientist named

"Ling et al" [121] [122]found that 42.6% bled when examining betel quid chewers and high packet death. betel quid chewers have a reddish-brown color in the mouth, as well as a tendency to miscarry and urinate. The most common site of contact with betel quid, the buccal mucosa, is where the wound develops[123][124]. Although the disease is often associated with other mucosal lesions such as leukoplakia and sub-mucous fibrosis, both of which are known to progress to cancer, the lesion is not considered a cancer. Oral submucous fibrosis is an incurable condition that can develop and appear from time to time. Patients with severe conditions have difficulty eating, swallowing, and speaking, and many have lingual papillae atrophy[125].

S. maltophilia is a "newly developing pathogen of concern" that is frequently being isolated [126][127][128]. The World Health Organization now recognizes it as one of the most significant multi-drug resistant pathogens in hospitals (WHO)[129][130]. According to British microbiologists, it is the ninth most important pathogen and one of the most difficult pathogens to study in the infectious disease community[131]. It is commonly recognized as an opportunistic bacterium associated with high morbidity and fatality rates in immunocompromised people. *Stenotrophomonas maltophilia* is a non-fermenting gram-negative rod that is related to *Achromobacter xylosoxidans and Burkholderia cepacian* and is the third most prevalent after Pseudomonas aeruginosa and Acinetobacter. [125][132][133]. It is the only species of the seventeen Stenotrophomonas species that infects humans[134].

Chronic respiratory disorders, including cystic fibrosis, hematologic malignancy, chemotherapy-induced neutropenia, organ transplant patients, human immunodeficiency virus (HIV) infection, hemodialysis patients, and newborns are all risk factors for this infection[135][136]. moreover, hospital settings, prolonged intensive care unit stays, mechanical ventilation, tracheostomies, central venous catheters, severe traumatic injuries, significant burns, mucositis or mucosal barrier damaging factors, and the use of broad-spectrum antibiotic courses have all been shown to increase the risk of infection[137][138].

Furthermore, it can be found in a wide range of healthcare settings, including hospital tap water faucets, sinks, shower outlets, air-cooling systems, ice-making

and soda fountain machines, disinfectant solutions, intravenous fluids, catheters, ethylenediaminetetraacetic acid (EDTA) containing blood collection tubes, blood gas analyzers, dialysis machines, intra-aortic balloon pumps, nebulizers, oxygen humidifiers, breathing circuits, scopes [139][140][141][142][143][144][145].

Most *Stenotrophomonas maltophilia* infections are nosocomial, and several outbreaks have been reported in hospitals and intensive care units in past years. [26] [27] [26] Although patient-to-patient transmission has been observed, studies have shown genetic diversity across nosocomial infection isolates, implying many separate environmental routes of transmission [145][146][147].

Stenotrophomonas maltophilia is thought to be the most prevalent carbapenem-resistant gram-negative bacterial cause of bloodstream infections in US hospitals, accounting for around 1% of all nosocomial bacteremia cases[148][149][150].

Since the 1970s, the frequency of infection cases has been estimated to range between 5.7 and 37.7 cases per 10,000 hospital discharges, which has been increasingly higher than previously recorded[151] [152][153][154][155]. This increased infection rate is thought to be caused mostly by a rise in the number of immunocompromised patients as well as the frequent use of broad-spectrum antibiotics.

Chapter 5

Conclusions and Recommendations

More than 300 germs live in the mouths of healthy people, most of which are commensals that play a key role in maintaining homeostasis by protecting against pathogenic strains, down-regulated inflammation, including the production of proinflammatory cytokine, and converting nitrate and nitrite into nitrogen oxide. And other intermediate nitrogen compounds. Betel-nut chewers often experience poor oral hygiene and chronic periodontitis, both of which have been linked to changes in oral mucosa and an increased risk of developing oral cancer. Polyphenols, which include tannins and alkaloids, are the main chemical components of betel nut.

The first objective of this study was to differentiate the oral bacterial microbiome from betel quid chewer vs. non-chewer.

For this purpose 200 samples were collected (60 for chewers and 60 for non-chewers), bred with nutritious agar. Of the 200 enlarged samples, a total of 120 samples of total and non-chewing individuals showed growth within 24 hours.

Based on the second objective, the samples were bacterial organisms. In the blood agar were collected 200 samples, only 120 samples showed growth while 80 samples showed contamination or immaturity.

Mannitol salt agar experiments show a high proportion of phenol red color in chewing and less in chewing while yellow color is found more in chewing and less

chewing. In starch hydrolysis tests high number of gram-positive bacteria were found in the non-chewers and less number in the betel quid chewers. While a large number of gram-negative bacteria were found in betel quid chewer and less in the non-chewers. In catalase experiments, large numbers of gram-positive bacteria were extracted from non-chewer and a large number of gram-negative bacteria were extracted from betel quid chewers.

In MacCokney agar experiments, red color was more commonly found in betel quid chewers than in non-chewer while peach color was more commonly found in non-chewers than chewers. A large number of gram-positive bacteria are found in the non-chewer and small in the chewing while gram-positive bacteria are most commonly found in betel quid chewer and less in the non-chewer. In urease tests, a large number of gram-positive bacteria are found in the non-chewer and small in the chewers while gram-negative bacteria are found mainly in the non-chewer and small in the chewing gum. The third objective was to identify individual bacterial strains in 16sRNA sequences.

In the 16sRNA sequence, a single sequence of chewing and other non-chewing fluids was submitted to the NCBI. The non-chewer entry ID number is OK896990. The following results show a similarity of 99% baseline with bacterial specie '*streptococci*" in non-chewer while betel quid chewers indicates the similarity of the base pair bacterial strain *Stenotrophomonas* spp. "*Stenotrophomonas* "is a gram-negative, aerobic, non-fermentative *Bacillus* closely related to *Pseudomonas* species. Gram-positive, non-motile, non-spore-forming, catalase-negative from in pairs or chains known as "*Streptococci*". The throat, nasopharynx, and mouth are the most common places to be found. Gram-positive coccus also a facultative anaerobe and catalase negative.

For future recommendations, metagenomics of chewer and non-chewer metagenomics were performed using a large number of samples to identify differences in the chewer and non-chewer oral microbiome to determine the association of healthy and pathogenic microbiome with post health. The chemical analysis of betel quid leaves performed and their biological impact on the microbiome in the laboratory should be evaluated.

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