Isolation and Detection of biofilm forming bacteria from Oral Cavity

Dissertation

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DEDICATION

We dedicate our dissertation work to our families and many friends.

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In performing our assignment, we had to take the help and guideline of some respected persons, who deserve our greatest gratitude. The completion of this assignment gives us much Pleasure. We would like to show our gratitude Ms. Dalal Mohammed, and Hawraa M.AL-rafyaia , Course Instructors, University Babylon for giving us a good guidelines for assignment throughout numerous consultations. We would also like to expand our deepest gratitude to all those who have directly and indirectly guided us in writing this assignment.

Abstract:

The mouth is a unique micro-environment that is characterized by water, temperature fluctuations, hard surface, and carbon and nitrogen input. Changes in the local environment effect the biofilm bacterial composition. Dental plaque is actually composed of mixed biofilms. However, the composition of the healthy plaque biofilm is significantly different from disease-associated plaque biofilms. This study included the isolation of bacterial strains from oral cavity for the period from November to December of the year 2022 AD. Where 50 samples were collected from healthy people and people with dental carries and were cultured on nutrient broth, then cultivated on different media to obtain pure colonies. All bacterial strains were grown on Congo Red Agar to estimate the ability of generating biofilm. The results showed the presence of *Lactobacillus* and *Streptococcus mutans* in large number, which are contributed in the biofilms formation. Depending on phenotypic features of bacterial strains, most of isolates producing biofilms appeared as black colonies on the surface of Congo Red Agar, while the other strains are non-producing biofilm emerged as white colonies.

Chapter One: Introduction

1-Introduction

Biofilm can be defined as an aggregation of one or more groups of different microorganisms, embedded in a self-produced matrix and adhering to a firm surface. Biofilms are ubiquitous. Bacteria can form them on the greatest variety of surfaces, living or non-living, in humid natural conditions, on medical equipment and living tissue, but also in the most extreme living conditions. Recent studies have described bacterial biofilms found in the extreme subzero temperatures of the Antarctic seawaters, thermal waters ranging from 35 to 50 degrees Celsius and conditions of extreme acidity, high metal content and lack of nutrients (Plančak, Musić, and Puhar 2015).

Both Gram-negative and Gram-positive bacteria can form biofilms on indwelling medical devices such as catheters, mechanical heart valves and prosthetic joints. The most common biofilm-forming bacteria associated with human disease are *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (Markowska, Grudniak, and Wolska 2013).

Biofilms can be formed from a single-species bacterial community or, which is more typical, represent a community derived from several different microbial species living an interdependent lifestyle. A fact worth mentioning is that even in the mono-species biofilms, phenotypic heterogeneity exists as a response of the individual bacterial cells to local microenvironment conditions. Dental plaque biofilm and biofilm in periodontal diseases are among the best described multi-species biofilms(Plančak, Musić, and Puhar 2015).

1-1 Biofilm structure

The structure of the extracellular polymeric substance (EPS) matrix of biofilm is composed of one or more of extracellular polysaccharides, DNA and proteins as shown in Figure 1. Channels in the biofilm allow for water, air and nutrients to get to all parts of the structure.

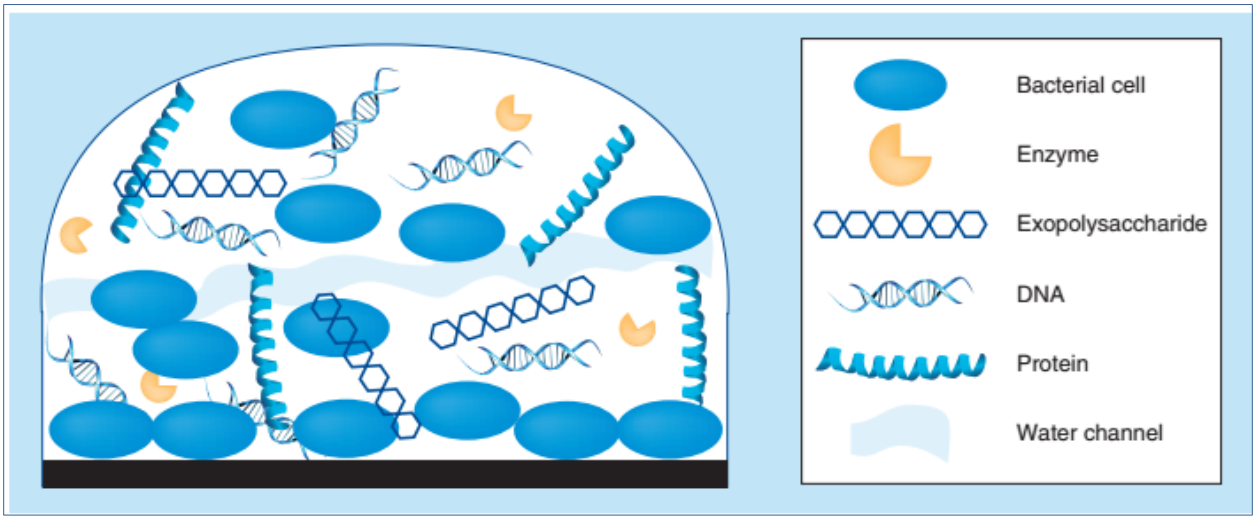

Figure 1: Biofilm structure(Koch 2017)

- Exopolysaccharides are either synthesized extracellular or intracellular or secreted into the outside environment. In electron microscopy, they look like linear or branched long strands that are attached to cell surfaces and stretched out to form large networks. Exopolysaccharides serve as scaffolds for other carbohydrates, proteins, nucleic acids and lipids to adhere. The components, structures and properties of the exopolysaccharides differ from one another(Koch 2017).

- Extracellular proteins are another major EPS matrix component. Some proteins are attached to cell surfaces and polysaccharides to help with biofilm formation and stabilization. One example is the glucan binding proteins (Gbps) in *S. mutans* biofIm. Gbps play an important role in biofilm architecture maintenance by linking bacteria and exopolysaccharides. Biofilms produced by Gbps mutants have significantly reduced height. Amyloids are also insoluble fibrous proteins that play a supportive role in biofilm architecture. One example is the Fap amyloids in *Pseudomonas* spp. Overexpression of Fap amyloids leads to cell aggregation and increased biofilm formation. Amyloid protein TasA is one of the major components of *B. subtilis* biofilms. TasA forms strong fibers that can hold biofilm cells together and tolerate harsh destructive forces. Another example is the biofilm associated protein (bap) family. The bap family includes Bap protein from *S. aureus* and Esp protein from *E. faecalis*. The bap family is involved in biofilm formation and infection processes(Koch 2017).
- Extracellular DNAs (eDNAs) were previously considered as leftovers from lyzed cells until Mattick and coworkers found that DNase I could prevent *P. aeruginosa* biofilm formation. The fact that eDNA not only comes from lyzed cells but also is actively secreted indicates that eDNA has an important role in biofilm formation. It is found to be critical for biofilm attachment. Its negative charge works as a repulse force in the initial attachment, but when the distance between cell and surface becomes a few nanometers, eDNA interacts with receptors on substratum surface to facilitate adhesion. Also, eDNA was found to coordinate cell movement in the twitching motility mediated *P. aeruginosa* biofilm expansion. Due

to its negative charge, eDNA is able to chelate metal cations and some positively charged antibiotics. eDNA can chelate Mg2+ and activate PhoPQ/PmrAB two component systems, leading to antimicrobial peptide resistance in *P. aeruginosa*, *Salmonella enterica* and other Gram-negative bacteria. In *S. epidermidis*, eDNA was also found to inhibit the transportation of vancomycin within bioflms and hence protect the bacteria embedded within the bioflm(Koch 2017).

Recent studies have reported that DNA from lysed cells functions as a structural component in biofilms: in *Pseudomonas aeruginosa* biofilms, DNA coated the biofilm "mushrooms" as a physical scaffold of the EPS matrix. The important function of extracellular DNA in the biofilm structure has been confirmed in experiments with DNA-aseI. The addition of this enzyme broke down DNA – inhibited biofilm formation, while it did not affect bacterial growth in the planktonic state(Cate 2006).

1-2 The stages of Biofilm formation

Biofilm formation is initiated when bacterial cells attach and adhere to a surface. The switch between a planktonic and sessile lifestyle is associated with the recognition and transmission of particular signals from the environment. Signals favoring the early settlement of bacteria may include:

(i) the presence of an appropriate surface

(ii) Increased levels of extracellular iron and ferritin that induce the biofilm phenotype in *P. aeruginosa* in the sputum of cystic fibrosis patients.

(iii) the presence of compounds such as indole that stimulate biofilm formation of many Gram-negative bacteria including *E. coli, Klebsiella*

oxytoca, Citrobacter koseri or other chemicals including polyamines, calcium or bile salts that modulate biofilm formation by *Vibrio cholera, Yersinia pestis, pseudomonas putida and S.aureus*(Markowska, Grudniak, and Wolska 2013).

Characklis and Marshal later described an eight-step process of biofilm information which included the formation of an initial conditioning layer, reversible and irreversible adhesion of bacteria, and the eventual detachment of cells from a mature biofilm for subsequent colonization as exhibited in Figure **2**.

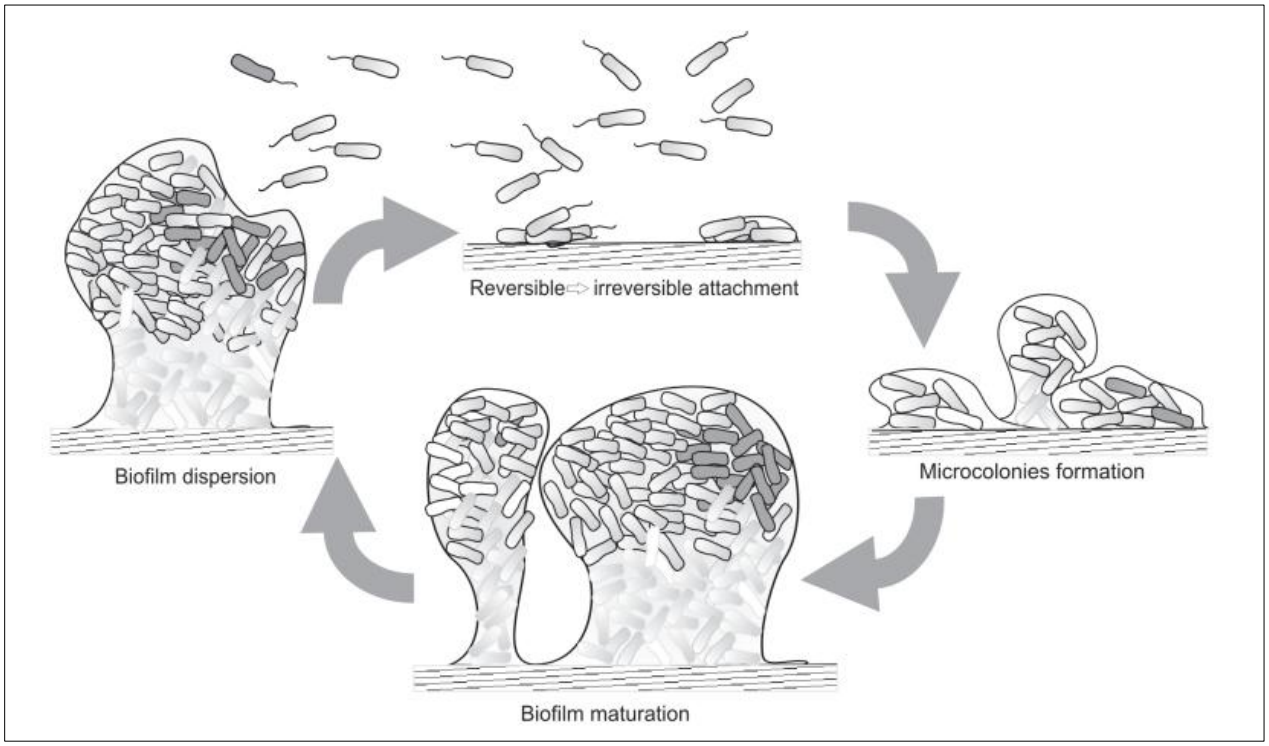

Figure 2:Subsequent stages of biofilm development (Markowska, Grudniak, and Wolska 2013).

The steps of biofilm formation include:

1. The conditioning layer is the foundation on which a biofilm grows, and can be composed of many particles, organic or inorganic. Anything that may be present within the bulk fluid can through gravitational force or movement of flow settle onto a substrate and become part of a conditioning layer(Roger, Bhakoo, and Zhang 2008).
2. Reversible adhesion : Initially, planktonic microbial cells are transported from bulk liquid to the conditioned surface either by physical forces or by bacterial appendages such as flagella. A fraction of the cells reaching the surface reversibly adsorbs(Roger, Bhakoo, and Zhang 2008).
3. Irreversible adhesion: In real time, a number of the reversibly adsorbed cells remain immobilized and become irreversibly adsorbed. It has been argued that the physical appendages of bacteria (flagella, fimbriae and pili) overcome the physical repulsive forces of the electrical double layer(Roger, Bhakoo, and Zhang 2008).
4. Population growth: As the stationary cells divide (binary division), daughter cells spread outward and upward from the attachment point to form clusters. Typically, such interactions and growth within the developing biofilm form into a mushroom-like structure. The mushroom structure is believed to allow the passage of nutrients to bacteria deep within a biofilm(Roger, Bhakoo, and Zhang 2008).
5. Final stages of biofilm development: The stationary phase of growth describes a phase where the rate of cell division equals the rate of cell death. At high cell concentration, a series of cell signalling mechanisms are employed by the biofilm, and this is collectively termed quorum sensing. Quorum sensing describes a process where a number of auto

inducers (chemical and peptide signals in high concentrations, e.g. homoserine lactones) are used to stimulate genetic expression of both mechanical and enzymatic processors of alginates, which form a fundamental part of the extracellular matrix(Roger, Bhakoo, and Zhang 2008).

1-3 The general resistance of biofilms mechanisms

There are three mechanisms, which have been proposed to explain the general resistance of biofilms to biocidal agents.

- The first is the barrier properties of the slime matrix. This mechanism might be more relevant for reactive (bleach or superoxides), charged (metals) or large (immunoglobulin) antimicrobial agents that are neutralized or bound by the EPS and are effectively 'diluted' to sublethal concentrations before they can reach all of the individual bacterial cells within the biofilm. The barrier properties of the EPS hydrogel might also protect against UV light and dehydration, and might localize enzymatic activity. For example, extracellular β-lactamase enzymatic activity against *P. aeruginosa* occurs within the matrix(Hall-stoodley, Costerton, and Stoodley 2004).
- The second protective mechanism could involve the physiological state of biofilm organisms. Although many antibiotics can freely penetrate the EPS, cells within the biofilm are often still protected. The creation of starved, stationary phase dormant zones in biofilms seems to be a significant factor in the resistance of biofilm populations to antimicrobials, particularly against antibiotics such as β-lactams, which are effective against rapidly dividing Gram-positive bacteria by interruption of cell-wall synthesis.

However, arguably all antibiotics require at least some degree of cellular activity to be effective, because the mechanism of action of most antibiotics involves disruption of a microbial process. Therefore, pockets of cells in a biofilm in stationary phase dormancy might represent a general mechanism of antibiotic resistance(Hall-stoodley, Costerton, and Stoodley 2004).

- A third mechanism of protection could be the existence of subpopulations of resistant phenotypes in the biofilm, which have been referred to as 'persisters'. Persisters comprise a small fraction of the entire biomass, whether in planktonic or biofilm culture, but as distinct phenotypes have yet to be cultured, it remains unclear if these organisms do indeed represent a distinct phenotype or are simply the most resistant cells within a population distribution. Although the relative contribution of each of these mechanisms (and possibly others) varies with the rapidly changing weather conditions and changes in local currents due to variations in bed morphology or flow channel. Over short periods (seconds), biofilms can absorb elevated shear by behaving elastically, but over longer periods loading stresses in the biofilm can be dissipated through viscous flow, so that instead of detaching, the biofilm can either flow over the surface or become streamlined to reduce drag (Hall-stoodley, Costerton, and Stoodley 2004).

1-4 Oral biofilms

Microbial communities within the oral cavity are polymicrobial and exist primarily as biofilms on the surfaces of the teeth, prostheses, and mucosal surfaces. These biofilms of oral bacteria and yeasts can cause several localized diseases in the oral cavity, including dental caries, periodontal diseases, candidosis, and endodontic, orthodontic, and implant infections. The survival of micro-organisms within the oral cavity is dependent on their ability to adhere to surfaces and subsequently develop into a biofilm, a process influenced by the physico-chemical properties of the underlying surface. On the tooth surface, the initial colonizers adhere to the acquired pellicle, a salivary-/dietary-derived proteinaceous layer, which can then influence the subsequent sequence of microbial colonization(Allaker 2010).

The occurrence of shifts in the composition of oral biofilms as a cause, but also as a result of tissue pathology, agrees with the ecological plaque hypothesis. Formulating this hypothesis bridged the gap between two schools of thought: the first group proposed a specific plaque hypothesis, in which the oral disease is associated to a limited number of specific pathogens, e.g., *S. mutans* and *Lactobacilli spp.* for dental caries. The second group postulated environmental changes (in particular, high levels of fermentable carbohydrates) as the cause of an outgrowth of microorganisms responsible for pathology. In fact, metabolic "waste" produced by the bacteria induces tissue changes in the underlying substratum including demineralization ,and inflammation(Cate 2006). There are studies suggesting that *S. mutans* isolates have a greater ability to form biofilm than the isolates of other *Streptococcus* species, which colonize the human oral cavity environment. The studies focused on *S. mutans* cells ,which

form biofilm and proved that they exhibit a different expression of some proteins in comparison to planktonic cultures, e.g., an increase in exopoly phosphatase expression and a decrease of lactate dehydrogenase or pyruvate kinase expression. Increased virulence of cells forming biofilm can also be associated with a higher tolerance to low pH, as compared to planktonic cultures(Nishimura et al. 2012).

Interestingly, the cariogenic bacteria make up less than 1% of the oral biofilm and include members of oral streptococci, mainly *Streptococcus mutans*, Streptococcus salivarius, and Streptococcus sanguis. Other cariogenic bacteria like *Lactobacillus acidophilis, Lactobacillus casei, Actinomyces naeslundii, Actinomyces viscusus, Enterococcus faecalis,* and *Candida albicans* also contribute to biofilm formation. Under extended acidic conditions, aciduric bacteria dominate along with strains of non-mutans streptococci, *Actinomyces, Bifidobacteria*, and yeasts. Apart from these initial colonizers the microbial co-aggregation exists among bacterial species like *Fusobacterium, Veillonella, Haemophilus, Campylobacter, Neisseria, Gemella, Granulicatella, Capnocytophaga,* and *Bacteroides.* These secondary colonizers and their association with tertiary colonizers, comprising of Gram negative anaerobic microbes, play a major role in oro-dental infections(Chenicheri et al. 2017).

The initial communities of bacteria found within the supra gingival plaque biofilm are of a relatively low diversity in comparison with those present in the mature communities of both supra gingival and sub gingival plaque. Initial colonizers include *Streptococcus oralis*, *Streptococcus sanguinis,* and *Streptococcus mitis*. The coaggregating partners with these bacteria would then include

predominantly Gram-negative species, for example, *Eikenella corrodens*, *Veillonella atypica,* and *Prevotella loescheii*. Co-aggregation bridges between these early colonizers are common to those with *Fusobacterium nucleatum*, which then coaggregates with numerous late colonizers(Allaker 2012). The late colonizers include *Prevotella intermedia*, *Treponema denticola,* and *Porphyromonas gingivalis*. The interactions among oral bacteriaare integral to the development and maturation of the biofilm. Such interactions occur at several levels and include physical contact, metabolic exchange, molecule communication, and genetic material exchange. A key feature of interaction is the ability to co-aggregate, which is mediated by specific adhesions and receptors that occur on the surfaces of genetically distinct cell types(Krzyściak et al. 2014).

Oral biofilms will accumulate on both the hard and soft tissues and are made up of a community of microbial species embedded in a matrix of bacterial components, salivary proteins/peptides, and food debris. Extracellular polymeric substances, produced by bacteria in a mature biofilm, contain large amounts of polysaccharides, proteins, nucleic acids, and lipids. These maintain the structural integrity of the biofilm and provide an ideal matrix for bacterial cell growth and survival. The biofilm mode of growth is thus clearly distinguished from planktonic growth by several features, which include the resistance to antimicrobial agents at concentrations that approach 1000 times greater than that required to kill planktonic micro-organisms(Cate 2006).

1-5 Quorum sensing (QS)

Many bacteria are known to regulate their cooperative activities and physiological processes through a mechanism called quorum sensing (QS), in which bacterial cells communicate with each other by releasing, sensing and responding to small diffusible signal molecules. The ability of bacteria to communicate and behave as a group for social interactions like a multi-cellular organism has provided significant benefits to bacteria in host colonization, formation of biofilms, defense against competitors, and adaptation to changing environments (Tan et al. 2018).

Bacteria in a community may convey their presence to one another by producing, detecting, and responding to small diffusible signal molecules called auto inducers. This process of intercellular communication, called quorum sensing, was first described in the marine bioluminescent bacterium Vibrio fischeri ,which lives in symbiotic associations with a number of marine animal hosts (Plančak, Musić, and Puhar 2015). In these partnerships, the host uses light produced by V. fischeri for specific purposes such as attracting prey, avoiding predators, or finding a mate. In exchange for the light it provides V. fischeri obtains a nutrient-rich environment where it resides. A luciferase enzyme complex is found to be responsible for light production in V. fischeri. Bioluminescence occurs only when V. fischeri is at high cell density, which is controlled by quorum sensing. Specifically, the production and accumulation of, and the response to, a minimum threshold concentration of an autoinducer regulate density-dependent light production in V. fischeri, and enable V. fischeri to emit bioluminescence light (Yung-Hua Li 2012).

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It has long been known that in infectious diseases the invading bacteria need to reach a critical cell density before they express virulence and overwhelm the host defense mechanisms before they initiate an infectious disease. In particular, many bacteria are capable of using a quorum sensing mechanism to regulate biofilm formation and other social activities.Under such complex conditions, bacteria could benefit from division of labor, collective actions, and other forms of cooperative activities with their neighbors. For example, dental plaque is a well-recognized biofilm community characterized by its vast biodiversity (>700 species) and high cell density (10^{11} cells/g wet wt). The high cell density and species diversity within dental biofilms coupled with environmental fluctuations should create an environment that is conducive to inevitable intra- and inter-species interactions(Cate 2006).

Indeed, cooperative interactions among oral bacteria have been well studied, including bacterial co-aggregation that facilitates co-adhesion of bacterial pairs to the tooth surface, nutritional synergy and complementation to enable cell growth in saliva, and formation of food chains through metabolic cooperation between two or more species. These cooperative interactions probably play very important roles in the development of dental biofilms(Plančak, Musić, and Puhar 2015) .

1-5-1 The Quorum sensing mechanism

Microbiologists have discovered an unexpectedly high degree of coordinated multi-cellular behaviors that have led to the perception of biofilms as “cities” of microorganisms. Especially,many bacteria have been found to regulate diverse physiological processes and group activities through a mechanism called quorum

sensing, in which bacterial cells produce, detect and respond to small diffusible signal molecule(Yung-Hua Li 2012).

Quorum sensing relies upon the interaction of a small diffusible signal molecule with a sensor or transcriptional activator to initiate gene expression for coordinated activities. Quorum sensing systems in bacteria have been generally divided into at least three classes:

(1) LuxI/LuxR–type quorum sensing in Gram-negative bacteria, which use acyl-homoserine lactones (AHL) as signal molecules.

(2) oligopeptide-two-component-type quorum sensing in Gram-positive bacteria, which use small peptides as signal molecules.

(3) luxS-encoded autoinducer 2 (AI-2) quorum sensing in both Gram-negative and Gram-positive bacteria. Each type of signal molecule is detected and responded by a precise sensing apparatus and regulatory network(Yung-Hua Li 2012).

Chapter two: Materials &Method

2-The materials and methods

2-1- The instruments and the materials used in the experiments

- Refrigerator
- Incubator
- Laminar air flow hood
- Autoclave
- Sensitive electronic balanced
- petri dishes
- Tubes, beakers, flask
- Loop
- Nutrient broth
- Chromogenic Candida agar
- MRS broth
- MRSagar
- Blood base agar
- Manitol salt agar
- Tryptic soy agar
- Sucrose
- Congo red stain

2-2 Preparation the cultural media

The variety selective and differential media were prepared for isolating and cultivating the gram positive and gram negative from oral cavity.

2-2-1 Preparation Nutrient broth

Nutrient Broth is a liquid medium used for the cultivation of a wide variety of organisms from clinical specimens and other materials. Upon to the synthesized company, suspend 13 g of the powder in 1 liter of distilled or deionized water. Mix well. Heat to boil shaking frequently until completely dissolved. Sterilize in autoclave at 121°C for 15 minutes.

2-2-2 Preparation Chromogenic Candida agar

According to synthesized company, suspend 21.02 grams in 500 ml purified / distilled water. Heat the media to boiling to dissolve the medium completely. Cool to 45-50°C and aseptically add rehydrated contents of 1 vial of HiCrome™ Candida Selective Supplement. Mix well and pour into sterile Petri plates.

2-2-3 Preparation MRS broth

According to synthesized company, suspend 55.15 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Distribute in tubes, bottles or flasks as desired. Sterilize by autoclaving at 15 lbs. pressure (121°C) for 15 minutes.

2-2-4 Preparation of MRS agar

For isolation, enumeration and cultivation of Lactobacillus spp., dissolve 68.2 g in 1 l of purified water. Heat in boiling water, and agitate frequentlyuntil completely dissolved. Autoclave 15 minutes at 121 °C The prepared medium is clear and brown.

2-2-5 Preparation of Blood base agar

According to synthesized company ,suspend 40.0 grams in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add 5% v/v sterile defibrinated blood. Mix well and pour into sterile Petri plates.

2-2-6 Preparation of Mannitol salt agar

Mannitol Salt Agar is a selective medium used for isolating pathogenic staphylococci from clinical samples, food and other materials of sanitary importance. Depending on synthesized company, suspend 111 g of the powder in 1 liter of distilled or deionized water. Mix well. Heat to boil for 1 minute shaking frequently until completely dissolved. Sterilize in autoclave at 121°C for 15 minutes.

2-2-7 Preparation of Tryptic Soy Agar

Tryptic Soy Agar is a general purpose culture medium for cultivation, isolation of fastidious or nonfastidious microorganisms or for maintenance of stock culture. Suspend 40 g of dehydrated media in 1 litre of purified filtered water. Sterilize at 121°C for 15minutes. Cool to 45- 50°C. Mix gently and dispense into sterile Petri dishes or sterile culture tubes.

2-2-8 Preparation of Congo Red Agar

Prepare100 ml of Tryptic soy agar according synthesized company than add 0.08% (w/v) Congo red and 5% (w/v) sucrose. Mix well. Heat to boil for 1 minute shaking frequently until completely dissolved. Sterilize in autoclave at 121°C for 15 minutes(Kaiser et al. 2013).

2-3 Collection of samples

The samples were collected from 50 individuals' covers male and female individuals at different age groups. The swabs were taken from gingival, buccal cavity, sub gingival and the entire teeth. Then the swab was inoculated MRS broth and nutrient broth medium. The bacterial strains grown on broth were streaked

on MRS agar, manitol salt agar, Blood agar, Candida chromogenic agar and incubated for 24 h.

2-4 Determination the strains producing biofilm on Congo Red Agar

For isolating the biofilm secreting microorganisms, the purified bacterial strains were further sub cultured on Congo red agar and incubated at 37 °C under aerobic conditions for 24 h. The biofilm producer strains formed black colonies, while the non-biofilm producer strains formed white colonies.

Chapter two: Materials &Methods

Chapter three: result & discussion

3- Result and discussion

3-1 Distribution of bacterial strains isolated from oral cavity

The current study is involved collecting 50 samples of mouth swabs from people with or suspected of having dental caries, and inoculating them on the various selective and differential media as shown in Figure 3.

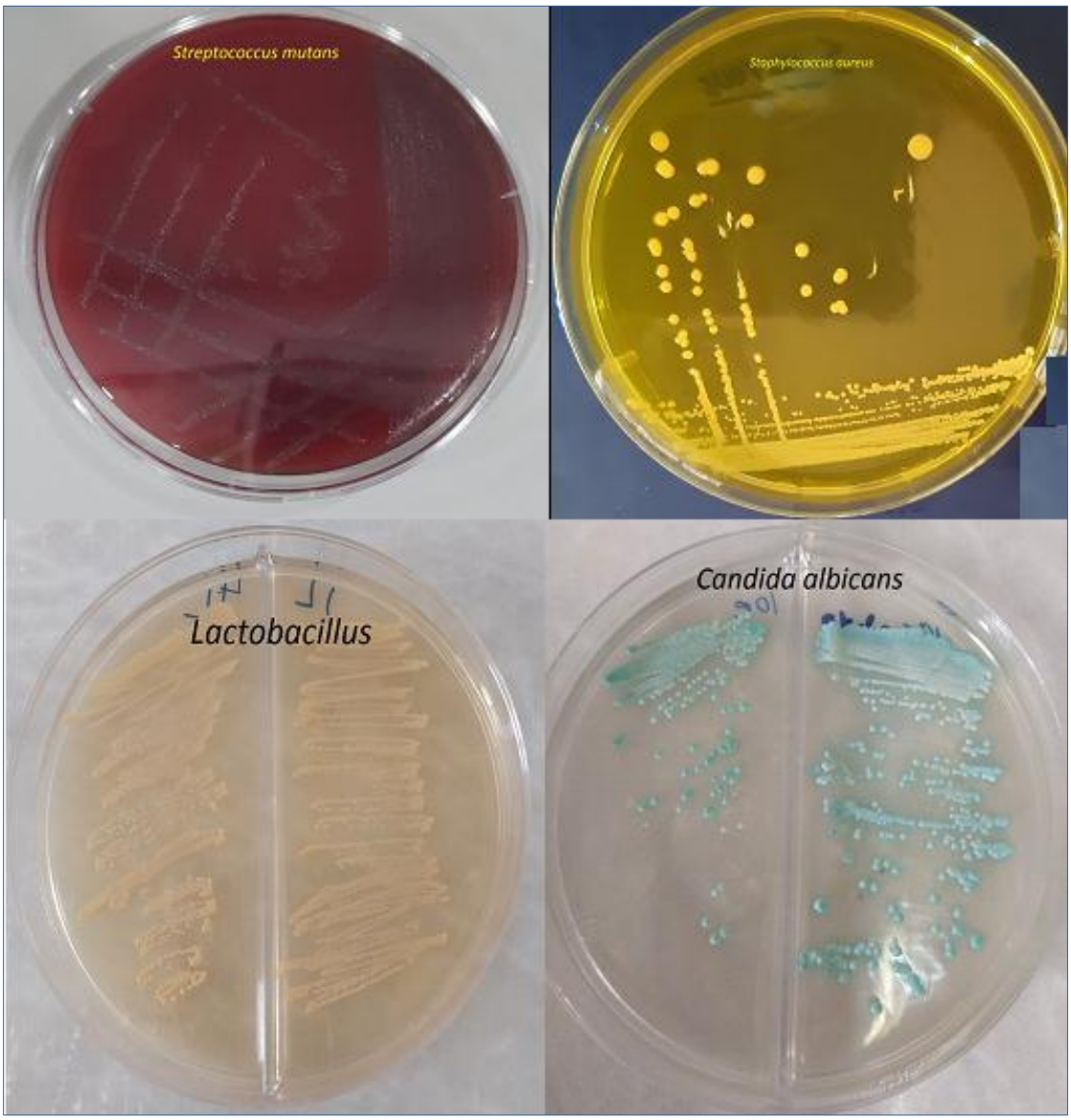

Figure 3: Different images of bacterial strains isolated from oral cavity

As it's demonstrated in Figure4 , species and *Streptococcus mutans* made up the large propotion of the strains isolated from oral cavity, compared to other gram positive such as *S. aureus* and *S. pyogens* that were found in small in number. S. mutans is considered the main etiological factors of the oral cavity infectious disease in which an important causative role is played by biofilms formed by microorganisms on the teeth and gums surface is dental caries. (Krzyściak et al. 2014). On the other hand the yeast isolated from oral cavity such as *Candida albicans,* and *Candida tropicalis* were with proportion of 17(34%) and 4(8%), respectively. In this study, it was noticed that the gram positive strains isolated from 50 samples are representing the large numbers compared to the gram negative strains.

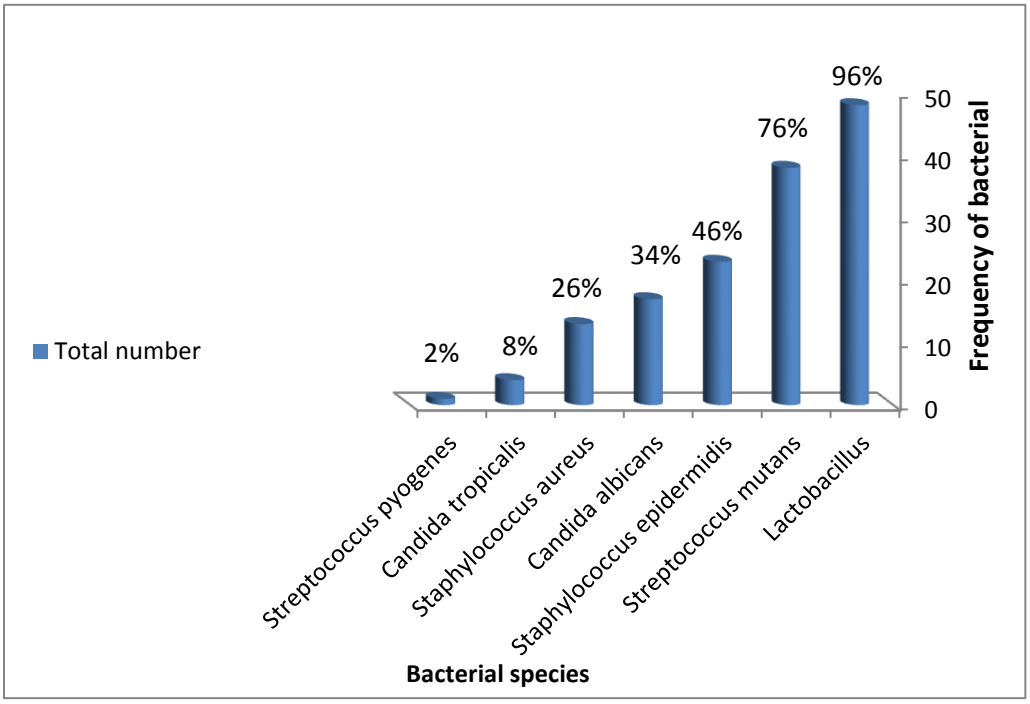

Figure4 **:** Frequency of bacterial strains isolated from the oral cavity.

3-2 Determination the strains producing biofilm

Investigation of the ability of bacterial to generate biofilm can be carried out using various phenotypic methods as illustrated in figure 4. The Congo red agar (CRA) test is based on the subculture of the bacterial strains on brain heart infusion agar (BHIA), supplemented with sucrose and Congo red dye. Studies have demonstrated that this method has low accuracy, but it is cheap and easy to perform and the evaluation criteria is based on visual analysis of the color of the colonies that grow on the agar(Kaiser et al. 2013).

Figure 5: Different samples of *S. mutans* and *S.aureus*

The ability of bacteria of the *S. mutans* species to form biofilms is significant from a clinical point of view, mainly in the context of carries etiology; however, there are also single casuistic cases of infective endocarditis (IE) with the involvement of these bacteria. The development of IE is observed when endocardial damage occurs followed by the formation of a very small blood clot, in which platelets play a crucial role. If, at the same time, microorganisms enter the bloodstream, they may use these favorable growth conditions for deposition and biofilm formation(Krzyściak et al. 2014)(Nishimura et al. 2012). In this study most of the bacterial strains taken from mouth have the capacity for generating biofilm in different proportion as exhibited in Table1 . The results showed *S. mutans* of 71% and *S.aureus* of 77% appeared as black colonies on Congo red agar due to their ability to form biofilm. The percentage of forming biofilm for *Lactobacillus ,* and *S.epidermidis* were the same (48%).

Table1 the proportion of biofilm-forming and non-forming strains.

Bacterial strain	Total number	Percentage of forming biofilm strains	Percentage of non-forming biofilm strains
Lactobacillus	48	48%	52%
Streptococcus mutans	38	71%	29%
Staphylococcus epidermidis	23	48%	52%
Candida albicans	17	41%	59%
Staphylococcus aureus	13	77%	23%
Candida tropicalis	4	0%	100%
Streptococcus pyogenes	1	100%	0%

For bacteria, the advantages of biofilm formation are numerous. These advantages include: protection from antibiotics, disinfectants, and dynamic environments. Intercellular communications within a biofilm rapidly stimulate the up and down regulation of gene expression enabling temporal adaptation such as phenotypic variation and the ability to survive in nutrient deficient conditions. About 99% of the world's population of bacteria are found in the form of a biofilm at various stages of growth and the films are as diverse as the bacteria are numerous(Roger, Bhakoo, and Zhang 2008).

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