THE *IN VITRO* STUDY ON THE EFFECT OF SELECTED COMMERCIALLY AVAILABLE MOUTHRINSES ON SINGLE- AND MIXED–SPECIES BACTERIAL BIOFILMS

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FACULTY OF DENTISTRY
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Field of Study: Oral Biology

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ABSTRACT

Oral cavity, like other locales of human body harbors the micro-florae which are distinctively distributed in various sites and surfaces of the mouth. The normal ecology of the oral cavity should be maintained and hence, the use of oral rinse with antibacterial agent should be used without completely killing the bacteria or compromising the normal oral ecology. Commercially available oral rinses contain active ingredient(s) with concentration that is claimed by manufacturers to be effective as antiplaque agent. To date there has been no mention of the effect of oral rinse on the adherence of early plaque colonizers in plaque formation. There is also no mention of suitable time to use the oral rinse (before or after meals). The aim of the study was to determine i) the bacterial adherence to the experimental pellicle pretreated with oral rinse and ii) the bacterial retention in the biofilm treated with oral rinse compared with their negative controls. An artificial mouth (NAM) system was used for the development of single-species biofilm (*Actinomyces viscosus*, *Streptococcus mitis*, and *Streptococcus sanguinis* respectively) and dual-species biofilm (*A. viscosus* + *S. mitis* and *A. viscosus* + *S. sanguinis* respectively). Three commercially available oral rinses containing active ingredients [Essential oils (EO), Chlorhexidine gluconate (CHX) and Cetylpyridinium chloride (CPC)] were used. The effect of the oral rinse was assessed using two concentrations (100% and 50%) for CPC and CHX and one concentration (100%) for EO. For the adherence study the experimental pellicle on the beads (in the capillary tubes of the NAM system) was pretreated with the oral rinse before the inoculation of bacteria, this would resemble the use of rinse before meal. In the retention study, the biofilm was formed first before treating with the oral rinse and this corresponds to the use of oral rinse after meal. The bacterial population of the biofilm was determined using serial dilution assay and expressed as colony forming unit per ml. Deionized distilled water was used in place of oral rinse and served as a negative

control. For the qualitative study of bacterial population viewing was carried out using Scanning Electron Microscope (SEM). It was observed that on treatment with the oral rinses (100%) the bacterial population of *S. mitis*, *S. sanguinis* and *A. viscosus* of both studies (adherence and retention) was significantly reduced where the reduction was less for EO-based oral rinse compared with that of CHX and CPC in the following sequence EO<CHX<CPC) ($p<0.05$). Moreover it was observed that bacterial population in the respective biofilms where the experimental pellicle was pretreated with the oral rinse before bacterial inoculation (bacterial adherence) was higher compared with that where the oral rinse was used after the bacterial inoculation (bacterial retention). From the results, it appears that oral rinses at their commercially available concentration (100% of the original concentration) with respect to those containing CPC and CHX to be preferably used before meal and EO after meal. A 50% of the original commercial concentration of oral rinses containing CPC and CHX can be recommended for use after meals.

ABSTRAK

Kaviti mulut, seperti di bahagian lain badan manusia menyorokkan mikroflora yang bertaburan di pelbagai tempat dan permukaan mulut. Ekologi normal kaviti mulut perlu dikekalkan dan dengan itu, penggunaan ubat kumuran mulut yang mempunyai agen anti-bakteria perlu digunakan tanpa membunuh sepenuhnya bakteria atau menjejaskan ekologi normal mulut. Ubat kumuran mulut boleh didapati secara komersial yang mengandungi bahan aktif dengan kepekatan yang didakwa oleh pengeluar berkesan sebagai agen antiplak. Setakat ini tidak ada lagi laporan tentang kesan ubat kumuran berhubung pelekatan bakteria plak awal dalam pembentukan plak. Turut tidak terdapat laporan tentang masa yang sesuai untuk menggunakan ubat kumuran mulut (sama ada sebelum atau selepas makan). Tujuan kajian ini adalah untuk menentukan i) pelekatan bakteria pada lapisan pelikel eksperimen yang dirawat dengan ubat kumuran mulut dan ii) pengekalan bakteria dalam biofilem yang dirawat dengan ubat kumuran. Kedua-dua kajian dibandingkan dengan kawalan negatif. Sistem mulut tiruan (NAM) telah digunakan untuk pembentukan biofilem spesies-tunggal (*Actinomyces viscosus*, *Streptococcus mitis*, dan *Streptococcus sanguinis* masing-masing) dan dwi-spesies biofilem (*A. viscosus* + *S. mitis* dan *A. viscosus* + *S. sanguinis* masing-masing). Tiga ubat kumuran mulut yang terdapat secara komersial yang mengandungi bahan-bahan aktif [Minyak pati (EO), Chlorhexidine gluconate (CHX) dan Cetylpyridinium klorida (CPC)] telah digunakan. Kesan ubat kumuran mulut dinilai menggunakan dua kepekatan (100% dan 50%) bagi ubat kumuran masing-masing mengandungi CPC dan CHX dan satu kepekatan (100%) untuk ubat kumuran mengandungi EO. Untuk kajian pelekatan bakteria, lapisan pelikel eksperimen pada manik (dalam tiub kapilari sistem NAM) dirawat dengan ubat kumuran mulut sebelum diinokulasi dengan bakteria. Ini akan menyerupai penggunaan ubat kumuran mulut sebelum makan. Manakala dalam kajian pengekalan bakteria dalam biofilem, biofilem dibentuk sebelum dirawat dengan

ubat kumuran mulut. Ini merujuk kepada penggunaan bilas mulut selepas makan. Populasi bakteria biofilem yang telah ditentukan dengan menggunakan asei siri pencairan dan dinyatakan sebagai unit pembentukan koloni setiap militer (CFU/ml). Air suling ternyahion telah digunakan mengganti ubat kumuran mulut sebagai kawalan negatif. Kajian kualitatif populasi bakteria dijalankan dengan melihat populasi bakteria di bawah mikroscop pr imbasan electron. Keputusan kajian telah menunjukkan bahawa rawatan dengan ubat kumuran mulut (yang 100% kepekatan asal), populasi bakteria (*S. mitis*, *S. sanguinis* dan *A. viscosus* masing-masing) bagi kedua-dua kajian (pelekatan dan pengekalan bakteria) adalah berkurang dengan ketara dengan pengurang yang sedikit bagi perawatan menggunakan ubat kumuran yang mempunyai EO berbanding dengan yang mempunyai CHX dan CPC dalam turutan seperti berikut: EO<CHX <CPC ($p < 0.05$). Selain itu, populasi bakteria dalam biofilem yang lapisan pelikel dirawat sebelum inokulasi (pelekatan bakteria) adalah lebih tinggi berbanding yang dirawat selepas biofilem terbentuk (pengekalan bakteria). Daripada keputusan yang diperolehi, ternyata bahawa ubat kumuran mulut pada kepekatan yang boleh didapati secara komersial (100% daripada kepekatan asal) bagi yang mengandungi CPC dan CHX akan digunakan sebaik-baiknya sebelum makan dan EO selepas makan. Lima puluh peratus (50%) daripada kepekatan komersial asal ubat kumuran mulut bagi yang mengandungi CPC dan CHX boleh disyorkan untuk digunakan selepas makan.

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LIST OF SYMBOLS AND ABBREVIATIONS

ATCC	:	American Type Culture Collection
A.viscosus	:	*Actinomyces viscosus*
BHI	:	Brain Heart Infusion
CHX	:	Chlorhexidine gluconate
CFU	:	Colony forming unit
CPC	:	Cetyl pyridinium chloride
CPD	:	Critical point desiccation
ddH_2O	:	Deionized distilled water
DTT	:	1,4-Dithio-D,L-Threitol
EO	:	Essential oils
GCF	:	Gingival crevicular fluid
Hr	:	Hour
MBC	:	Minimum bactericidal concentration
MIC	:	Minimum inhibitory concentration
min	:	Minutes
NAM	:	Nordini`s artificial mouth
PBS	:	Phosphate buffered saline
SEM	:	Scanning electron microscopy
S. mitis	:	*Streptococcus mitis*
SM	:	Serially diluted oral rinse + sterile BHI
SMB	:	Serially diluted oral rinse + bacteria
S.sanguinis	:	*Streptococcus sanguinis*
SWS	:	Stimulated whole saliva

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CHAPTER 1: INTRODUCTION

1.1. Oral cavity a "habitation" for microflora

Oral cavity, like other locales of human body encloses the distinguishing micro-florae which are distinctively distributed in various sites and surfaces of the mouth (Faust *et al*., 2012). So, in the mouth a specific site or surface has a characteristic microbial populace (Arrieta *et al.*, 2014; Christensen and Brüggemann, 2013; Cogen *et al.*, 2008; Crielaard *et al*., 2011; Sampaio-Maia and Monteiro-Silva, 2014). For instance, gram-positive *streptococci* (facultative anaerobes) are among the major component of the flora, residing on the occlusal surfaces of teeth and are predisposed by the saliva. In comparison, obligate anaerobes are the main species, residing gingival crevice and are chiefly influenced by the gingival crevicular fluid (GCF) (Marsh and Martin, 2009). The biological environment is defined by the metabolism and composition of its microbial populaces which are interactive, metabolically and structurally well-organized (Vincent Zijnge *et al*., 2010). As a reaction to any change in the oral environment, a momentous relocation will occur in the structure and metabolism among its microflora which live in a dynamic equilibrium with their habitation. Phenomena like tooth eruption, salivary flow, individual's life style, habits like smoking, any ailment and therapeutic side effects can generate variation in the oral environment (Crielaard *et al*., 2011; Sampaio-Maia and Monteiro-Silva, 2014).

1.2. Role of whole saliva in oral biofilm formation

Whole saliva is a clear complex bio-fluid which surrounds the oral tissues and contains a mixture of glandular and non-glandular secretions. Glandular secretions are secreted from salivary glands. Non-glandular secretions include crevicular fluids, which contain oral microorganisms and host cells (Cunha-Cruz *et al*., 2013). Saliva does the conditioning of the oral cavity. It contains proteins such as glycoproteins,

phosphoprotein, proline-rich proteins, histidine-rich protein, α-amylase; glucosyltransferases (derived from bacteria). Bacteria adhere to the tooth by adhesion receptor and charge interactions among other mechanisms (Fatin-Majdina *et al.*, 2014; Nobbs *et al.*, 2009; Song *et al.*, 2015).

Classically, in the formation of oral biofilm, which is also known as dental plaque, the pioneer bacteria will first adhere to the tooth surface in a reversible manner. This is then followed by an irreversible interaction forming a substratum for the adhesion of secondary bacteria.

In the oral cavity, *Streptococcus* species like *Streptococcus mitis*, *Streptococcus sanguinis* and *Actinomyces* species like *Actinomyces viscosus* are among the primary/early colonizers of oral biofilm/dental plaque. They have a primary attachment role by binding to the acquired pellicle on the tooth surface, providing the substratum for the attachment of secondary colonizers (Diaz *et al*., 2006; Fatin-Majdina *et al*., 2014; Rahim *et al.*, 2008).

1.3. Formation of dental plaque biofilms

Distinct stages in plaque formation include:

1.3.1. Acquired pellicle formation

Within minutes after cleaning of the teeth, their surface rapidly become coated with acquired pellicle (acellular layer containing albumin, glycoproteins, acidic proline-rich proteins, mucins, cell debris, exoproducts (such as amylase and lysozyme), and sialic acid) which provides a variety of receptors that are recognized by early colonizing bacteria (Marsh *et al.*,2009).

1.3.2. Transport of microorganisms and reversible attachment

The primary colonizers of dental plaque/biofilm are mostly gram-positive cocci species, among which are the *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus oralis* and gram-positive rod species, such as *Actinomyces viscosus*, *Actinomyces israeli*. These bacteria are transported passively through saliva flow and attach to the receptors of acquired pellicle. This attachment is reversible, involving weak, long range of physicochemical interactions between the cell surface and the acquired dental pellicle (Marsh *et al*., 2009).

1.3.3. Pioneer microbial colonization and irreversible attachment

This reversible adhesion of early colonizers, alter the attachment surface by developing the new surface-attached phenotype with distinct metabolic activity, thus altering their surroundings and creating new niches for other bacteria to colonize (Davey and Costerton, 2006). Many oral microbial species have multiple adhesion types on their cell surface, hence simultaneously participate in various interactions with other bacteria and also with the host surface molecules (Marsh *et al*., 2009). The interaction between specific molecules (adhesions) on the bacterial cells and the complementary receptor proteins on the pellicle surface resulted in a much stronger and irreversible attachment.

1.3.4. Co-aggregation

Most oral bacteria adhere to one another. This cell-to-cell adherence is known as coaggregation. Over the time, bacterial colonization becomes diverse and there is a shift away from initial predominance of gram-positive species to the gram-negative species and makes the environment more suited for the fastidious bacteria. A series of complex interactions called “microbial succession” occurs and composition of plaque microflora changes with time (Marsh *et al*., 2009).

1.3.5. Mature biofilm formation

The bacterial cells continue to divide until a three-dimensional mixed-culture biofilm develops. Some bacteria produce extracellular polymers like hetero-polymers, soluble and insoluble glucans, fructans that contribute to plaque matrix formation. Plaque matrix plays an important role in structural integrity and general tolerance of biofilm to environmental factors and antimicrobial agents. The bacterial stratification is organized according to metabolism and aero-tolerance, with the number of gram-negative cocci, rods and also filaments increasing as more anaerobic bacteria appear (Sbordone and Bortolaia, 2003). As the biofilm thickens and becomes more mature, anaerobic bacteria live deeper within the biofilm which protects them from the aerobic environment within the oral cavity (Figure 0.1) (Marsh *et al*., 2009).

1.3.6. Detachment from the surfaces

The structure of the climax community of plaque is diverse, with many species being detected at individual sites. Shear forces can remove bacteria, as the detachment of bacteria from biofilms is essential to allow colonization of new habitats. Some bacteria have the ability to detach themselves from the biofilm. This phenomenon has been observed from *in vitro* studies that in un-favorable conditions, bacterial cells detach in different ways such as detachment of single cell or a group of cells or by intermediate process in which cluster of bacteria are detached (Marsh *et al*., 2009).

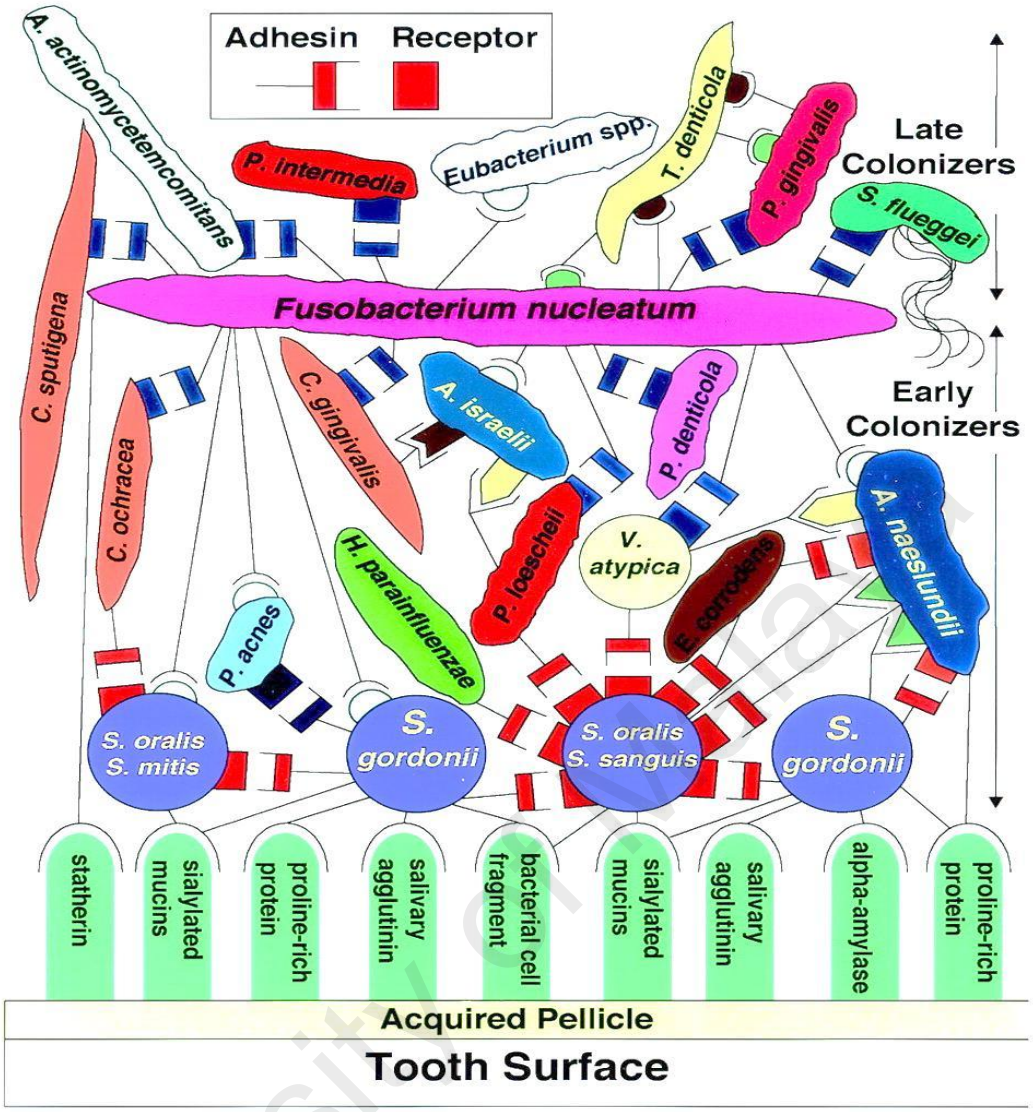


Figure 0.1: Schematic representation of the patterns of coaggregation (co-adhesion) in human dental plaque. Early colonizers bind to receptors in the acquired pellicle; subsequently, other early and later colonizers bind to receptors on the surface of these already attached cells (co-adhesion). Adhesions (symbols with stems) are cell components that are heat or protease sensitive; the receptor (complementary symbol) is insensitive to either treatment. Identical symbols do not imply identical molecules. The symbols with rectangular shapes represent lactose-inhibitable coaggregations. (Adapted from (Marsh *et al*., 2009)**.**

Oral micro-biome forms plaque (Marsh, 2012) which if maintained in healthy proportion, has a significant role in the preservation of good and over-all wellbeing of the oral cavity (Hezel and Weitzberg, 2015; Marsh, 2012). However, if the plaque is allowed to accumulate for a longer period of time it has the potential to induce gingivitis (Lee *et al*., 2012). Mechanical removal of plaque by tooth brushing and dental flossing is among the recommendations by the dentist for healthy oral environment. However, if

the mechanical removal technique cannot provide optimal maintenance of oral health, oral rinses containing antimicrobial agents are prescribed as an adjunct treatment. It is has been reported that these activities (brushing, flossing, gargling with antibacterial oral rinse) can inhibit or limit the bacterial adhesion and the subsequent growth of plaque on tooth surface (Fatin-Majdina *et al*., 2014). Antibacterial agents used should have an effective formulation to balance the microbes of the mouth in a healthy proportion rather eradicating them completely causing imbalance (Marsh, 2012; ten Cate and Zaura, 2012).

1.4. Possible targets for study when exploring the use of oral rinses in controlling plaque development

The antimicrobial effect of oral rinse can be achieved better without compromising the normal oral flora if used at the right time and in the right concentration. Two potential times for oral rinse application were suggested, that is before and after biofilm formation. Before the biofilm started to form, which is related to immediately after tooth brushing, the use of oral rinse could act as a protective layer to minimize the adherence of bacteria to the salivary pellicle, a stage considered to have a leading role in biofilm formation. When used after biofilm formation such as after meal, the oral rinse may affect the bacterial population in the plaque by modifying the organization of plaque matrix that may favor bacterial detachment.

1.5. Rationale of the study

The active ingredient(s) in various commercially available oral rinses such as those that contain Essential oils (EO), Chlorhexidine gluconate (CHX) and Cetylpyridinium chloride (CPC) is/are in concentration that have potential to kill bacteria as claimed by the manufacturers (Colgate, 2016; Oradex, 2016; Listerine, 2016). The amount of the respective antimicrobial agent/active ingredient incorporated in the commercially

available oral rinses is stated but there is no mention of when to use the oral rinses, (before or after meals). There are many bacterial species in the oral cavity. In this study we chose three species which are among the early colonisers of dental plaque. The normal ecology of the oral cavity should be maintained and hence, a need to know the minimum effective concentration of the oral rinses towards the oral flora. We are targeting the effect of the oral rinses towards the early colonizers (*S. mitis*, *S. sanguinis* and *A. viscosus*) without completely killing them.

In this study, the antibacterial effect of three commercially available oral rinses (those that contain CHX, CPC and EO as active ingredients) in the control of plaque development were investigated. The antibacterial effect of the oral rinses was investigated *in vitro* with respect to the i) bacterial adherence toward oral rinse-treated experimental pellicle and ii) bacterial retention in oral rinse-treated biofilm.

Aim

To investigate the anti-plaque effect with respect to anti-adherence and anti-retention activities of the various commercially available mouth/oral rinses.

Specific objectives

i) To determine the growth curve of selected bacteria.

ii) To determine the minimum inhibitory concentration of commercially available oral rinses.

iii) To validate the bacterial population of biofilm formed on glass beads at different locations (near Inlet, at the Middle, near Outlet) in the glass capillary tube of the Nordini`s artificial mouth system.

iv) To compare the effect of the different oral rinses on the adhering capacity (adherence) of selected oral bacteria to the pellicle (experimental pellicle).

v) To compare the effect of the different oral rinses on the retention of selected oral bacteria in the biofilm (simulated biofilm).

vi) To demonstrate the anti-adhering and anti-retention activities of the different oral rinses qualitatively using Scanning Electron Microscope.

CHAPTER 2: LITERATURE REVIEW

2.1. Oral Environment

The oral cavity is the beginning of the gastro-intestinal system. It houses hard and soft oral tissues, saliva and oral microflora creating the oral environment. The environment is constantly affected by changing conditions associated with food intake and it's processing – mechanical and biochemical - the beginning of its digestion. The oral cavity possesses a distinctive ecosystem as a host environ it supports the growth and establishment of a variety of microflorae. The hard and soft structures in oral cavity include cheeks, tongue and gingivae and teeth which provide the sites for the adherence and subsequently growth of microbes.

Initially the composition of nascent microbes is quite simple taxonomically during the former few days of infancy, with time-phase the bacterial diversity rises through microbial settlement/migration (Favier *et al.*,2002). Subsequently, in the infant the microbial-ecology on all the colonisable surfaces like oral cavity and intestine is established (Beerens *et al.*,1980).

An extensive variety of bacteria (500-700) inhabit and colonize the oral cavity (Marsh and Martin, 2009c). Different populations of bacteria are concomitant with different sites within the mouth and their compositions also reliant on the anatomical locality. For instance, the bacterial consortium which colonizes teeth is different to that which colonizes the soft tissues like tongue, buccal mucosa (Mager *et al.*, 2003). The

population of these commonly found bacteria with respect to their anatomical location remains stable (Marsh and Martin, 2009c) and are non-pathogenic under normal conditions until any discrepancy happens which leads to the impairment of host`s defences due to any underlying illness/pathology.

2.1.1. Tooth Surface

The hard structure in oral cavity is tooth which plays a vital role in mastication of food. It is suitable structure for the colonization of oral microbes as it allows large masses of microbes to accumulate and form the oral biofilm. A natural tooth comprises of pulp, cementum, dentine and enamel (Fehrenbach and Popowics, 2015; Nanci, 2014). Being the outermost layer of the tooth "enamel" is the only part that is exposed to the oral environment under normal condition. Tooth offers surfaces such as pits and fissures that influence the microbial growth and colonization. The tooth surface normally favours the habitation of aerobic, facultative and anaerobic microflora. (Aruni *et al.*, 2015; Samaranayake, 2002a).

2.1.2. Mucosal Surface

The oral mucosal surfaces contribute to a variety of oral microbes present in the specialized areas, for example, cheek mucosa, provides the establishment of predominantly the facultative types, especially streptococci (Wan *et al.*,2013; Ross and Holbrook, 1984). Tongue, a soft tissue, consist of papillary surface and provides situates for colonization. These sites are protected from the washing action of saliva (Marcotte

and Lavoie, 1998). Another anatomical location of tongue is its dorsum , a study reported that the dorsum of tongue was frequently colonized by *Candida albicans* (Zahir and Himratul-Aznita, 2013).

2.1.3. Saliva

Saliva is an exocrine secretion and a primary source of nutrition for oral microbes (Kidokoro *et al.*, 2014; Knosp *et al.*, 2012; Patel and Hoffman, 2014; Varga, 2015). It contains water (99%), electrolytes (such as calcium, sodium, potassium, magnesium, bicarbonate which contribute its buffering property), various proteins and mucins (Marsh & Martin, 2009b). Three major salivary glands mainly secrete the saliva, additional to these are minor glands which are present on the soft palate and on the sub-mucosal surfaces of the lips and cheeks (Marsh & Martin, 2009c; Berkovitz, 2011). The chemical composition of saliva varies and depends on its secreting gland, for instance, parotid secretes serous secretion, however submandibular and sublingual glands secrete more viscous secretion which contain glycoproteins in high contents (Carpenter, 2013; Dijkema, 2013). The type of secretion puts influence on the adherence capacity of oral bacteria to the oral surfaces; there is a study which shows that parotid saliva supported the adherence of *S. mutans* to the beads, whereas the whole saliva inhibited its adherence (Carlen *et al.*, 1996). The term "whole saliva" refers to the complex mixture of oral fluids which comprises of saliva secreted from major (including parotid, submandibular and sublingual glands) and minor salivary glands (such as the glands in labial mucosa), gingival crevicular fluid (GCF), desquamated (epithelial cells), free-

floating bacterial cells, dietary residues, exudates (mucous or naso-pharyngeal origin) and traces of introduced chemicals or curatives (Carpenter, 2013; Patel and Hoffman, 2014). The composition and flow rate of whole saliva is maintained by sympathetic and parasympathetic stimuli and predisposed by a number of physiological factors (Proctor, 2016; Proctor and Carpenter, 2014) like flow rate of saliva varies during the day, in resting (unstimulated) saliva flow rate ranges between 0.2-0.5 ml.min-1 while stimulated saliva flow rate ranges from 0.8 to 3 ml.min-1.

The main organic component of saliva are proteins and mucins (glycoproteins) which aid in cleansing, aggregation and attachment of the oral microbes and contribute to dental plaque metabolism (Humphrey and Williamson, 2001). Mucins are present as two molecular weight types; MG1 (high molecular weight) and MG2 (low molecular weight), which perform biologically distinct functions. For instance, MG1 has a tissue-coating role (both soft and hard tissues) by forming a thin, protecting layer on oral tissue surfaces known as the “acquired pellicle” which works as host receptors that can interact with the adhesins on the bacterial surface. Thus, the acquired pellicle plays an important role to enhance the attachment and colonization of the pioneer bacteria on the tooth surface, therefore involved in the selective attachment of microbes and contributing to colonization of bacteria. This saliva layer is approximately 100µm in depth and provides nutrients to adherent microbes, its organic components can also carry the lysozymes like antimicrobial factors (Samaranayake, 1998). The existence of these antimicrobial factors is significant to screen and regulate the microbial population

in the mouth. The type MG2 is more soluble and boosts aggregation of free floating oral microbes thus facilitating their clearance from the oral cavity. Salivary proteins and glycoproteins affect the oral ecosystem in many ways and promotes the maintenance of oral health (Ali and Creanor, 2016).which it contributes to by providing physical protection and by balancing the oral microecology (Humphrey and Williamson, 2001)

2.1.4. Gingival Crevicular Fluid

A serum like fluid, secreted by the junctional epithelium into the gingival crevice which is located between the junctional epithelium of the gingiva is known as GCF or gingival creviular fluid. This area also offers a unique site for the colonization of microbes as it includes both (hard and soft) tissues (Bosshardt and Lang, 2005; Darout, 2014). A suitable environ is provided by GCF for anaerobic and as well as facultative bacterial populations (Faran Ali and Tanwir, 2012; Marsh, 2000). The composition of the gingival fluid is similar to that of plasma, as it is originated from plasma and comprises of proteins like albumin other components are leucocytes, antibodies and complements (Koregol *et al.*, 2015; Loo *et al.*, 2010; Proctor, 2016). The subgingival microbes are responsible for the degradation of proteins and glycoproteins by their enzymes who breakdown these proteins into amino acids, peptides and carbohydrates and make GCF rich in these sources (Marsh & Martin, 2009c). In periodontal disease, enzymes like trypsin, elastase and collagenase can be detected in GCF. These enzymes have been accepted as potential diagnostic indicators (Marsh and Martin, 2009c). GCF comprises of antibodies which are directed to the microbial organisms and aids in

keeping the flora in check either by inhibiting their colonization and acting as opsonins, or by initiating the complement system (Schonfeld, 1992).

2.2. The Oral Microflora

The cohabitation of microbes with a variety of demands and requirements in the oral biofilm is accommodated by numerous physicochemical-interactions inside the bacteriological population.

2.2.1. Streptococcus bacteria species

Among the oral microflora, streptococci bacteria are the largest group of the resident microbes (Wan *et al*., 2013; Willett *et al.*, 1991). Facklam (2002) reported haemolysis as the most useful feature for the true identification of streptococci. To bind to the salivary components which are already deposited on oral surfaces, streptococci have a variety of mechanisms. These mechanisms also help and promote the adherence of one bacterium to another bacterium in a process called coaggregation, a process in which protein-carbohydrate interactions occur and lead to the plaque formation.

Oral Streptococci bacteria are distributed into 4 groups; 1) *mutans*-group, 2) *salivarius*-group, 3) *anginosus*-group and 4) *mitis*-group (Marsh and Martin, 2009d; Samaranayake, 2002b). The *mutans*-group has been related to the human dental caries (Gross *et al*., 2012; Samaranayake, 2002b). The *salivarius*-group commonly colonizes mucosal surfaces (like tongue) and vestibular mucosa of the human mouth (Maiden *et al.*, 1992). The *anginosus*-group is the carbon dioxide – reliant group (Maiden *et al*.,

1992; Samaranayake, 2002b) and are usually associated with abscesses of internal structures and organs, also reported to play an important role in causing purulent disease in humans (Marsh and Martin, 2009d). The *mitis*-group is known as an opportunistic pathogens and are often belongs to infective endocarditis (Marsh and Martin, 2009d; Samaranayake, 2002b). Facklam (2002) has proposed 5 major groups of oral streptococci which include *mutans*-group, *salivarius*-group, *anginosus*-group, *mitis*-group and *sanguinis*-group based on their phenotypic individualities.

2.2.1.1. *S. mitis* bacterial specie

Streptococcus mitis (*S. mitis*) was formerly known as "*Streptococcus mitior"* is a mesophilic, alpha hemolytic, gram-positive cocci. *S. mitis* is one of the bacterial species in the oral cavity which predominates with other streptococci (*S. sanguinis*) in colonizing the cleaned tooth surface (Caufield *et al*., 2000b; Wan *et al.,* 2013). *S. mitis* having a potential to adhere the non-keratinized mucosa, is usually found on the soft tissues of the oral cavity such as cheeks, lips and the ventral surface of the tongue (Samaranayake, 2002b; Wan *et al*., 2013).

S. mitis being facultative anaerobes, can make their metabolism versatile. They consume and synthesize intercellular glycogen and catabolize it to lactate. *S. mitis* can be differentiated from other oral streptococci by being negative for esculin hydrolysis, extracellular polysaccharide formation and insulin fermentation (Maiden *et al*., 1992). Its adherence potential is influenced by sucrose, as it can produce dextran in presence of

sucrose which promotes its adherence to the surfaces (Wan *et al.*, 2006; Melville and Russell, 1975). In relation to oral cavity, *S. mitis* resistance to penicillin is reported to be associated with endocarditis (Samaranayake, 2002b; Levy *et al*., 2001; Matsui *et al*., 2013). The endocarditis occurs due to lack of prophylactic treatments before the dental procedure. *S. mitis* being a commensal bacteria is closely associated to *Streptococcus pneumoniae*, the causal agent of pneumonia, otitis, sepsis and meningitis.

Researchers in current studies have reported high level of resistance of *S. mitis* to beta-lactams antibiotics, prescribed in dental treatments (Nakayama and Takao, 2003; Soares *et al*., 2012).

2.2.1.2. *S. sanguinis* bacterial specie

Streptococcus sanguinis (*S. sanguinis*) is a gram-positive, non-motile and non-spore forming cocci bacteria which was formerly called as *Streptococcus sanguis* (Caufield *et al*., 2000a; Trůper and De'clari, 1997). It is a member of *mitis*-group (Samaranayake, 2002b) and one of the first bacteria to colonize newly erupted tooth or freshly cleaned tooth surfaces (Caufield *et al*., 2000a; Li *et al*., 2004; Magalhães *et al*., 2016). *S. sanguinis* is not considered as cariogenic in human; however it was found to induce fissure caries in gnotobiotic rats fed with diet containing high levels of sucrose. *S. sanguinis* has potential to hydrolyze arginine and esculin. It produces water-insoluble glucan α-1,3- + α-1,6- and water insoluble dextran α-1,6 when sucrose is present as substrate (Marsh and Martin, 2009d). *S. sanguinis* was found to be involved in subacute bacterial endocarditis (Facklam, 2002). In infants, *S. sanguinis* starts colonizing the tooth surfaces at the age of nine months, the population was observed to increase with age of the infants (Caufield *et al*., 2000a; Lucas *et al.*, 2000). In a study conducted on

children by Lucas *et al*. (2000) it is reported that among the other oral micro-flora *S. sanguinis* is about 39.4%. Oral cavity is continuously coated with saliva and thin layer of saliva is formed on the teeth called acquired pellicle (Samaranayake, 2002b) which carries receptors to facilitate the adherence of *S. sanguinis* (Kreth *et al.*, 2009; Rosan and Lamont, 2000). The acquired pellicle contains receptors that facilitate the binding of the *S. sanguinis* involving specific interactions between bacterial cells and the constituents of pellicle (Rahim *et al.*,2014; Gong *et al.*, 2000).

S. sanguinis has hydrophobic characteristics in its cell wall with the existence of several amino acids having hydrophobic side chains (Black *et al.*, 2004; Fives-Taylor and Thompson, 1985; Nesbitt *et al.*, 1982) due to which bacteria exhibits a high affinity for hydrocarbon solvents (Nesbitt *et al*., 1982). A study showed *S. sanguinis* more hydrophobic when grown as biofilms compared to planktonic form (Black *et al*., 2004) the difference in the affinity could be due to the difference in protein expression. This affinity was found diminished when *S. sanguinis* was subcultured in an *in vitro* study (Westergren and Olsson, 1983).

2.2.2. Actinomyces bacterial species

Actinomyces are gram-positive bacilli, belong to the commensal flora of human and usually colonize the approximal sites and the gingival crevice (Marsh and Martin, 2009d; Maiden *et al*., 1992). *Actinomyces* cells are short rods or long filaments in appearance. They have a characteristic to ferment glucose and also produce succine, acetic, formic and lactic acids (Marsh and Martin, 2009d; Samaranayake, 2002b)

2.2.2.1. *A. viscosus* bacterial specie

Actinomyces viscosus (*A. viscosus*) are among the bacterial strains which grow well in the presence of oxygen being catalase-positive and facultative aerobes. On culture plates, during early stage before division they appear as long hyphal elements and later as irregular rod-shaped forms (Melville and Russell, 1975). *A. viscosus* has been found to be associated with the development of supra- and sub- gingival plaque which can further lead to periodontal diseases (Melville and Russell, 1975).

A.viscosus have fibrils on their cell surface, these fibrils are thought to play an important role in adhesion of the bacterial cell to the surface. Wheeler and Clark (1980) reported that the adhesion of *A. viscosus* to the substratum (coated with saliva) was arbitrated by fibrils present on the bacterial cell. Whereas Rozen *et al.,* (2001) reported that the adherence was fructan-dependent. This is due to the potential of *A. viscosus* to produce fructosyltransferase (FTF) which produces fructans with sucrose (substrate) (Rozen *et al*., 2001). Compared to glucans, *A. viscosus* were observed to show higher affinity towards fructans. Further Rozen *et al.*,(2001) proved a reduction in *A. viscosus* adhesion to the substratum when fructan synthesis was inhibited.

2.3. Oral Biofilm

2.3.1. Biofilm

The term biofilm can be defined as a layer of a well-organized community of microorganisms growing on a surface (Branda *et al.*, 2005; Marsh and Martin, 2009b). It exists in the form of multicellular aggregates of bacterial species embedded in an extracellular matrix (Branda *et al*., 2005; Stephens, 2002). The extracellular polymer matrix is derived from the bacterial cells themselves and the surrounding environment (Marsh and Martin, 2009c). A moist environment with rich nutrient supply favors the development of biofilm and fulfills the metabolic needs of the microbiota ((Sarjit *et al.*, 2015).

2.3.2. General Properties of biofilm

The biofilms are developed by the aggregation of either same or mixed bacterial species (Singh *et al.*, 2006). Therefore the nature and features of the dynamic biofilm may be different from those in planktonic state. Usually the biofilm comprises of cooperating microorganisms, arranged in micro-colonies and surrounded by protective matrix (Costerton *et al.*, 1999). The protective matrix known as extracellular polysaccharide (EPS), comprises of polymers which have a potential to develop a thick, continuous, hydrated and charged layer around the cells (Marsh and Martin, 2009a; Sutherland, 2001).

The EPS matrix gives bulk to the volume of the biofilm and responsible for its macroscopic characteristics (Stephens, 2002) also maintaining the integrity of biofilm (Sutherland, 1999). This matrix is insoluble and can not be separated easily from bacterial cells (Branda *et al*., 2005). The biofilm is protected by the EPS matrix from challenges like host defenses (Marsh & Martin, 2009a), desiccation and the antimicrobial therapy. A study by Stoodley *et al* (1999) showed that the biofilm is contained masses of microbial cells within an EPS matrix, there are penetrating, interstitial voids/channels which separate the micro-colonies and permit the movement of nutrients, metabolites, enzymes all over the biofilm structure (Sutherland, 2001).

2.3.2.1. Resistance to antimicrobial treatment

Biofilms are associated with the resistance to antibiotic treatment. Various antibiotics drugs have been used against microbes. Among those, aminoglycosides being positively charged and hydrophilic are the common one, against which microbes have shown resistance.

This resistance of bacteria in the biofilm was about 1000 times greater when compared to the same cell but grown planktonically (Costerton *et al*., 1999; Hogan and Kolter, 2002). Doron *et al* (2001) has reported that the S*treptococcus sobrinus* biofilm was more resistant to parabens than those in planktonic phase. Although the antimicrobials eliminate the bacterial cells in liquid state but if these cells are in sessile form, they continue to populate and spread the infection. In that case antigens and

antibodies are released that ultimately originate more destruction to the neighboring tissues.

It was also reported that the multi-species biofilm (of four bacterial species) displayed to be more resistant to tetracycline and hydrogen peroxide in comparison with the single-specie biofilm (Burmølle *et al*., 2006). In another study by Kara *et al* (2006) it is reported that dual species biofilm of *S. mutans* and *Veilleonella parvula* was observed to be more resistant to treatments (antimicrobials) than the single-species biofilms. It has been proposed that the interactions between the different bacterial species and their matrix polymers may promote the formation of more viscid matrix, reducing the penetration of the antimicrobials (Burmølle *et al*., 2006) and encourage the survivability of the biofilm against antimicrobial agents (Filoche *et al.*, 2004). This is due to the EPS matrix, which is being an initial barrier either hinders the surrounding chemicals/antibiotics to diffuse into the biofilm (Jenkinson and Lappin-Scott, 2001; Mah and O'Toole, 2001) or incapacitate the particular antibiotic agent by altering the enzymes (Chadha, 2014).

The mechanism in which oral bacteria transfer their genes with each other called conjugation. In relation to the occurrence of drug-resistant microbes; research on gene transfer in natural environs has been started. It has been proposed that these genes in the biofilm may have proficiency to vary its phenotype which results in resistance by limiting the penetration of the antimicrobials (Marsh, 2005). A hypothesis is that the

resistance capability of some biofilms to antibiotics is due to the existence of 'persister cells' (Keren *et al.*, 2004), accumulated in low-nutrient area of the biofilms which are assumed to be inactive but capable to relapse quickly and allow the regrowth of biofilm (Roberts and Stewart, 2005).

2.3.2.2. Defense Character

Within a biofilm, bacterial populations are actively adapting to environmental stress. In addition to protection against chemical agents, The EPS provides protection to its microbial flora against therapeutic agents; physical forces (blood flow and saliva), (Jefferson, 2004) and shear forces (Donlan and Costerton, 2002). EPS defends the bacteria from phagocytosis by making them difficult to dislodge (Donlan and Costerton, 2002; Stephens, 2002). Another important feature of bacteria has been reported by Novick (Novick, 2003) and quoted by Fux *et al.,* (2005) that in a biofilm, bacteria can activate the genes for stress-response which make their phenotypes more tolerant during environmental conditions like, nutritional deficiency, temperature, pH, and cell density. Bacterial cells in the biofilm also produce catalase, superoxide dismutase like defensive enzymes that shield them from oxidizing ions, (Costerton *et al*., 1999). This protective behavior of biofilm in the protection of its resident flora sometimes gives more harm to the neighboring tissue than to the biofilm for example; in order to replace the biofilm phagocytes may engulf other tissues which is nearby (Jefferson, 2004).

2.3.3. The Development of Oral Biofilm

The oral biofilm also known as dental plaque, is an adherent material which develops naturally on the tooth surface. Dental plaque comprises of diverse microbial community, extracellular polymers and adsorbed salivary proteins (Do *et al.*, 2013). Accumulation of dental plaque beyond the normal levels leads to the commencement of oral and dental infectious ailments, depending upon the locations, such as dental caries and gingivitis (Doron *et al*., 2001). The understanding about the formation of the oral biofilm is decisive so that an effective approach can be deliberated to control its development.

The development of oral biofilm is a biological phenomenon involved with the attachment, detachment and proliferation of oral microbial communities on the tooth surface (Takeshita *et al*., 2015). Following are the stages:

i) Formation of acquired pellicle

ii) Adhesion, reversible and irreversible interactions

iii) Multiplication and co-aggregation

iv) Detachment from surface

The ultimate outcome of these stages is the development of a multifaceted, multi-species, spatially and organized biofilm.

2.3.3.1. The formation of acquired pellicle

A thin, acellular layer composed of salivary proteins is known as acquired pellicle. These salivary proteins provide receptors for the binding of bacterial species (Cole and Eastoe, 1988). The formation of acquired pellicle occurs in two stages i) Adsorption of discrete proteins to the enamel surface and ii) Adsorption of protein aggregates (contributing to rapid increase in pellicle layer thickness) (Do *et al*., 2013). Hannig, (1999) reported that on exposure to saliva a detectable pellicle is adsorbed onto the enamel surface within minutes. Similarly Smith and Bowen, (2000) observed glycoproteins adsorb on the Hydroxyapatite (HAp) disc within one minute on exposure to the oral environment.

2.3.3.2. Adhesion, reversible and irreversible interactions

Development of oral biofilm is initiated on adhesion of the bacterial cells present in the saliva to the cleaned tooth surface (enamel) (Zijnge *et al*., 2010). For the adhesion of bacterial cells on the enamel surface two stages are involved i) reversible adsorption and ii) iireversible adsorption (Scheie, 1994). During the first stage of biofilm development, the long-range physico-chemical forces start to operate between bacterial cell surface and the pellicle-coated tooth. Hydrophobic interactions are initiated as the lipophilic adhesins on bacterial cell come in contact with hydrophobic receptors on the epithelial cells (Schonfeld, 1992). Similarly the negatively charged adhesins on the bacterial cells via a divalent cation “bridge” bind to the glycoproteins (negatively charged) (Schonfeld, 1992). Later the van der Waals attractive forces and electrostatic repulsion produce a

weak area of attraction, facilitating reversible adhesion. *Streptococcal* species are reported to be the predominant organism during early phase of biofilm formation (Li *et al*., 2004). Marsh and Martin (2009d) reported that *streptococcal* species rely on various adhesins via lectin-like, hydrophobic and specific protein interactions for its binding to the saliva-coated surface, for example *S. oralis* and *S. gordonii* bind to the salivary protein receptors such as mucoglycoprotein (MG2) (Murray *et al.*, 1992). Costerton *et al*., (1999) reported that there are some wild bacterial species which rely on the fimbriae on the cells for adhesion.

During the second phase the stereo-chemical activities are initiated between the adhesins (bacterial cell surface) and the receptors (acquired pellicle) leading to irreversible adsorption.

2.3.3.3. Multiplication and co-aggregation

Co-aggregation encompasses a range of interactions happening between the residents microbial species within the biofilm, it is a cell to cell recognition of inherently different partner cell-types (Rickard *et al.*, 2003). A study has been reported by Filoche *et al.* (2004) showing that during the early stages multi-species (planktonic cells) aggregated to expedite biofilm accretion. Another study on the early dental plaque also demonstrated the co-aggregation *S. oralis* with *S. gorndonii* (interspecies) and interactions between *S. sanguinis* SK163 and *S. sanguinis* SK1 (intraspecies), (Palmer *et al.*, 2003). Some species among the early plaque colonizers co-aggregate through the

co-aggregation bridges, in which two species that are not co-aggregation partners come together with the aid of other bacteria as a "bridge". Kolebbrander *et al*., (2002) have reported this type of co-aggregation, happened among *P. loeschii-S. oralis* and *P. loeschii-A. israelli*, here, *P. loeschii* is a bridge. Consequently, other genera also co-aggregate with the early colonizers like the presence of *A. viscosus* in subgingival plaque enhanced the ability of *P. gingivalis* to colonize these areas (Schonfeld, 1992). The aggregation between the species performs a vital role in the formation and spread of the anaerobic biofilm.

2.3.3.4. Detachment from surfaces

Within the established biofilm, the bacteriological component can still disengage themselves from the surface or even within the biofilm into the planktonic phase (commonly saliva) before setting down to colonize new situates and sites. According to Hunt *et al.*, (2004), detachment or disengagement refers to the discharge of microbial cells as well as their respective matrix (polymers) from the biofilm to the bulk fluid bathing a film. There are various factors which have been regarded as imperative in this biofilm detachment, like enzymes responsible for matrix degradation (Kaplan *et al.*, 2003), multivalent cross-linking cations (Caccavo *et al.*,1996), nutrients levels and bacterial growth status (Jackson *et al*., 2002), shear stress in the fluid (Stoodley *et al*., 2001), role of nutrient starvation (Hunt *et al.*, 2004), and the stimulation of a lytic bacteriophage (Webb *et al*., 2003). Stoodley *et al*. (2001) have reported that in the biofilm of mixed-species detachment of microbial cells among cell clusters occurred in

a steady manner, which was further supported by Kaplan *et al.* (2003) who displayed that process of detachment of biofilm is distinctive involving the formation of non-aggregated cells within the biofilm that are meant to be released from the colony (biofilm). An endogenous enzymatic activity was observed in mono layer biofilm of *S. mutans*, in the presence of minimal shear force, biofilm showed the ability to detach itself from the surface (Lee *et al.*, 1996). This indicates that during biofilm formation, the composition as well as the metabolism of the biofilm can be influenced by the interaction between detachment and growth forces (Liu *et al.*, 2003).

2.3.4. **Development of simulated oral biofilm and its advantages**

The inquisitiveness is in the nature of human being, to see what happens in the oral cavity (normal to diseased like caries or gum diseases) and need to comprehend the intricacy and diversity of oral environ became the reason to develop a simulated mouth replica (Wade, 2013). An artificial mouth system should offer the advantage to develop the biofilm in the artificial situation (mimicking oral cavity) simultaneously allows the researcher to observe and monitor (Tang *et al.*, 2003).To investigate the food processing in mouth (Morell *et al.*, 2014) effect of any chemical agent on oral biofilm (Giacaman *et al.*, 2013) or to observe the change in the normal ecology of oral cavity all have become easier due to the development of artificial mouth models. The developed biofilm can be viewed using latest microscopy tools or by evaluating the facts and figures obtained through laboratory trials (Montelongo-Jauregui *et al.*, 2016). A wide range of mouth replicas has been developed and still being developed by the investigators from various

researches disciplines like pharmacy dentistry and nutrition (Morell *et al*., 2014). Earlier in 1976 a model reported by Coulter and Russell (1976) consisted of glass incubation chamber where extracted teeth and glass surface were used as substratum for biofilm formation. Similarly, another mouth model called "Column System" has been described by Sudo (1977). Later in 1986, De jong and Van Der Hoeven reported a model consisted of culture vessel linked to a medium pump through this they developed the biofilm of *Streptococcus sanguis* biotypes 1 and 2. To study the long term growth of biofilm, a model called "A Multi-Plaque Artificial Mouth" contained five stations for the growth of plaque was reported by Sissons (1991). Many other artificial mouth models have been used to observe the bacterial growth on different samples type (glass surfaces, glass beads, glass slides, hydroxyapatite discs, acrylic discs, enamel discs, enamel slides) (Touger-Decker and Van Loveren, 2003).

NAM (Nordini`s artificial mouth) model was used to develop simulated oral biofilm. The model was developed in 2008 by Rahim and co-workers (Rahim *et al.*, 2008). This model consists of 1) A glass capillary tube which represents the mouth, 2) Glass beads which mimic teeth, 3) Saliva reservoir to supply the saliva into the system, 4) Bacterial reservoir to supply the bacteria into the system, 5) Peristaltic pump to control the flow rate of saliva/bacterial inoculum into the system at 0.3ml/min, 6) Water bath to maintain the temperature of the artificial mouth (glass capillary tube) at 37˚C. The NAM model is a bench scale artificial mouth system, appropriate for the current study as it encountered all the parameters for example maintained temperature, flow rate and the sterility of the

whole set-up in the system which ensured the accuracy and reproducibility of results. This has been validated in various studies like determining the effect of plant extracts (Nordin *et al.*, 2013; Wan *et al.*, 2013) and oral rinses on the adhering and retaining capacities of oral bacteria (single- and mixed-species) to the experimental pellicle (Rahim *et al.*, 2014).

2.3.5. Mechanical and chemical plaque control

Oral health maintenance can only be attained by taking an effective oral hygiene measures on regular basis. A toothbrush is the most used device or tool to eradicate the dental plaque, although its proper use is not trivial and requires quite some handiness. When performed with an adequate skill and duration of time, manual brushing is highly effective. However, for most reasons neither of these criteria is fulfilled like in case of pits and fissures, interproximal spaces, around orthodontic appliances for that dental floss, toothpicks, mini brushes, interdental brushes are used to compensate an improper brushing. Powered toothbrushes with a rotating, oscillating or sonic action are also available in the market and able to efficiently remove plaque and reduce gingivitis (Biesbrock *et al.*, 2008; Ho and Niederman, 1996; Moritis *et al.*, 2002; Tritten and Armitage, 1996).

Chemotherapeutic agents have been formulated in order to contribute in the control of gingivitis and plaque and accepted only according to the established guidelines by the American Dental Association.

Toothpastes or dentifrices encourage good oral health in many ways. The ingredients in toothpaste commonly include fluorides which have an ability to inhibit enamel demineralization and promote remineralization (Almeida Ayres *et al.*, 2015; Li and Tanner, 2015; ten Cate, 2013) abrasives and detergents to enhance plaque removal and antimicrobials to kill remaining plaque organisms on the tooth surface. Nowadays, toothpastes also carry cosmetic component, such as to whiten the teeth as well.

Oral rinses are also available as cosmetic and therapeutic products and are used to remove bad breath, to assist in prevention of tooth decay, to eliminate plaque, to reduce gingivitis, or to produce a combination of these effects. For these chemical agents low toxicity and substantivity are vital as their efficiency should last after brushing/rinsing (DePaola and Spolarich, 2007).

The oral rinses which are recommended by dental professionals to the patients are those which are proven to be effective and safe formulations. Among these formulations few are being used very commonly. These rinses contain respective active ingredient like chlorhexidine gluconate (CHX), cetylpyridinium chloride (CPC) and essential oils (EO). Chlorhexidine gluconate, known to be a salt of chlorhexidine and gluconic acid, is very famous for its antimicrobial activity (Klompas *et al.*, 2014). Another rinse which is based on cetylpyridinium chloride (CPC) as an active ingredient is being used to treat plaque bacteria. The CPC is a cationic quaternary ammonium compound which is

prescribed for treatment of throat and nasal infections as an antiseptic formulation. It has been claimed to be able to kills bacteria (Elias-Boneta *et al*., 2015; Pekovic, 2016).

Essential oils based oral rinse is famous for its antiplaque effectiveness (DePaola and Spolarich, 2007; Ghapanchi *et al.*, 2015; Vlachojannis *et al.*, 2015). These active ingredients with various concentrations as well as in combinations are commercially available. These oral rinses have the potential to kill bacteria as claimed by the manufacturers (Colgate, 2016, Oradex, 2016**).** Studies have reported the bactericidal effect of these oral rinses (Al-Bayaty *et al.*, 2011). These rinses have been either separately used in different researches (Klompas *et al*., 2014); some studies reported their anti-bacterial potential as a combination formulation for example, cetylpyridinium chloride with essential oils in a fixed combination has been revised by the Food and Drug Administration (FDA) advisory committee which put it in the Category I recommendation and confirmed its safety and effectiveness to control supra-gingival plaque and gingivitis (Gurenlian, 2007). Like (CHX+CPC) (Slot *et al.*, 2015) / (CHX+EO) / (EO+CPC) or each one is combined with any other chemical agent to formulate new rinse to get more potent results (Jhingta *et al*., 2013; Kang *et al.*, 2015). The quantity of the antimicrobial/active agents which are used in the formulation of oral rinses is usually stated without declaring the right time to rinse (before or after meals). In order to manage the oral microbial load rather than killing them completely, there is a need to know the minimum effective concentration of these rinses towards early plaque colonizers settlers. Thus, daily tooth brushing, interdental flossing and use of an

effective antimicrobial oral rinse have been accepted as strategies to decrease the bacterial load in the plaque to avoid periodontal diseases (Gurenlian, 2007). Strategies to manage the dental plaque to promote oral and general health are being made. Although dental biofilm can not be avoided but its pathogenicity can be reduced by taking right measures which are proven to be effective.

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipment

- Autoclave, Hirayama (HVE-50) (Japan)
- Autoclave, Tomy High Pressure Steam Sterilizer (SX-500) (USA)
- Dryer, WTC Binder (7200 Tuttlingen) (Germany)
- Balance, Mettler Toledo (JP105DUG) (Switzerland)
- Laminar Flow, Thermo Scientific, (USA)
- Freezer (-80˚C) ,Telstar Igloo (U830) (Spain)
- High Speed Refrigerated Centrifuge (SUPREMA21),Tomy (Japan)
- Control Shaking Incubator ,IKA Works (KS 4000 I Control) (Malaysia)
- UV-Spectrophotometer, Shimadzu Corporation (UV-1700) (Japan)
- Elisa Plate Reader, BioTek Instruments (218740) (USA)
- High Speed Refrigerated Microcentrifuge, Tomy (MX-305) (Japan)
- Hotplate (Magnetic stirrer), Corning (PC-420D) (USA)
- Hotplate (Magnetic stirrer), Thermo-Line (TL-A550) (Malaysia)
- Hotplate (Magnetic stirrer), Cimarec (SP131010-33) (USA)
- Thermometer, Chemo-Lab(Malaysia)
- Pump Drive Master-flex, Cole Parmer (USA)
- Ultrasonicator, Selecta (Segar Alantan Sains) (Malaysia)
- Vortex Mixer, Stuart (USA)
- Ductless Fume Hood, Labcaire (FE 4850) (USA)
- Colony Counter, Rocker (Galaxy230) (Taiwan)
- Scanning Electron Microscope (SEM) (Quanta 250 FEG, Japan)

3.1.2. Chemicals

All chemicals and reagents used were of analytical grade unless otherwise stated. They were obtained from the following sources.

- Decon 90, Decon Laboratories Ltd (UK)
- Minisart, Syringe Filter (0.2 µm), Sartorius Stedim Biotech (Germany)
- Terumo Syringe (10 cc/ml), Terumo Corporation (Philippines)
- 1,4- Dithio-DL-threitol powder, Fluka, Biochemika (Switzerland)
- Brain Heart Infusion Agar, Brain Heart Infusion Broth and Phosphate buffered saline (PBS) tablets, Oxoid Ltd., Hampshire (England)
- Centrifuge tubes (50ml), Microcentrifuge tubes (1.5ml) and Magnetic stirrers, Chemolab (Malaysia)
- Pipette tips (100µl, 1000µl, 5000µl), Eppendorf AG (Germany)
- Pasteur Capillary Pipettes, Witeg (Germany)
- Masterflex Silicone tubing (L/S 16), Cole-Parmer Instrument Co (USA)
- *Streptococcus sanguinis* (BAA-1455), *Streptococcus mitis* (ATCC 49456) and *Actinomyces viscosus (*ATCC 43146), American Type Culture Collection (ATCC), (USA)
- EO (essential oils) containing mouth wash (Johnson & Johnson, Malaysia) This mouth wash contained four essential oils comprising of eucalyptol, thymol, methyl salicylate and menthol.
- CPC (cetylpyridinium chloride) containing mouth wash (Colgate-Palmolive, Malaysia)
- CHX (chlorhexidine gluconate) containing mouth wash (Fortune Laboratories, Malaysia)

- Glass beads (3mm diameter), Hydrochloric acid (37%), Glutaraldehyde (4%) and Ethanol (99.99%), (Merck, Germany)
- Acetone (99.98%), (Fisher Scientific, UK)
- Osmium oxide and Sodium cacodylate powder, (Agar scientific, UK)
- Glycerol, (Sigma-Aldrich, USA)

3.2. Methods

3.2.1. Research Outline

The research was carried out according to the flow chart as shown in Figure 3.1.

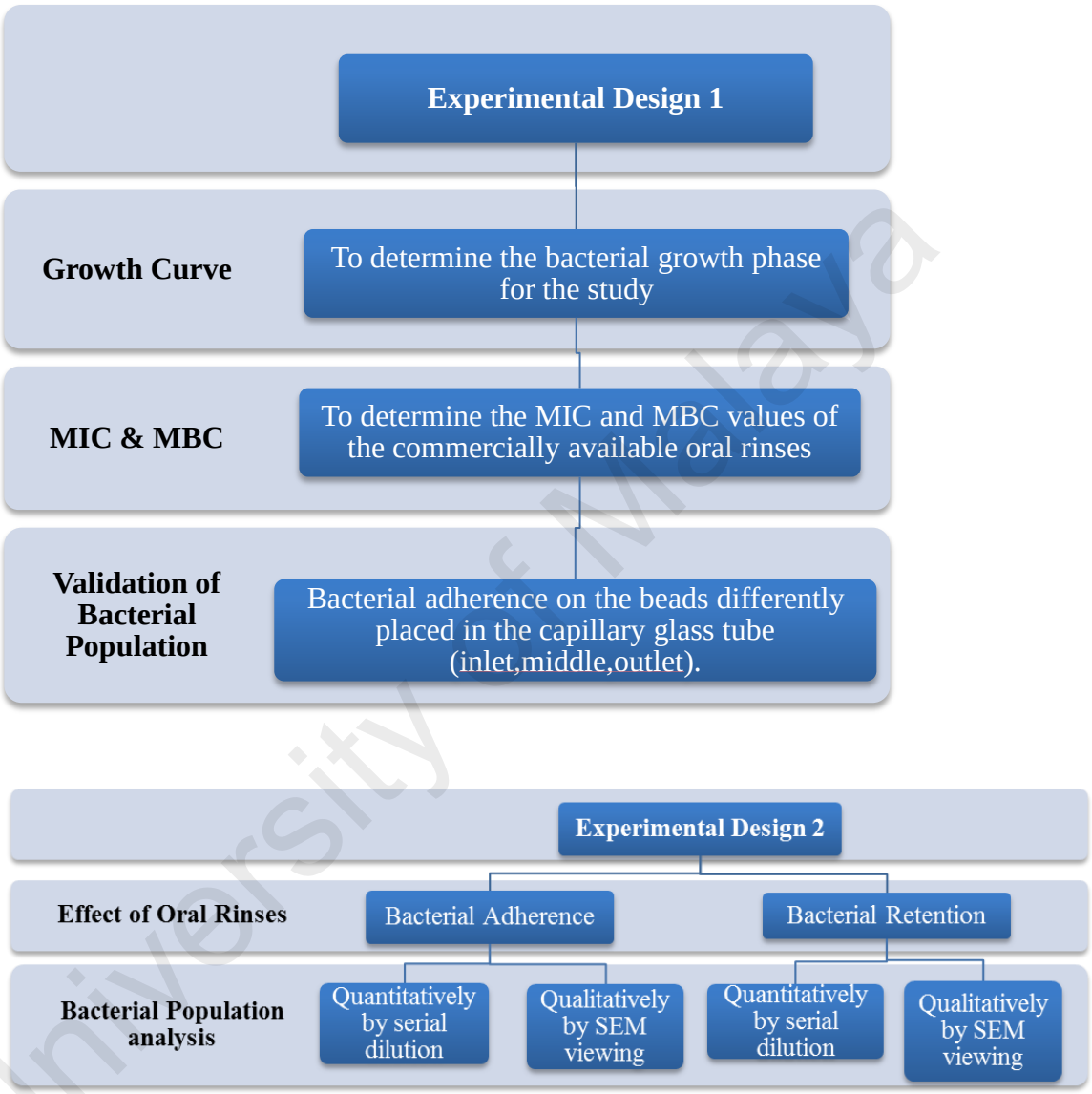


Figure 0.1: Schematic flow chart showing the design of the study

3.2.2. Washing procedure for washable apparatus

Washable apparatus like glass wares were soaked for overnight in a diluted solution of Decon 90 (10% v/v). The glass wares were then sonicated in an ultra-sonicator for 5-10 minutes which was followed by scrubbing with suitable cleaning brushes. The apparatus was then rinsed properly with tap water first and then, re-rinsed with the deionized distilled water. All of the apparatus was air- or oven-dried prior to sterilization.

3.2.3. Sterilization procedure

Heat sterilization method was used. Moist heat sterilization was carried out by autoclaving the whole apparatus at 121℃ and 15 p.s.i for 20 mins. All microbiological media (BHI Agar/ Broth), solutions, glass-wares, glass beads, tubing, pipette tips and other items that can bear the high temperature and pressure were sterilized using this method. Direct flaming was done to sterile the inoculating loops, hockey sticks and glass spreaders. All of the procedures that involve aseptic techniques employed, according to the method described by Benson (Benson, 2001).

3.2.4. Culture Media preparation

3.2.4.1. Preparation of Brain Heart Infusion Broth (BHI Broth)

Brain heart infusion (BHI) broth was used to culture the bacteria (*Streptococcus mitis, Streptococcus sanguinis, Actinomyces viscosus*) for this study. It was prepared according to the manufacturer`s instruction (Oxoid Ltd, Hampshire, England). Thirty seven grams of BHI broth powder was weighed and dissolved in 1 liter of distilled water and sterilized in an autoclave at 121℃ and 15 p.s.i for 20 mins. Sterilized BHI broth was then kept in the fridge at 4℃ until further use. Prior to use it was normalized to room temperature (25˚C).

3.2.4.2. Preparation of Brain Heart Infusion Agar (BHI Agar)

The BHI agar plates were used to grow bacteria and for the enumeration of the colony forming units (CFU). The plates were prepared according to the manufacturer`s instruction (Oxoid Ltd, Hampshire, England). Forty seven grams of BHI agar powder were weighed, dissolved in 1 liter of distilled water and then sterilized in an autoclave at 121 ℃ and 15 p.s.i for 20 mins. The sterilized mixture was kept in a laminar flow cabinet. The mixture in hot liquid form was poured into the sterilized petri dishes (30ml/dish) and left in the cabinet to solidify the agar. The agar plates were then stored in the fridge at 4℃ until further use. Prior to use the plates were normalized to room temperature (25˚C).

3.2.5. Preparation of the stock culture of bacteria

The respective oral bacterial species (*Streptococcus sanguinis* BAA-1455, *Streptococcus mitis* ATCC 49456 and *Actinomyces viscosus* ATCC 43146*)* were obtained from the Balai Ungku Aziz Laboratory, Faculty Dentistry, University of Malaya. The stock culture of the respective bacteria was prepared according to the following procedure. The sterilized BHI broth (1:100 v/v) was first inoculated with the bacteria and then placed in an incubator at 37℃ for 18-24 hrs (*Streptococcus mitis and Streptococcus sanguinis*) and 24-36 hrs (*Actinomyces viscosus*). Three hundred microliters (µl) of the growth suspension were mixed with two hundred microliters (µl) of glycerol were used as the stock culture and stored in 1ml eppendorf tubes at -80℃ until further use (Koo *et al.*, 2006; Percival *et al.*, 2006; Rahim *et al*., 2014).

3.2.6. Preparation of Phosphate Buffered Saline (PBS) Solution

PBS was prepared according to the manufacturer instructions (Oxoid Ltd, Hampshire, England). One Phosphate-Buffered Saline (PBS) tablet was dissolved in 100 ml of distilled water; its pH was then adjusted to 7.4 by using 0.1M hydrochloric acid (HCl) and then sterilized in an autoclave at 121 ℃ and 15 p.s.i for 10 mins. The sterilized PBS solution was stored in the fridge at 4℃ until further use. The PBS solution was used as a medium for bacterial serial dilution for bacterial population determination.

3.2.7. Preparation of sterile saliva

Ethical approval for saliva collection was obtained from the Ethics Committee, Faculty of Dentistry, University Malaya [DF OB1507/0071(P)]. Sterile saliva was prepared according to the method described by De Jong and Van Der Hoeven, (1987; 1986). About 40 ml of stimulated whole saliva (SWS) was collected for single experiment. Single volunteer was asked to chew a sugar free gum to stimulate saliva production. The SWS was collected in an ice-chilled test tube. The saliva was collected from a single healthy female volunteer (27 years old) to minimize any variation that may arise between individuals. The aggregation of proteins in SWS samples was minimized by adding 1,4-Dithio-D,L-Threitol (DTT) to a concentration of 2.5 mM. Following the addition of DTT, the saliva was stirred slowly for 10mins before it was centrifuged at 864 g for 30 mins at 4℃ (Rahim *et al*., 2008; Rahim *et al*., 2014). The supernatant obtained was then filter-sterilized using a disposable syringe filter of 0.2µm pore size (Minisart, Syringe filters,) into sterile test tube. The sterile SWS was kept at -20℃ until further use. Prior to use, the saliva was first thawed and then centrifuged (to remove any precipitate). The sterile SWS was used to coat the glass beads to mimic the layer of acquired pellicle on the tooth surface which was referred as the experimental pellicle.

3.2.8. Preparation of the bacterial suspension

The stock culture of the respective oral bacteria (*Streptococcus sanguinis*, *Streptococcus mitis* and *Actinomyces viscosus)* which was kept at -80 ℃ was thawed at room temperature, inoculated into sterilized BHI broth (1:100 v/v) and then incubated at 37 ℃ for a duration in accordance with the bacterial species (18-24 hrs for *Streptococcus sanguinis* and *Streptococcus mitis* and 24-36 hrs for *Actinomyces viscosus)*. The bacterial suspension was adjusted spectrophotometrically at 550nm to 0.144 absorbance during their growth phase which is equivalent to 10^6 cells/ml (Rahim *et al*., 2014; Razak and Rahim, 2003). This is important to standardize the number of cells in the suspension to be used in the study.

3.2.9. Determination of the growth curve of bacteria

Two milliliters (ml) of the bacterial stock culture *(Actinomyces viscosus*) were inoculated into 198 ml of sterile BHI broth in a sterile bottle. This bottle (containing the bacterial suspension) was then placed in a shaking incubator (100 rpm) at 37 °C and the absorbance or turbidity of the bacterial suspension was measured spectrophotometrically at one-hour intervals for 24 hours at 550nm. Before measuring, the spectrophotometer was first standardized to zero at 550nm using BHI broth. The dynamics of the bacterial growth were then studied by plotting the cell growth (absorbance) versus the incubation time. The experiment was repeated using *S. mitis* and *S. sanguinis*. For *A. viscosus* suspension, the one hour intervals measurement of absorbance was extended for another 24 hours. The different duration for the absorbance measurement of the different bacterial species was to allow for the growth period to attain stationary phase. Each individual experiment was carried out in triplicate.

3.2.10. Determination of the Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentration (MBC) of the oral rinses

The MICs of the commercially available oral rinses (based on their active ingredients) against selected oral bacteria were determined according to the broth microdilution method (Moon *et al.*, 2011) and were carried out in triplicate. Prior to the determination, the absorbance of the respective bacterial suspensions was adjusted to 0.144 at 550 nm as described in 3.2.8. In a sterile 96-well micro-titer plates (Rahim *et al.*, 2014) as shown in Figure 3.2 a series of two-folds dilutions were prepared with sterile BHI broth for the respective oral rinses: Chlorhexidine gluconate (CHX) (Fortune Laboratories, Malaysia), Cetylpyridinium chloride (CPC) (Colgate-Palmolive, Malaysia) and essential oils (EO) (Johnson & Johnson, Malaysia). The first two oral rinses have one active ingredient whereas the EO contains four main ingredients that include Eucalyptol, Thymol, Methylsalicylate and Menthol. The 2-folds serially diluted oral rinse was pipetted into well # 1 to well # 8 over a dilution range giving the approximate final concentrations of CHX (1200-9.37 µg/ml), CPC (750-5.86 µg/ml) and for EO (mixture of the Eucalyptol (920-7.19µg/ml); Thymol (640-5.0µg/ml); Methylsalicylate (600-4.69µg/ml) and Menthol (420-3.28µg/ml)) (For conversion from % to µg/ml please refer to Appendix B). The final concentration of the individual ingredient of the respective oral rinse in µg/ml was calculated according to the dilution of the original concentration stated on the bottle.

One hundred µl of inoculum was added to each well containing the serially diluted oral rinse and written as SMB (which is the serial dilution of oral rinse and bacteria) in Figure 0.2. A parallel series of 2-fold dilution for oral rinse was also prepared with sterile BHI broth written as SM which is the serial dilution oral rinse and BHI broth

used as, blank control (Figure 3.2). After incubation for 24hrs at 37°C, the absorbance determined spectrophotometrically at 550nm using a microtitre plate reader.

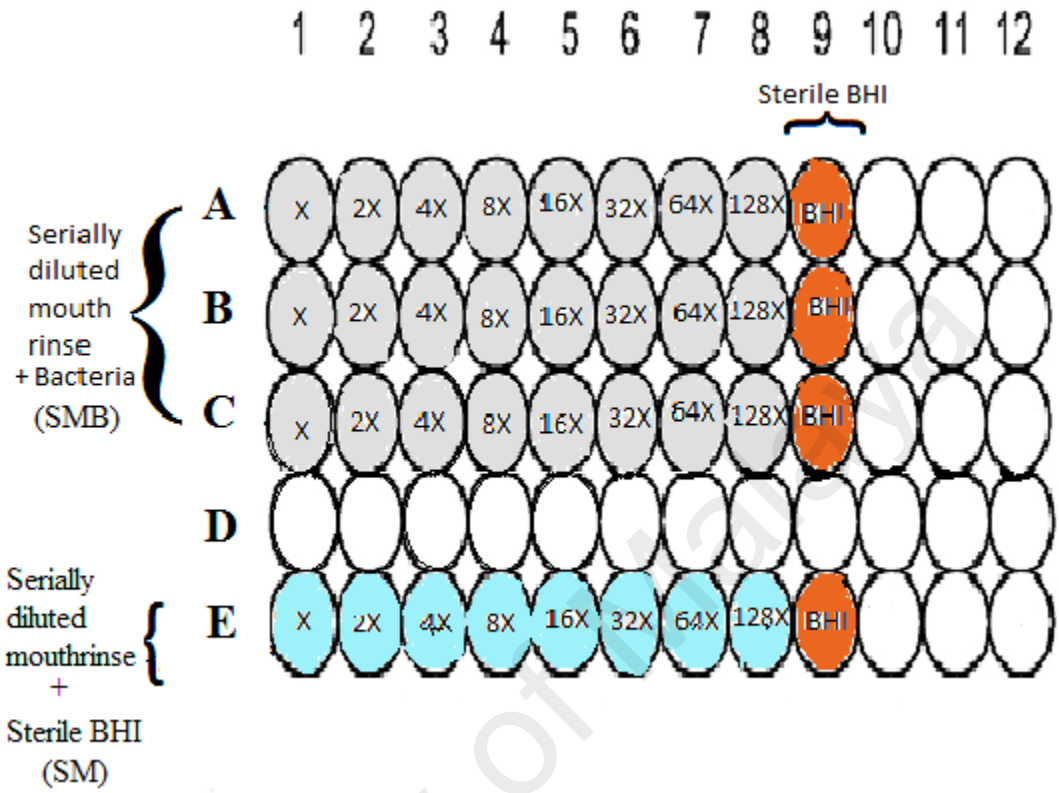


Figure 0.2: Schematic diagram showing the microtitre plate used in the study.

Microtitre plate showing the first three rows (grey circles) from well #1 to well #8 containing bacteria with the respective 2-fold serial dilutions of an oral rinse in 1:1 ratio (v/v). The row E (sky blue circles) from well #1 to well #8 contained the oral rinse in 2-fold serial dilution and sterile BHI broth (without bacteria) in 1:1 ratio (v/v), referred to as blank control. Wells #9 in rows A-C and E (brown circles) contained sterile BHI broth to ensure no contamination during serial dilution.

The final reading (absorbance) of the 2-folds serially diluted oral rinse was calculated using the following formula:

Absorbance of the bacterial population in the respective dilutions (wells) = SMB – SM

where SMB (serially diluted oral rinse + bacteria) in respective rows A to C and SM (serially diluted oral rinse + sterile BHI) in row E.

For the Minimum Bactericidal Concentration (MBC) determination, aliquot (50µl) from the well containing no visible growth was diluted 100-fold with sterile distilled water and sub-cultured into BHI agar. The plates were incubated at 37°C for 24hrs. The lowest concentration of the oral rinse that did not permit any visible growth on the appropriate agar plate after the incubation period was referred as the MBC.

3.2.11. Validation of the bacterial population of biofilm on the beads located at the inlet, middle and outlet of the capillary glass tube

The development of biofilm was carried out using the Nordini`s Artificial Mouth (NAM) model (Rahim *et al*., 2008). The NAM model allows the development of oral biofilm in a dynamic state. It consists of a glass capillary tube (mimicking mouth) containing seven glass beads (simulating teeth in the mouth used as the substratum for biofilm formation), the openings at both ends of the capillary tube were close-fitted with rubber tubings, with one end serving the inlet and the other end serving the outlet of the system. The glass capillary tube was immersed in a water bath, maintained at 37°C (mimicking mouth temperature). A peristaltic pump linked the rubber tubings and was used for to control flow rate.

Figure 0.3: A) Four sets of Nordini's Artificial Mouth Model to develop four individual oral biofilms, B) Glass tube containing seven glass beads mimicking oral cavity and teeth respectively.

Sterile stimulated saliva was pumped into the glass capillary tube (artificial mouth) at 0.3 ml/min forming experimental pellicle on the glass beads. Following that, sterile deionized distilled water was pumped to remove the excess saliva (Rahim *et al*., 2008; Rahim *et al*., 2014). Bacterial suspension of *A. viscosus* which was first standardized to

an absorbance of 0.144 at 550 nm (Hasnor *et al.*, 2008) was then allowed to pass through the capillary glass tube and the experiment was run for 24 hours at 37°C to form a 24 hour simulated oral biofilm. The glass beads with the biofilm were then taken out with care and their location in the glass tube was noted. The glass beads nearest to the entrance of the glass tube were referred as Inlet beads, the ones at the middle of the glass tube were referred as the Middle beads and those nearest to the outlet/exit of glass tube were referred as the Outlet beads (Figure 0.4). One bead from the different location was then selected for use in the bacterial population determination as discussed below (Section 3.2.11.1).

The procedure was repeated with *S. sanguinis* and *S. mitis.* The individual experiment was carried out in triplicate. The data on the bacterial population for the respective biofilm was referred as 100% bacterial adherence, representing the negative control and would be used later for comparison with the bacterial population in biofilm where the experimental pellicle was pretreated with oral rinse.

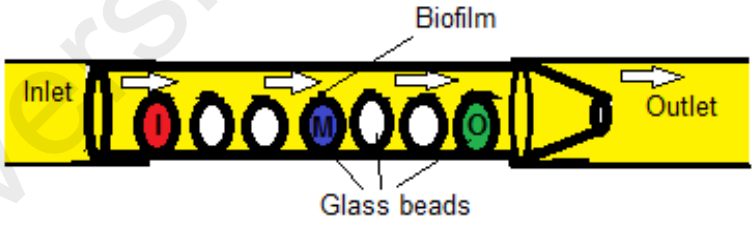


Figure 0.4: A schematic diagram showing the flow of solution (oral rinse/water) into the glass capillary tube (mimicking the oral cavity) and the location of the glass beads (mimicking the teeth). The Red bead (I), located near the inlet; Blue bead (M), located in the middle and Green bead (O), near the outlet of the glass capillary tube.

3.2.11.1. Determination of the bacterial population in the 24hr oral biofilm on the glass bead

Each of the selected beads (inlet, middle and outlet beads respectively) was immersed separately in a micro-centrifuge tube containing 1000 µl phosphate-buffered solution (PBS). This was followed by sonication in an ultrasonicator for 10 seconds and vortexed for 1min in order to detach the adhered bacteria into the PBS solution. The PBS solution was then diluted serially up to T^6 where T^1 (10^1) was referred to the first tube and T^6 (10^6) the sixth tube of the serial dilutions (Figure 3.5). One hundred µl of the respective serially diluted bacterial suspensions from the T^1 to T^6 tubes were pipetted out and streaked onto separate BHI agar plates as shown in Figure 3.5. The procedure was repeated thrice for each dilution (Figure 0.5)

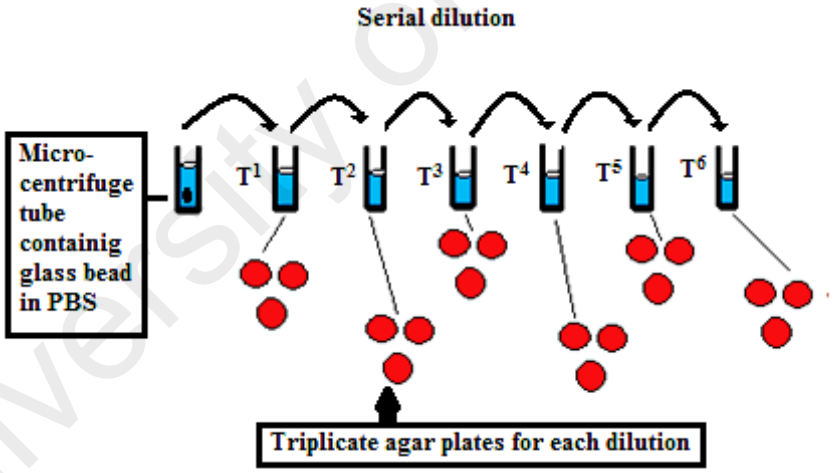


Figure 0.5: Serial dilution and agar plates' inoculation. Red circles represent agar plates (in triplicate) used for each dilution of the individual bead.

Culture plates were incubated for 24 hrs at 37 °C followed by Colony Forming Unit (CFU) determination. Culture plates of dilution factor which had the CFU number 30-300 were selected and the bacterial population was expressed as Colony forming unit/ml (CFU/ml) (Hasnor *et al.*, 2008). CFU of the bacterial population on every petri dish was determined using a Colony Counter (Rocker, Colony Counter Galaxy 230, Taiwan). Each individual experiment was carried out in triplicate. The CFU count of the bacterial population on the glass bead located at different positions (inlet, middle and outlet) was determined and analyzed for the percentage difference in the average bacterial population between the different positions.

3.2.12. Determination of the effect of the commercially available oral rinses towards bacterial adherence in single- and dual-species biofilms

The effect of oral rinses was determined on the adhering bacterial population for single and dual-species biofilms. In this experiment, the saliva coated glass beads were treated with the respective concentration of oral rinses prior to the development of 24-hrs single- and dual – species biofilms as described in the following subsections. Two concentrations of each of the respective oral rinses (CHX and CPC) were used, one at the original concentration (100%) and the other at half of the original concentration (50%) and hence the concentration of CHX being 1200µg/ml and 600µg/ml, respectively and that of CPC being 750µg/ml and 375µg/ml, respectively. For EO the original concentration was used (EO comprising of Eucalyptol (920µg/ml); Thymol (640µg/ml); Methylsalicylate (600µg/ml); Menthol (420µg/ml). The following formula was applied to calculate the adherence potential of bacteria.

% of bacterial adherence = $(A_{OR} / A_W) \times 100$

where A_{OR} is the mean of bacteria (CFU count) treated with respective oral rinse, Aw is the mean of bacteria (CFU count) treated with deionized distilled water (negative control).

3.2.12.1. Bacterial adherence in single-species biofilm

The experiment was carried out using four artificial mouth models (NAM system) running simultaneously, each connected to a peristaltic pump (Figure 0.3). Sterile saliva was pumped separately into the glass capillary tubes of each of the NAM system at the 0.3ml/min for 2mins. This would allow the saliva to coat the glass beads and created the experimental pellicle. This was followed by allowing a)- oral rinse containing CHX

(100%) to flow for 2mins into the capillary glass tube of one of the NAM systems: b) oral rinse containing CPC (100%) in to the capillary tube of another NAM system: c) oral rinse containing EO (100%) into the capillary tube of another NAM system and d) sterile deionized distilled water into the capillary tube of the last NAM system. In that way the experimental pellicle on the glass beads in the respective capillary tubes of the NAM systems were treated with the 3 oral rinses respectively and sterile deionized distilled water (negative control). After that, deionized distilled water was pumped to remove any excess oral rinses. Subsequently bacterial suspension of *A. viscosus* which was standardized first to the absorbance of 0.144 at 550 nm and representing $1x10^6$ bacteria population was pumped through the capillary tubes of the respective NAM systems at the same flow rate (0.3ml/min) for 24 hours at 37°C. After 24 hours, three glass beads (one near the inlet, one at the middle and one near the outlet) were taken out of the capillary tube with care and used for the determination of bacterial population as described in Section 3.2.11.1 The remaining four glass beads were stored in 4% glutaraldehyde for SEM viewing.

The above procedure was repeated with other bacteria which in this case *S. mitis* and *S. sanguinis* respectively. Each of the individual experiment was carried out three times.

The experiment was repeated using a lower concentration of two of the oral rinses (oral rinse containing CHX and oral rinse containing CPC) where the original concentrations was diluted to half (50%). Each individual experimental was carried out in triplicate.

3.2.12.2. Bacterial adherence in dual-species biofilm

Similar procedure as described in Section 3.2.12.1 was carried out but in this experiment two groups of a mixture of two bacterial species (i) a mixture of *A. viscosus* and *S. mitis* and ii) a mixture of *A. viscosus* and *S. sanguinis* respectively) were used to inoculate the treated experimental pellicle. In order to prepare mixture of dual species, initially the suspension for individual bacterial specie was standardized spectrophotometrically to an absorbance of 0.144 at 550 nm during their growth phase which is equivalent to 10^6 cells/ml (Rahim *et al*., 2014; Razak and Rahim, 2003). Later the bacterial suspension for the individual bacteria was mixed in equal quantities (1:1) to prepare a final mixture of dual-species. Each individual experiment was carried out in triplicate. On agar plate the CFU count for the two bacterial mixtures (*A. viscosus* + *S. mitis* and *A. viscosus* and *S. sanguinis*) was performed separately. The two bacterial species in a mixture can be differentiated from one another. *A. viscosus* appear to be in rod shape whereas *S. mitis* are cocci in short chain and *S.sanguinis* are also cocci but in long chain.

3.2.13. Determination of the effect of the commercially available Oral Rinses towards bacterial retention in single- and dual-species biofilms

This experiment was to check the potency of an oral rinse in detaching the bacteria in the biofilm on the glass beads. The respective single- and dual-species biofilm were allowed to develop on the glass beads for 24-hrs before treating them with the oral rinses. For CHX- and CPC-containing oral rinses, two concentrations were used, one at the original concentration and the other diluted to half of the original concentration. (CHX 1200µg/ml and 600µg/ml, CPC 750µg/ml and 375µg/ml). For EO the original concentration was used without dilution (EO comprising of Eucalyptol (920µg/ml); Thymol (640µg/ml); Methylsalicylate (600µg/ml); Menthol (420µg/ml).

The following formula was applied to calculate the retention of bacterial population in the biofilms.

% of bacterial retention = $(R_{OR} / R_W) \times 100$

where R_{OR} is the mean of bacteria (CFU count) treated with the respective oral rinse, Rw is the mean of bacteria (CFU count) treated with the deionized distilled water (negative control).

The experiment for the single and dual-species were described in the subsections below.

3.2.13.1. Bacterial retention in single-species biofilm

Similar procedure as described in Section 3.2.12.1 was carried out with the three respective bacterial species to develop biofilm. The modification carried out was that the oral rinses in this experiment were pumped after development of single specie biofilm rather than pumping before the bacterial inoculation on experimental pellicle.

3.2.13.2. Bacterial retention in dual-species biofilm

Similar procedure as described is Section 3.2.12.2 was carried out for the development of dual-species biofilms (1) *A. viscosus* and *S. mitis* and*;* 2) *A. viscosus* and *S. sanguinis*) with a slight modification. In this experiment the oral rinses were pumped into each of the respective NAM system after biofilm formation rather than treating the experimental pellicle.

3.2.1.4. Preparation of samples for Scanning Electron Microscopy (SEM) Viewing

The preparation of glass beads samples for SEM viewing was carried out according to the method described by Lagace *et al*., (2006) and also by Rahim and Thurairajah (2011). The glass beads with the biofilm on them were fixed in 4% glutaraldehyde in glass vials for 1hour at room temperature. Excess glutaraldehyde was discarded and the glass beads were rinsed once with distilled water. The washed glass beads were then fixed in 1% osmium tetraoxide and left overnight for 14hrs in tightly capped vials at 4°C in the refrigerator. After 14hrs, the vials were taken out from the refrigerator and left for 30mins at room temperature. The 1% osmium tetraoxide was gently pipetted out and the samples were washed with distilled water for 5 mins. The dehydration process was carried out by treating the samples with the ascending percentages of ethanol (10% to 100%). The samples were immersed in the different concentrations of ethanol for 15 mins. The samples were then immersed in 100% ethanol twice to ensure that most of the water in the sample was eliminated. Gradual displacement of ethanol with acetone was carried out (20 mins each) using the following ratios (v/v) Ethanol: Acetone 3:1, 1:1 and 1:3 respectively.

Following that, the samples were immersed in 100% acetone for 20 mins. This step was repeated four times followed by critical point desiccation (CPD). The samples were

then mounted on metal stubs and coated with gold. After gold coating process, the samples were ready for SEM viewing.

3.2.15. Statistical Analysis

The data obtained from validation of bacterial population on the selected beads and the data obtained from the treated and control group (for bacterial adherence and retention objectives, respectively) were analyzed and compared by One-way ANOVA and Bonferroni test in post hoc using IBM SPSS statistics software version 22 (Chicago, U.S.A).

CHAPTER 4: RESULTS

4.1. Growth Curve of Bacterial Species

Figure 0.1 shows the graphic representation of the growth curve for three bacterial species with absorbance value versus time. For *A. viscosus* before the growth phase it was the lag phase which remained till the end of the 13th hour. It was followed by the exponential growth phase (log phase) which started at about 14th hour after incubation and remained until 24th hour of incubation. After the log phase it entered the stationary phase showing a consistent value till the 38th hour of incubation. Whereas for *S. mitis* (Figure 4.1) it was shown that the exponential growth/log phase of this bacterium is between 7th hour to about 15th hours of incubation. The lag phase which is the earliest phase of growth was before the 6th hour of incubation. Bacteria continued to multiply till the 15th hour of incubation after that the growth became static (Stationary phase) which remained till the 24th hour of incubation. For *S. sanguinis* (Figure 4.1) the log/exponential growth phase started at the 6th hour and continued until the 14th hour of incubation. The lag phase which was before the log phase continued until the 5thhour of incubation. The stationary phase began at the 15th hour and remained static up to the 24th hour of incubation.

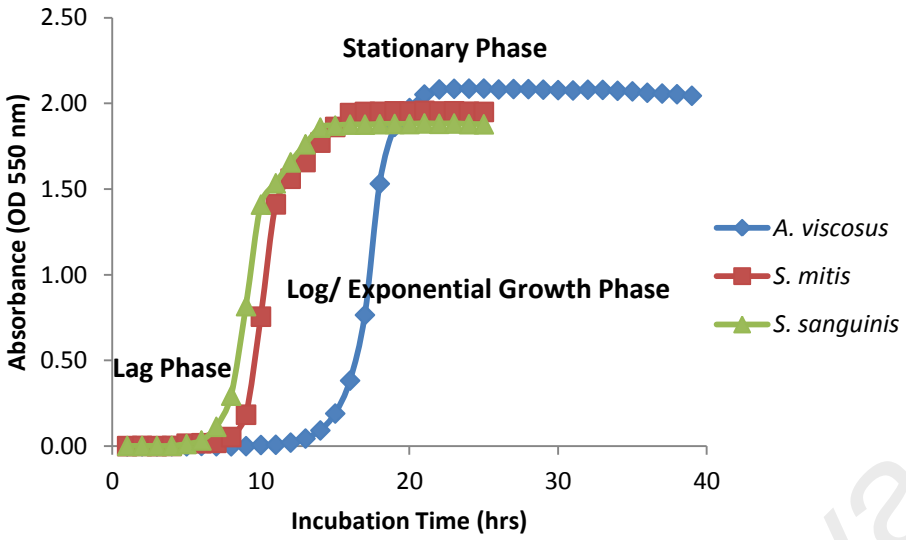


Figure 0.1: Growth curve of *A. viscosus* ATCC 43146, *S. mitis* ATCC 49456 and *S.sanguinis* BAA-1455.

The growth of *A. viscosus*, *S. mitis* and *S. sanguinis* was measured in absorbance at 550nm. The experiments were carried out in triplicate and the growth curve was plotted based on the average of (3 absorbance values) versus time.

4.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Table 0.1 shows the MIC and MBC values of three commercially available oral rinses towards three bacterial species for both single and dual species bacterial suspensions used in this study. The oral rinse containing EO which has eucalyptol, thymol, methyl salicylate and menthol as its active ingredients exhibited strong inhibitory effect towards the three single species of bacteria and in the mixed bacterial suspension of dual species. The MIC values of its individual active ingredient can be written in the following descending order: Eucalyptol> Thymol> Methyl salicylate> Menthol was based on the calculated values from the original proportions as stated on the label of the bottle. The MBC value of the EO-based oral rinse is similar to its MIC value for all three bacteria and the mixed bacterial suspensions (Table 0.1).

The MIC value of the CHX based oral rinse (600µg/ml) and CPC-based oral rinse (375µg/ml) was found to be half of its original concentration (two fold dilution). The MBC value for both the oral rinses corresponded to their original concentrations which was twice their MIC values (Table 0.1).

Table 0.1: MIC and MBC of Commercially Available Oral Rinses towards the Single- and Dual –Species Bacterial Suspensions

Oral rinses	*Single Species bacterial suspension*			*Dual Species bacterial suspension*	
	***Actinomyces viscosus* (*A.v*)**	***S. mitis***	***S. sanguinis***	***A.v* + *S. mitis***	***A.v* + *S. sanguinis***
	MIC (MBC)				
EO	100% (100%)	100% (100%)	100% (100%)	100% (100%)	100% (100%)
CPC	50% (100%)	50% (100%)	50% (100%)	50% (100%)	50% (100%)
CHX	50% (100%)	50% (100%)	50% (100%)	50% (100%)	50% (100%)

For EO (essential oil) the active ingredients as stated on the bottle are: Euc(Eucalyptal), Thy(Thymol), M.Salic (Methyl Salicylate) and Menthol. For CPC the active ingredient is cetylpyridinium chloride, For CHX the active ingredient is chlorhexidine gluconate. For the single bacterial specie suspension, the bacterial population was standardized to an absorbance of 0.144 at 550nm. For the dual species bacterial suspension (the bacterial population of each specie was first standardized to an absorbance of 0.144 at 550nm and then mixed in 1:1 ratio).

The MIC and MBC were expressed in %; 100 % original commercially available concentration (100%) and 50% half (two fold dilution) of the original concentration. The concentration of the ingredients in the respective oral rinses as stated on the bottle was in % and the values were then converted to µg/ml as stated in the Appendix B. All of the individual experiment was carried out in triplicate.

4.3. Validation of the Bacterial Population of the Biofilms Formed on the Saliva-Coated Glass Beads in the Capillary Glass Tube of the Nordini`s Artificial Mouth (NAM) Model

The bacterial population on the saliva-coated glass beads located at the inlet, middle and outlet of the capillary glass tube in the (NAM) was compared. The comparison was made between the respective bacterial populations of the single- and between the respective bacterial populations of the dual-species biofilms. Three differently positioned beads were selected to get an average population of bacteria.

4.3.1. Bacterial Population in single-species biofilm

Figure 0.2 shows the population of the early colonizers of dental plaque (*A.viscosus*, *S. mitis* and *S. sanguinis*) in single-species biofilms developed on the glass beads at the inlet, middle and outlet along the capillary glass tube in the NAM model over a period of 24 hrs.

It was observed that the maximum adherence of *A. viscosus* to the inlet bead was $279.33 \pm 3.39 \times 10^2$ CFU/ml followed by middle bead $273.44 \pm 2.13 \times 10^2$ CFU/ml and outlet bead $265.89 \pm 2.09 \times 10^2$ CFU/ml. A similar sequence in adherence was also observed for *S. mitis* and *S. sanguinis* (Figure 0.2). The results obtained were statistically significant (One-way ANOVA, Bonferroni post-hoc test: $p<0.05$).

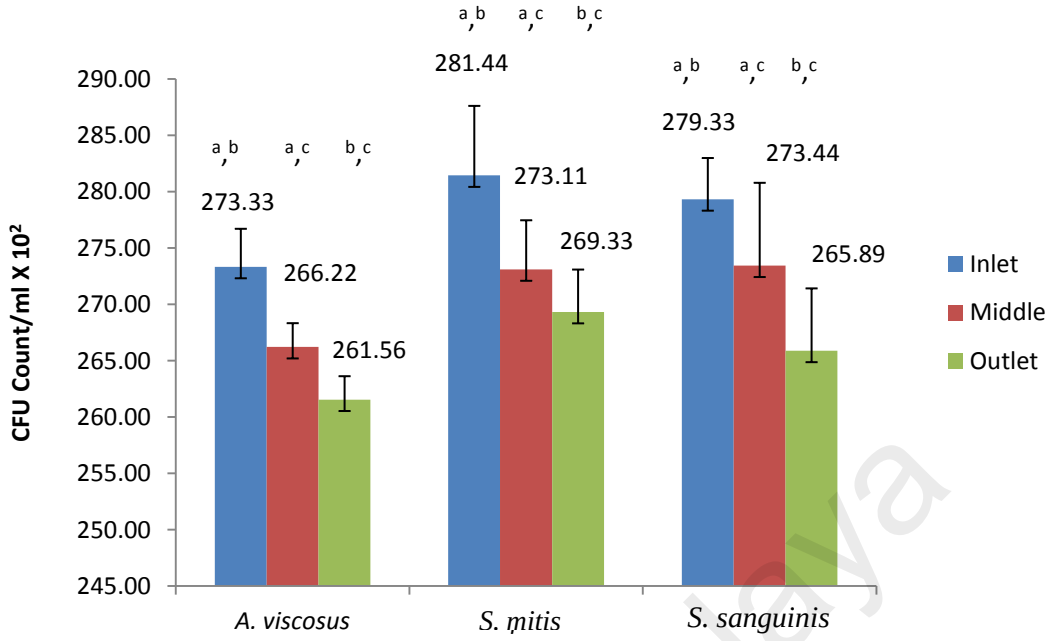


Figure 0.2: The bacterial population (CFU/ml) of single-species biofilms formed on the beads positioned at the inlet, middle and outlet. The population was expressed as mean ± SD where the number of determination (n) =27. "[a]" represents statistical difference between bacterial population of Inlet- and middle glass beads (p<0.05); "[b]" represents significant difference between the bacterial population of inlet and outlet glass beads (p<0.05); "[c]" represents significant difference between bacterial population of middle and outlet glass beads (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

4.3.2. Bacterial Population in Dual-Species Biofilm

Figure 0.3 illustrates the bacterial population of the respective dual-species biofilms [(i) *A. viscosus* - *S. mitis*, (ii) *A. viscosus* – *S. sanguinis*] adhered to the experimental pellicle on the glass beads at the inlet, middle and outlet along the glass capillary tube in the NAM model over a period of 24 hrs. The adherence was also found to be highest at the inlet and followed by middle and outlet beads.

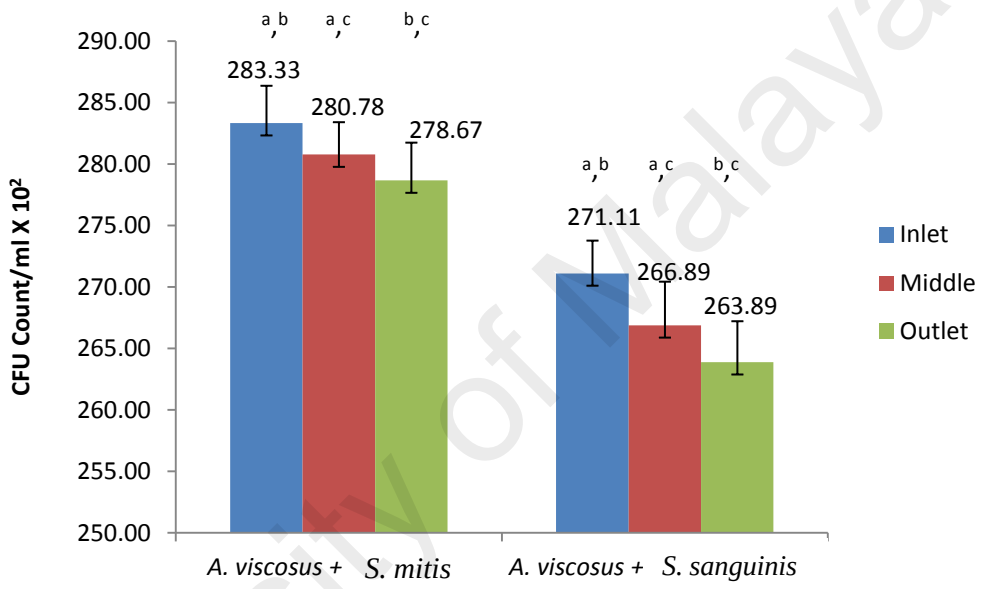


Figure 0.3: The bacterial population (CFU/ml) of dual-species biofilms formed on the beads positioned at the inlet, middle and outlet. The population was expressed as mean ± SD where the number of determination (n) =27. "[a]" represents statistical difference between bacterial population of Inlet- and middle glass beads (p<0.05); "[b]" represents significant difference between the bacterial population of inlet and outlet glass beads (p<0.05); "[c]" represents significant difference between bacterial population of middle and outlet glass beads (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

4.3.3. Percentage Difference of Bacterial Population in the Biofilm on the Beads at Different Positions (Inlet, Middle and Outlet) Along the Glass Capillary Tube

As discussed in Section 4.3.2 maximum bacterial cell adherence was observed on the inlet bead. The adhered bacterial cell population on the middle glass bead decreased slightly and the slight decrease was also for that of the outlet bead. Therefore the percentage differences in the bacterial population of the single- and dual-species biofilms adhering to the beads at different positions in the NAM model were evaluated. The population difference was observed to be less than 5% between the beads present at the three positions respectively expressed in percentage (%) difference (Table 0.2).

Table 0.2: The percentage difference of the average bacterial population on the glass beads at different positions along the glass capillary tube

Bacterial population (single-, dual- species)	**% Difference in the average bacterial population between the beads**		
	Inlet-Middle	**Inlet-Outlet**	**Middle-Outlet**
A. viscosus	2.60	4.31	1.75
S. mitis	2.96	4.30	1.38
S. sanguinis	2.11	4.81	2.76
A. viscosus, S. mitis	0.90	1.65	0.75
A. viscosus, S. sanguinis	1.56	2.66	1.12

Percentage difference was calculated based on the following formula:

$$\% \text{ Difference between Inlet-Middle} = \frac{\text{CFU count of inlet bead - CFU count of middle bead}}{\text{CFU count of inlet bead}} \times 100$$

$$\% \text{ Difference between Inlet-Outlet} = \frac{\text{CFU count of inlet bead - CFU count of Outlet bead}}{\text{CFU count of inlet bead}} \times 100$$

$$\% \text{ Difference between Middle-Outlet} = \frac{\text{CFU count of Middle bead - CFU count of Outlet bead}}{\text{CFU count of Middle bead}} \times 100$$

4.4. Effect of the commercially available oral rinses towards the bacterial adherence in single- and dual-species biofilms

Two concentrations (100%, 50%) of CHX and CPC oral rinses were used. For the EO oral rinse its original concentration (100%) was used. The effect of two different concentrations of respective oral rinses on the percentage of bacterial adherence in single- and dual-species biofilms is described in the following sub-section.

4.4.1. Effect of the 100% concentration of Oral Rinses With Respect To Bacterial Adherence in Single- And Dual-Species Biofilms

4.4.1.1. Bacterial Adherence in Single-Species Biofilms pre-treated with 100 % concentration of respective oral rinses

Figure 0.4 shows the percentage of adherence of *A. viscosus* towards the experimental pellicle pre-treated with the 100 % concentration of three respective oral rinses available commercially. The percentage of adherence was observed to be in the following decreasing sequence: EO > CHX> CPC. On statistical analysis using a One-way ANOVA, Bonferroni post-hoc test, significant difference was observed between the negative control and the respective treatment groups ($p<0.05$). On analyzing the difference in the adherence between the different treatment groups, statistically significant difference was observed between all the three treatment groups (EO, CPC and CHX) ($p<0.05$).

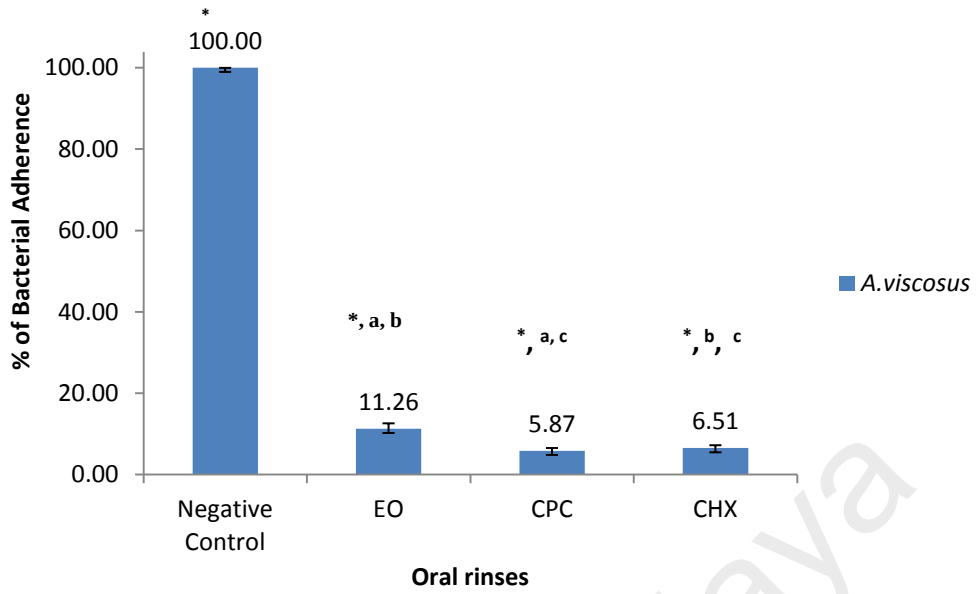


Figure 0.4: Percentage of *A.viscosus* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinse (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "b" represents significant difference between EO- and CHX-treatment groups (p<0.05); "c" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

Figure 0.5 shows the percentage of *S. mitis* adherence on the pre-treated experimental pellicle. The sequence of bacterial adherence was observed to be similar to that of *A. viscosus*: EO > CHX > CPC. On statistical analysis using a One-way ANOVA, Bonferroni post-hoc test, significant difference in bacterial adherence was observed between the negative control (ddH2O) and the treatment groups (p<0.05). The analysis between the treatment groups showed a statistically significant difference in the bacterial adherence to the pre-treated experimental pellicle with EO compared with that of the other two respective treatment groups (CPC and CHX) (p<0.05). The difference in bacterial adherence between CPC- and CHX-pre-treated experimental pellicle was not statistically significant.

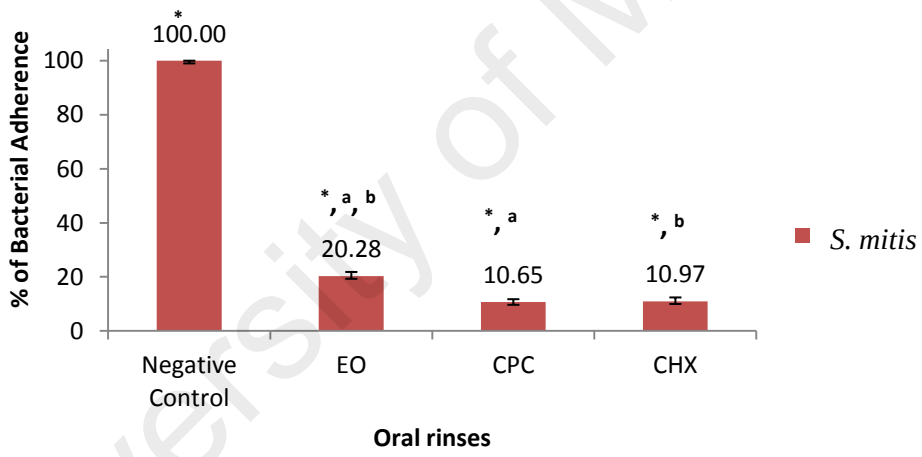


Figure 0.5: Percentage of *S. mitis* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "b" represents significant difference between EO- and CHX-treatment groups (p<0.05); "c" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

The percentage of adherence potential of *S. sanguinis* towards the experimental pellicle pre-treated with the respective oral rinses at their commercially available concentration (100%) was determined (Figure 0.6). The bacterial adherence to the experimental pellicle pre-treated with EO showed the maximum adherence of about 19.24%. A reduction in the adherence was observed for experimental pellicle pre-treated with CHX. A further reduction was demonstrated when CPC oral rinse was used to pre-treat the experimental pellicle. On performing a statistical analysis using One-way ANOVA, Bonferroni post-hoc test, a significant difference was observed in the adherence of *S. sanguinis* between the negative control (ddH2O) and the three respective treatment groups (p<0.05). A statistically significant difference was also observed between EO and other two treatment groups (p<0.05), similarly the difference between CPC- and CHX-treatment groups was also statistically significant (p<0.05).

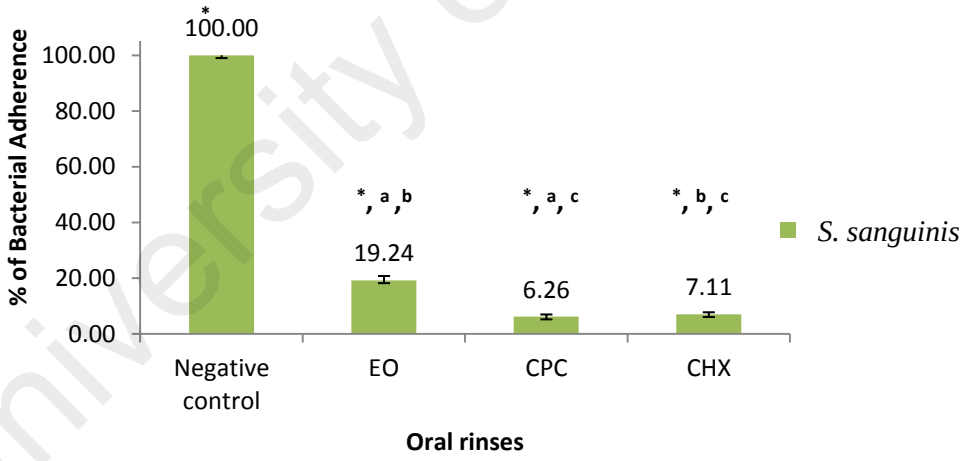


Figure 0.6: Percentage of *S. sanguinis* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "b" represents significant difference between EO- and CHX-treatment groups (p<0.05); "c" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

4.4.1.2. Bacterial Adherence in Dual-Species Biofilms pre-treated with 100 % concentration of the respective oral rinses

The percentage of adherence of *A. viscosus, and S. mitis* to the pre-treated experimental pellicle is shown in Figure 0.7. The adherence to the experimental pellicle pre-treated with EO was observed to be the highest (10.97%) and this was followed by CHX and CPC. When the results were analyzed using a One-way ANOVA, Bonferroni post-hoc test it was observed that there was significant difference between the negative control and the respective treatment groups (p<0.05). The difference in the percentage of adherence between all the three treatment groups was also found to be statistically significant (p<0.05).

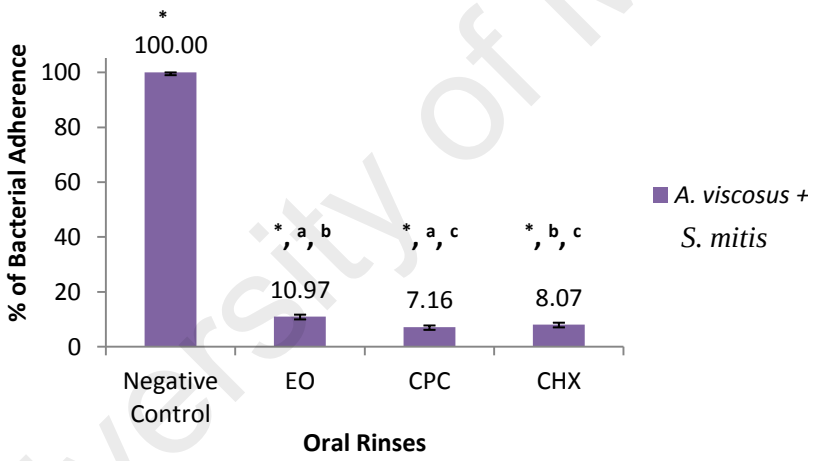


Figure 0.7: Percentage of *A. viscosus, + S. mitis* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "[a]" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "[b]" represents significant difference between EO- and CHX-treatment groups (p<0.05); "[c]" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

The percentage of *A. viscosus*, and *S. sanguinis* adherence (dual-species) to the pre-treated experimental pellicle is presented in Figure 0.8. The experimental pellicle pre-treated with EO appeared to allow the maximum adherence of the dual-species (10.46%) followed by CHX and CPC. On statistical analysis using One-way ANOVA, Bonferroni post-hoc test a significant difference was observed between the negative control and the respective treatment groups (p<0.05). On analyzing the difference between the three treatment groups, statistically significant difference was observed between EO and the other two treatment groups (CPC & CHX) (p<0.05). The difference between CPC- and CHX-treatment groups was also statistically significant (p<0.05).

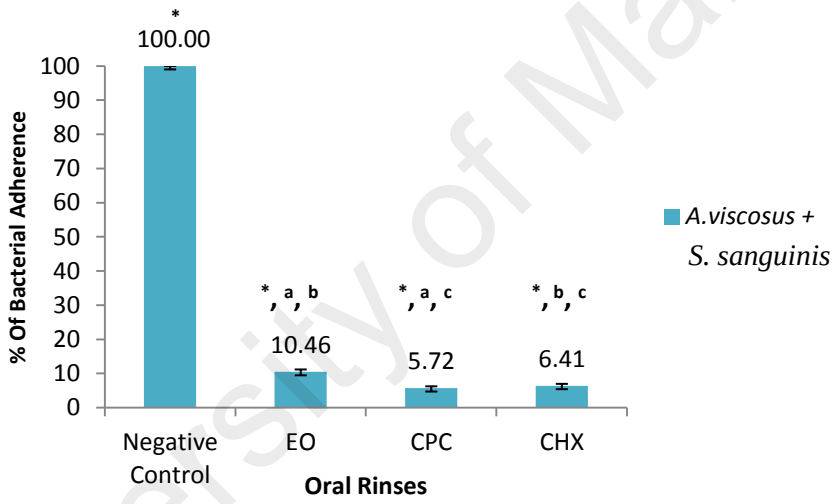


Figure 0.8: Percentage of *A. viscosus*, + *S. sanguinis* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "[a]" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "[b]" represents significant difference between EO- and CHX-treatment groups (p<0.05); "[c]" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

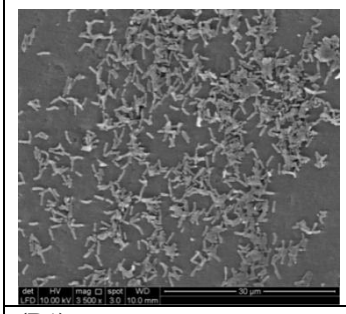
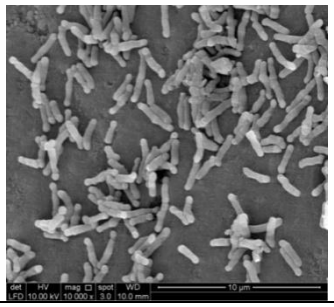
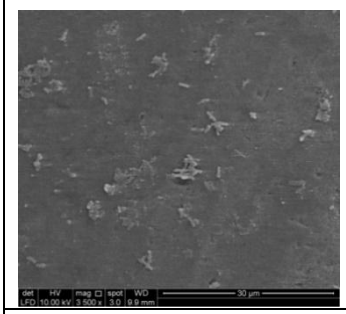
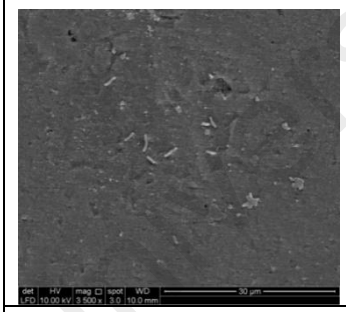
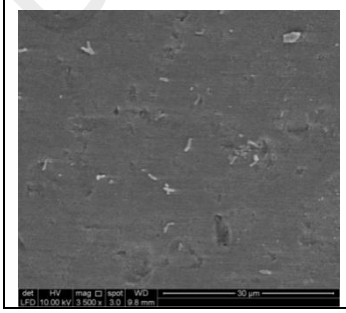
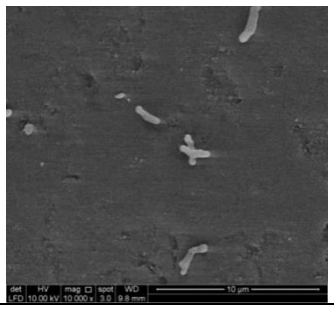
4.4.1.3. SEM study for the bacterial population adhering to the saliva-coated glass beads (experimental pellicle) pre-treated with commercially available oral rinses (at 100% concentration) in single- and dual-species 24 hours biofilms

4.4.1.3.1. Bacterial Adherence in Single-specie biofilm pre-treated with 100 % concentration of respective oral rinses

It was observed that the bacterial adherence to the experimental pellicle pre-treated with the respective oral rinse was less as compared to the negative control (Tables 4.3, 4.4 and 4.5). Micrographs of all the single-species in 24 hours biofilms showed high numbers of bacterial cells present on the glass beads (experimental pellicle) with negative control (ddH2O). Compared to the negative control less adherence of bacteria was allowed by EO treated experimental pellicle. After EO, CHX showed to affect the bacterial adherence and allowed lesser bacteria to adhere. CPC treatment showed to be the most effective oral rinse which allowed the least adherence of bacteria to the experimental pellicle.

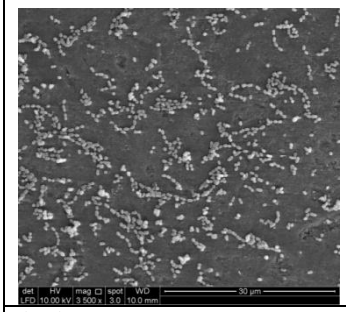
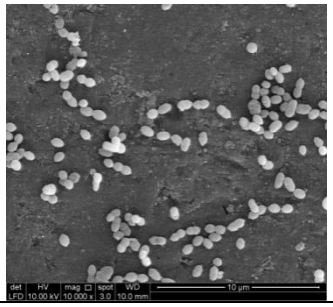
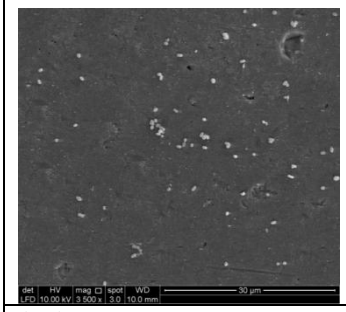
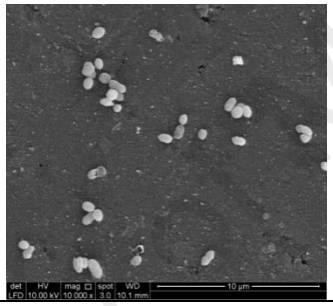
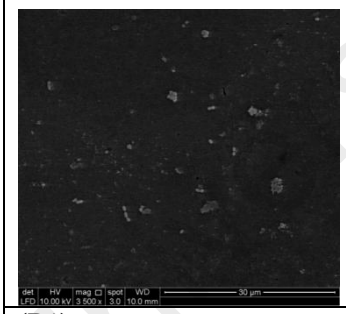
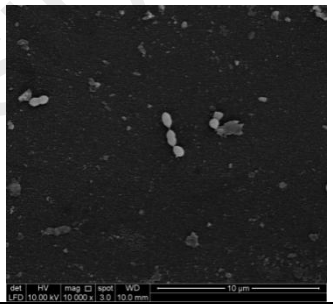
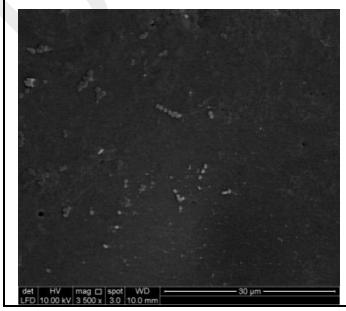
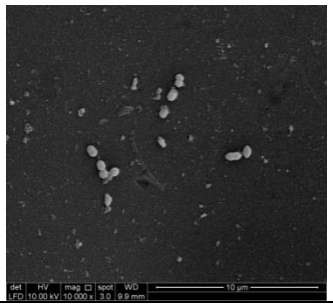
The characteristics of bacterial cells adhering to the saliva-coated glass beads pre-treated with the respective oral rinse observed under SEM in single-species biofilms and presented in the following tables.

Table 0.3: Population and characteristics of *A. viscosus* adhering to the saliva-coated glass beads pre-treated with 100% concentration of the respective oral rinses observed under SEM in single-species biofilm.

A. viscosus		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Large numbers of rod shaped cells were observed on the glass beads.
(B1) 	(B2) 	• Less numbers of rod cells were observed compared to the negative control treated experimental pellicle. • Cells were shorter in size compared to the cells in the negative control.
(C1) 	(C2) 	• Least number of rod shaped cells was present compared to other treatment groups including the negative control.
(D1) 	(D2) 	• Less number of rods was observed compared to EO and the negative control.

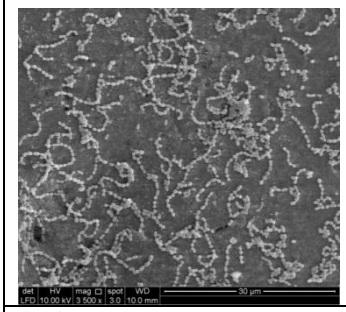
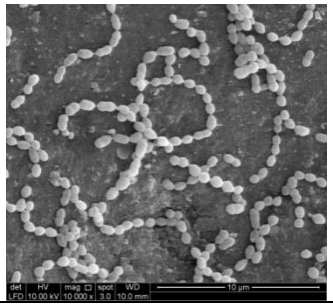
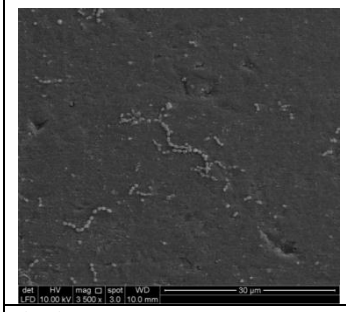
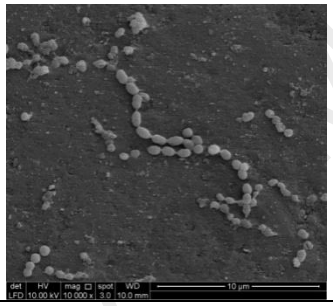
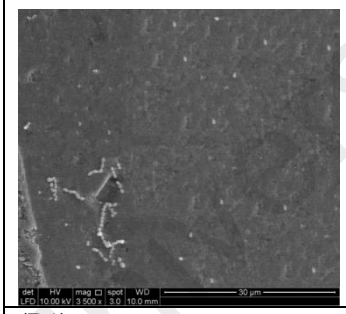
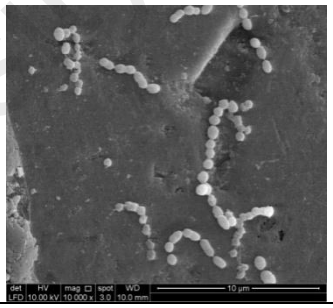
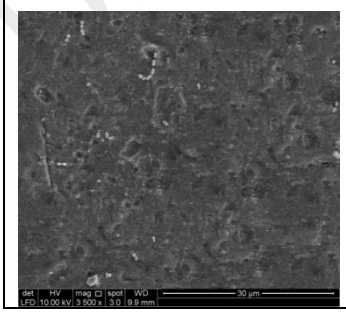
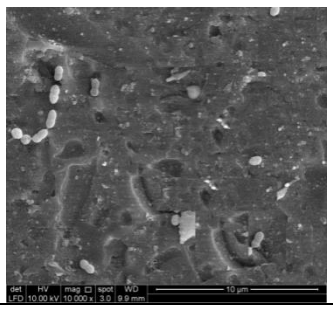
SEM micrographs (3500X and 10,000X) showing population of *A. viscosus* (rod) in 24hour biofilms: negative control (Micrographs A1 and A2), EO-treated experimental pellicle (Micrographs B1and B2), CPC-treated experimental pellicle (Micrographs C1 and C2) and CHX-treated experimental pellicle (Micrographs D1 and D2).

Table 0.4: Population and characteristics of *S. mitis* adhering to the saliva-coated glass beads pre-treated with 100% concentration of the respective oral rinses observed under SEM in single-species biofilm.

S. mitis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Large number of cocci cells was observed. • The cells were in spherical or elliptical in shape. • Cells were present very close to one another and forming chains.
(B1) 	(B2) 	• Less adhered cells were observed compared to the negative control treated glass beads.
(C1) 	(C2) 	• Least number of adhered cells was observed. • Cells were observed as single-cells.
(D1) 	(D2) 	• Less numbers of cells were observed in SEM viewing compared to EO. • Cells were present in the form of single-cells.

SEM micrographs (3500X and 10,000X) showing population of *S. mitis* (cocci) in 24hour biofilms: negative control (Micrographs A1 and A2), EO-treated experimental pellicle (Micrographs B1and B2), CPC-treated experimental pellicle (Micrographs C1 and C2) and CHX-treated experimental pellicle (Micrographs D1 and D2).

Table 0.5: Population and characteristics of *S. sanguinis* adhering to the saliva-coated glass beads pre-treated with 100% concentration of the respective oral rinses observed under SEM in single-species biofilm.

S. sanguinis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• A large numbers of round shaped, closely attached cells were present. • Cells were found to be in long chains.
(B1) 	(B2) 	• Less numbers of round shaped cells forming chains were seen. • Cells were found to be in shorter chains.
(C1) 	(C2) 	• Lesser numbers of cells were seen compared to EO. • Cells were found to be in shorter chains. • Some cells were found to be scattered as individual cells.
(D1) 	(D2) 	• Least numbers of cocci cells observed compared to other two treatments.

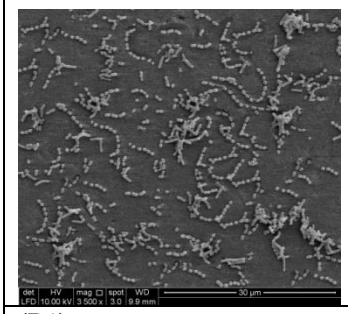
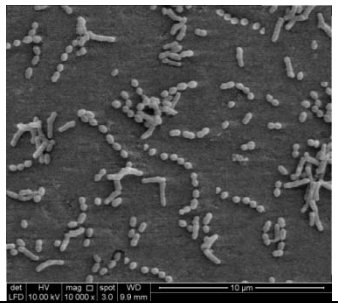
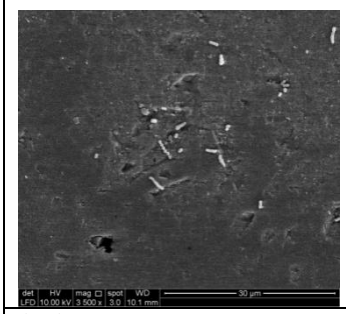
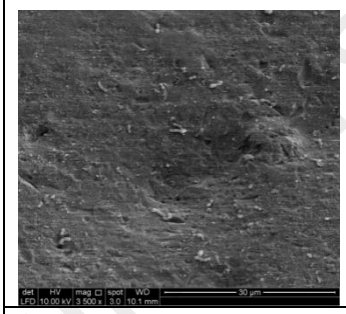
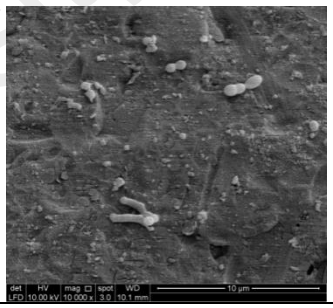
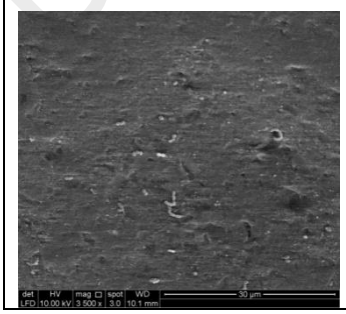
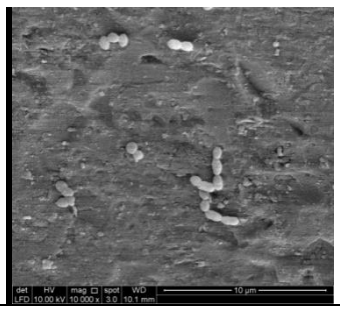
SEM micrographs (3500X and 10,000X) showing population of *S. sanguinis* (cocci) in 24hour biofilms: negative control (Micrographs A1 and A2), EO-treated experimental pellicle (Micrographs B1and B2), CPC-treated experimental pellicle (Micrographs C1 and C2) and CHX-treated experimental pellicle (Micrographs D1, D2).

4.4.1.3.2. Bacterial Adherence in Dual-Species Biofilm pre-treated with 100 % concentration of respective oral rinses

In SEM viewing, negative control bead was observed to have the highest adherence of bacterial population compared to the experimental pellicle pre-treated with the respective oral rinse (Table 4.6 and 4.7). Among the oral rinses, the experimental pellicle pre-treated with EO showed highest adherence of bacterial cells to the 24 hours dual-species biofilm and it was followed by CHX and CPC.

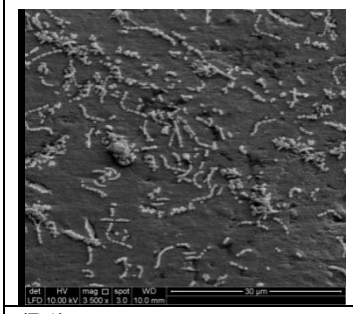
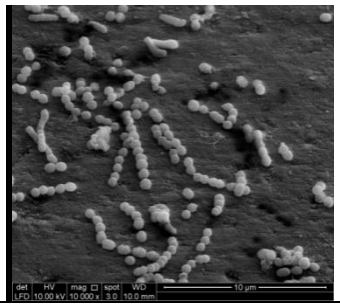
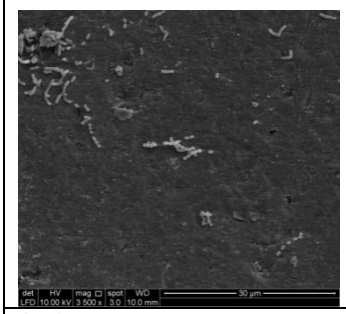
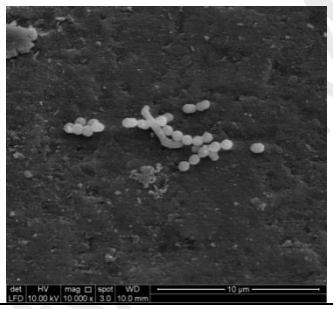
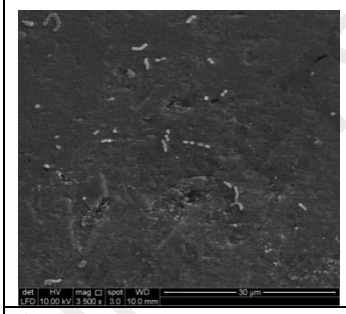
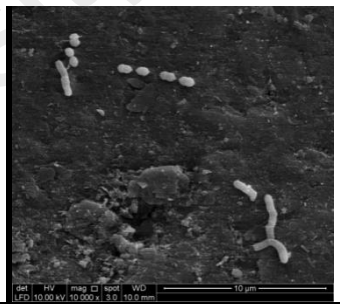
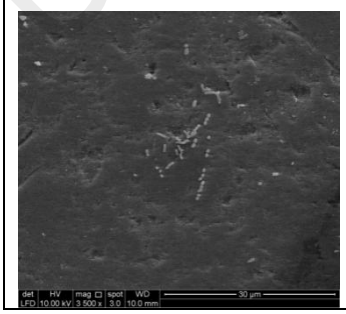
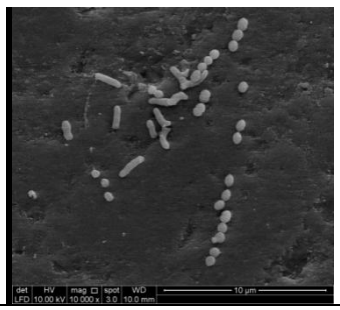
The characteristics of bacterial cells adhering to the saliva-coated glass beads pre-treated with the respective oral rinse observed under SEM in dual-species biofilms and presented in the following tables.

Table 0.6: Population and characteristics of *A. viscosus* and *S. mitis* adhering to the saliva-coated glass beads pre-treated with 100% concentration of the respective oral rinses observed under SEM in dual-species biofilm.

A. viscosus* and *S. mitis		
Viewing magnifications		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Large numbers of rod and cocci cells were observed on the glass beads. • Rod cells were found in groups. • Cocci were mostly present in chains
(B1) 	(B2) 	• Less numbers of rod and cocci cells were observed compared to the negative control. • Cocci cells were observed in chain like structure.
(C1) 	(C2) 	• Least numbers of rods were present compared to other treatment groups including the negative control. • Cocci cells were found as diplococci.
(D1) 	(D2) 	• Less numbers of cocci cells was observed compared to EO and the negative control. • Cocci were present in short chains.

SEM micrographs (3500X and 10,000X) showing population of *A. viscosus*,(rods) and *S. mitis* (cocci) in 24hour biofilms: negative control (Micrographs A1 and A2), EO-treated experimental pellicle (Micrographs B1and B2), CPC-treated experimental pellicle (Micrographs C1 and C2) and CHX-treated experimental pellicle (Micrographs D1 and, D2).

Table 0.7: Population and characteristics of *A. viscosus* and *S. sanguinis* adhering to the saliva-coated glass beads pre-treated with 100% concentration of the respective oral rinses observed under SEM in dual-species biofilm.

A. viscosus* and *S. sanguinis		
Viewing magnifications		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Large numbers of cocci cells were observed. • Cocci cells were present in the form of long chains. • Rods were also observed
(B1) 	(B2) 	• Less adhesion of cells was seen as compared to the negative control treated glass beads. • Cocci cells were present in short chains. • Few rods were observed.
(C1) 	(C2) 	• Least number of cells adhered to the CPC-treated saliva coated glass beads was observed. • Rods and cocci cells were observed in single or pairs.
(D1) 	(D2) 	• Less numbers of cells was observed in SEM viewing compared to EO.

SEM micrographs (3500X and 10,000X) showing population of *A. viscosus* (rods) and *S. sanguinis* (cocci) in 24hour biofilms: negative control (Micrographs A1 and A2), EO-treated experimental pellicle (Micrographs B1and B2), CPC-treated experimental pellicle (Micrographs C1 and C2) and CHX-treated experimental pellicle (Micrographs D1 and, D2)

4.4.2. Effect of the 50% concentration of Oral Rinses With Respect To Bacterial Adherence in Single- And Dual-Species Biofilms

The effect of two commercially available oral rinses containing respective active ingredient (CHX and CPC) at 50% concentration (two fold dilution) towards the experimental pellicle was determined and expressed as the percentage of bacterial adherence in single- and dual-species biofilms. The analysis of the results is defined in the following sub-sections.

4.4.2.1. Bacterial Adherence in Single-specie biofilm pre-treated with 50 % concentration of respective oral rinses

The percentage of adhering bacterial population on pre-treated experimental pellicle in developing single- species biofilms will be described individually with respect to the bacterial species.

Figure 0.9 shows the adherence potential of *A.viscosus* to the pre-treated experimental pellicle. The percentage of *A. viscosus* adherence was observed highest for the experimental pellicle pre-treated with CHX and was followed by CPC. On performing One-way ANOVA, Bonferroni post-hoc test analysis a statistically significant difference was found between the negative control (ddH2O) and the two respective treatment groups ($p<0.05$). The statistical difference was found to be significant between CPC and CHX ($p<0.05$).

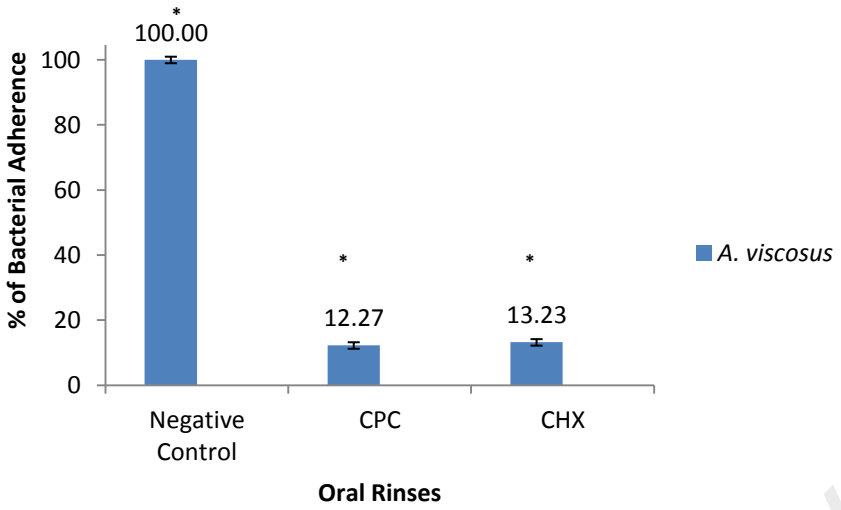


Figure 0.9: Percentage of *A.viscosus* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "[a]" represents statistical difference between CPC- and CHX-treatment groups (p<0.05); The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

The percentage of adherence of *S. mitis* to the pre-treated experimental pellicle was observed in the following decreasing sequence: CHX > CPC (Figure 0.10). On statistical analysis, a significant difference was observed in the *S. mitis* adherence between the negative control (ddH2O) and the two respective treatment groups (p<0.05). Similarly a significant difference was observed among CPC and CHX treatment groups (p<0.05).

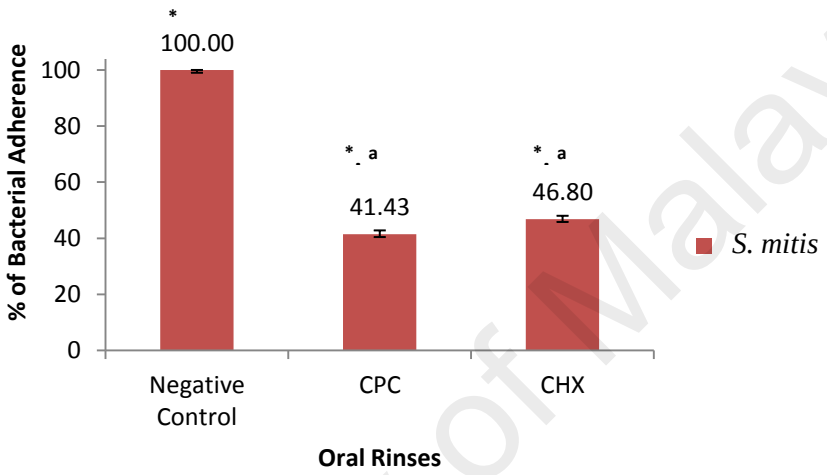


Figure 0.10: Percentage of *S. mitis* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between CPC- and CHX-treatment groups (p<0.05); The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

Figure 0.11 demonstrates the percentage adherence of *S. sanguinis* to the experimental pellicle pre-treated with oral rinses in the following sequence: CHX > CPC. On statistical analysis using One-way ANOVA, Bonferroni post-hoc test, significant difference was seen between the negative control and the two respective treatment groups (p<0.05). Similarly statistically significant difference was observed among all the treatment groups (p<0.05).

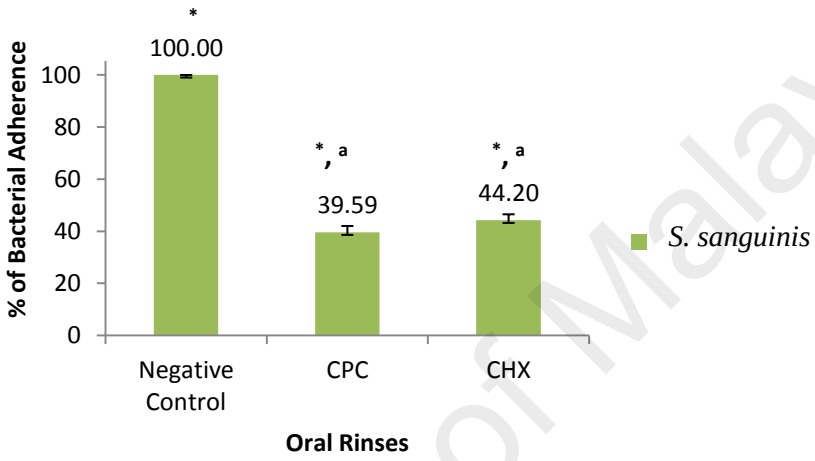


Figure 0.11: Percentage of *S. sanguinis* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between CPC- and CHX-treatment groups (p<0.05); The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

4.4.2.2. Bacterial Adherence in Dual-species Biofilm pre-treated with 50 % concentration of respective oral rinses

The percentage adherence of bacterial population to the pre-treated experimental pellicle in developing dual-species biofilm will be described independently with respect to the bacterial mixture.

The adherence of *A. viscosus* and *S. mitis* (dual-species) was determined on the pre-treated experimental pellicle with oral rinses at their 50% concentrations (Figure 0.12). The percentage of bacterial adherence observed in the following decreasing order: CHX > CPC. One-way ANOVA, Bonferroni post-hoc test was performed for Statistical analysis, significant difference was found between the negative control (ddH2O) and the two respective treatment groups (p<0.05). Similarly a significant difference was observed on statistical analysis among both treatment groups (p<0.05).

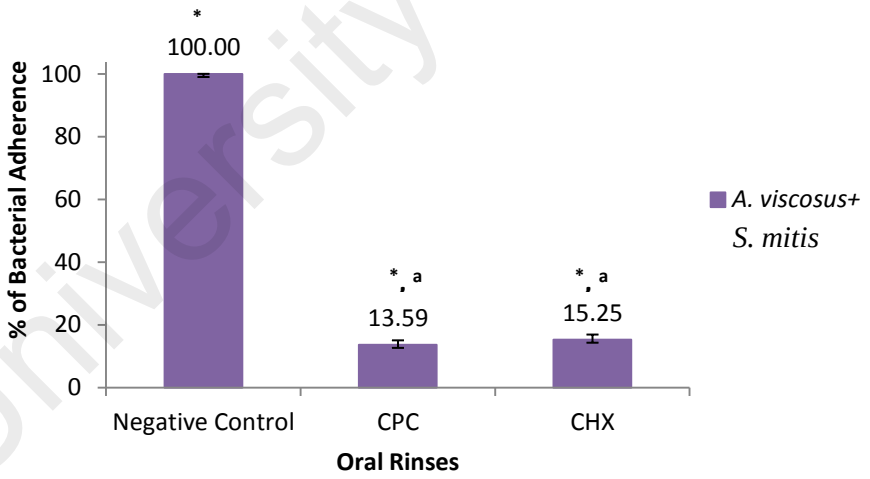


Figure 0.12: Percentage of *A. viscosus* + *S. mitis* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "[a]" represents statistical difference between CPC- and CHX-treatment groups (p<0.05); The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

Figure 0.13 shows the percentage of adherence of *A. viscosus* and *S. sanguinis* to the pre-treated experimental pellicle with 50% concentration of the respective oral rinses. Dual-species adherence showed the following manner: CHX > CPC. Statistical analysis showed significant difference between the negative control and the two respective treatment groups (p<0.05), similarly a significant difference was also observed in the percentage of bacterial adherence to the pre-treated experimental pellicle between both treatment groups respectively (p<0.05).

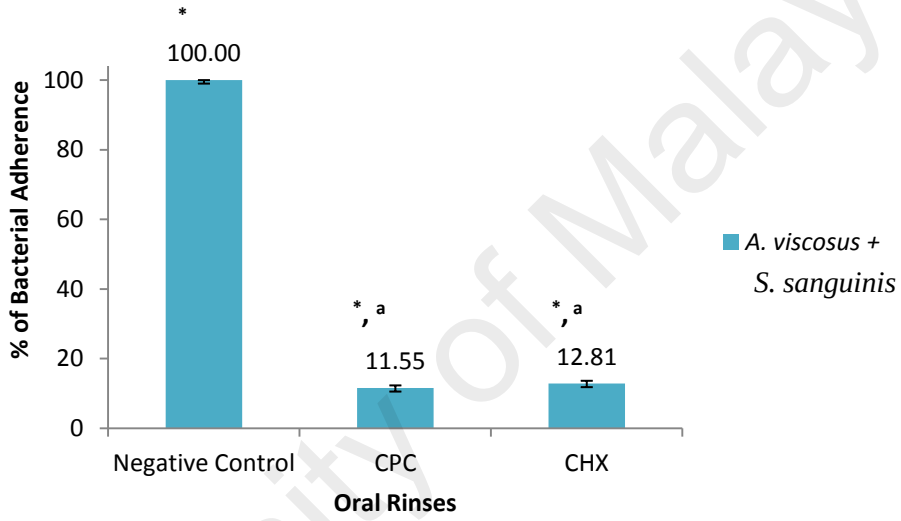


Figure 0.13: Percentage of *A. viscosus* + *S. sanguinis* adherence expressed in Mean ± SD towards the experimental pellicle pre-treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium) and CHX (Chlorohexidine gluconate). The percentage of adherence for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between CPC- and CHX-treatment groups (p<0.05); The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

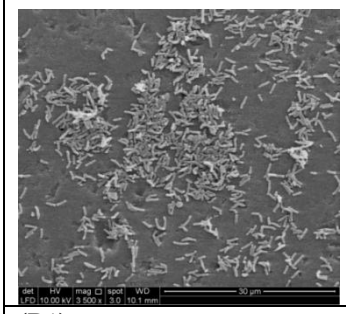
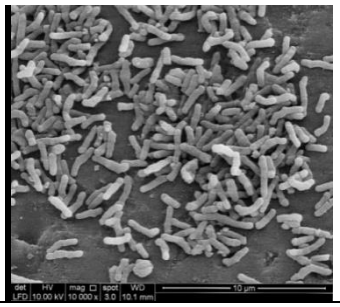
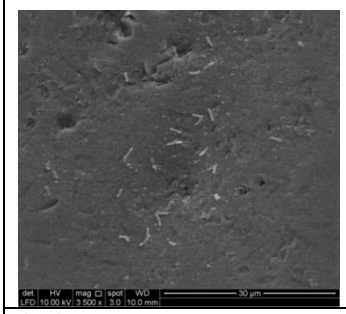
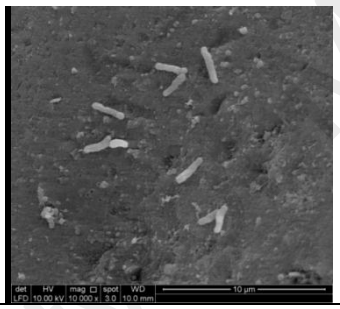
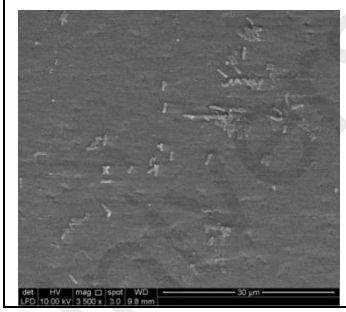
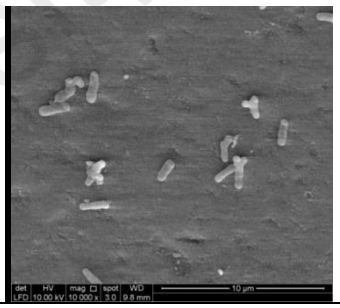
4.4.2.3. SEM study for the bacterial population adhering to the saliva-coated glass beads (experimental pellicle) pre-treated with commercially available oral rinses (at 50% concentration) in single- and dual-species 24 hours biofilms

SEM studies were carried out for single- and dual-species biofilms formed on the saliva-coated glass beads pre-treated with the respective oral rinse at 50% concentration. The micrographs were examined under the Scanning Electron Microscope at various magnifications (3,500x and 10,000x).

4.4.2.3.1. Bacterial Adherence in Single-Species Biofilm pre-treated with 50% concentration of respective oral rinses

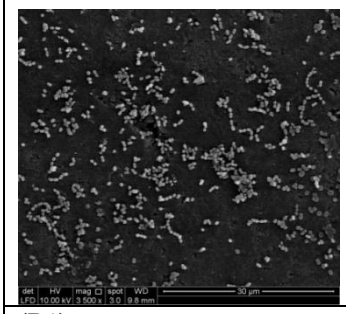
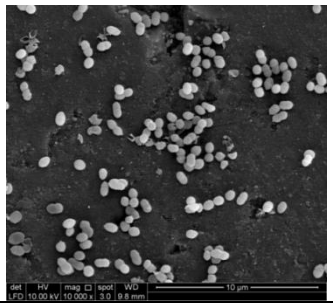
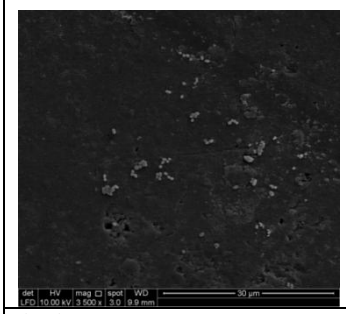
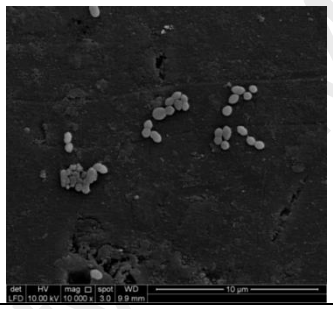
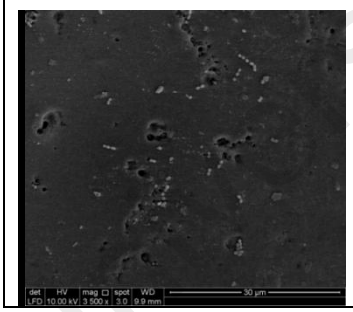
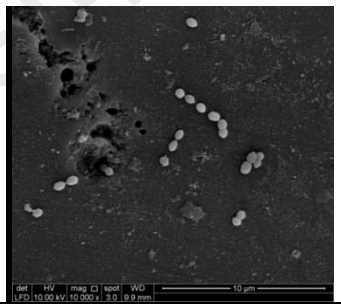
It was observed that the bacteria adhered to the experimental pellicle pre-treated with the respective oral rinse at 50% concentration, showed low adherence as compared to the negative control (Tables 4.8, 4.9 and 4.10). Micrographs of all the single-species in 24 hours biofilms showed high numbers of bacterial cells present on the negative control (ddH2O) glass beads (experimental pellicle). Compared to the negative control, the adherence of *A. viscosus* bacteria was observed to be less in CHX treated experimental pellicle and this was followed by CPC-treated experimental pellicle. Other single-species *S. mitis* and *S. sanguinis* showed moderately high adherence to CHX-treated pellicle followed by CPC.

Table 0.8: Population and characteristics of *A. viscosus* adhering to the saliva-coated glass beads pre-treated with respective oral rinses (50% of their original concentration) observed under SEM in single-species biofilm

A. viscosus		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Enormous numbers of rod cells were observed on the glass beads. • Cells were clustered together.
(B1) 	(B2) 	• Small number of rod cells was observed. The cells were scattered.
(C1) 	(C2) 	• Cells were found to be shortening in size as a result of breakdown.

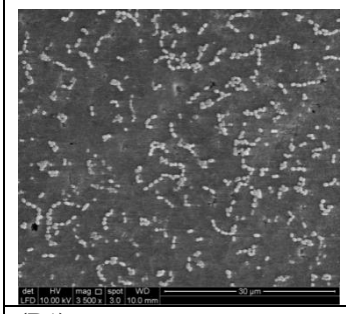
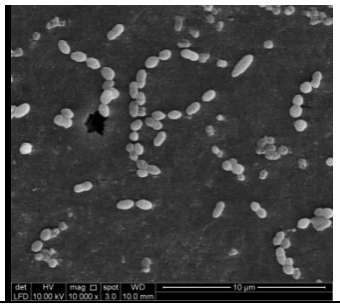
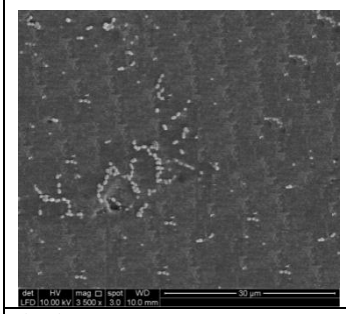
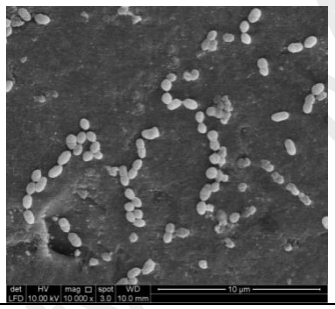
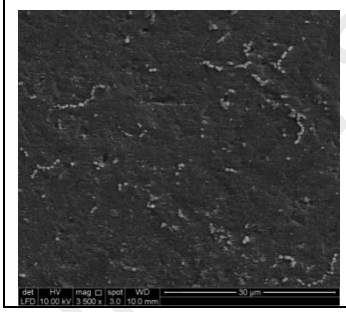
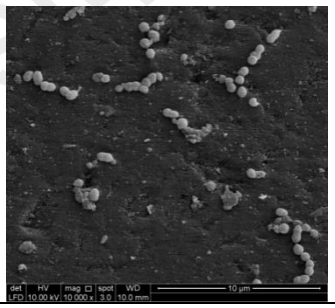
SEM micrographs (3500X and 10,000X) showing population of *A. viscosus* (rods) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated experimental pellicle (Micrographs B1 and B2) and CHX-treated experimental pellicle (Micrographs C1 and C2).

Table 0.9: Population and characteristics of *S. mitis* adhering to the saliva-coated glass beads pre-treated with respective oral rinses (50% of their original concentration) observed under SEM in single-species biofilm

S. mitis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Large number of round shaped cells was observed. • Cells were present in the form of groups. • Few cells observed to be present singly.
(B1) 	(B2) 	• Least adherence of cocci cells was seen. • Cells were present in the form of small groups.
(C1) 	(C2) 	• Less number of round shaped cells were seen • Cells were present in the form of broken chain

SEM micrographs (3500X and 10,000X) showing population of *S. mitis* (cocci) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated experimental pellicle (Micrographs B1 and B2) and CHX-treated experimental pellicle (Micrographs C1 and C2).

Table 0.10: Population and characteristics of *S. sanguinis* adhering to the saliva-coated glass beads pre-treated with respective oral rinses (50% of their original concentration) observed under SEM in single-species biofilm.

S. sanguinis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Enormous number of round shaped cells were seen • Fewer cells were observed in dividing state • Numerous cellular chains were observed
(B1) 	(B2) 	• Lesser number of bacterial cells observed • Various ruptured bacterial cells • Broken chains with 6-7 cells/chain
(C1) 	(C2) 	• Less bacterial cells observed • Fewer ruptured bacterial cells • Small broken chains

SEM micrographs (3500X and 10,000X) showing population of *S. sanguinis* (cocci) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated experimental pellicle (Micrographs B1 and B2) and CHX-treated experimental pellicle (Micrographs C1 and C2).

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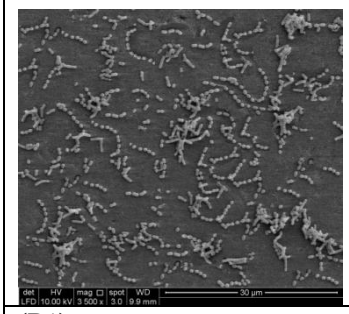
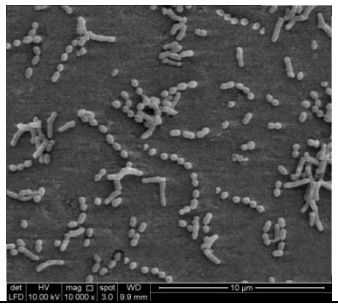
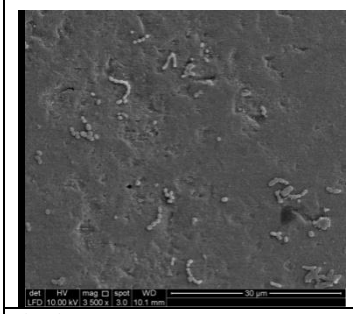
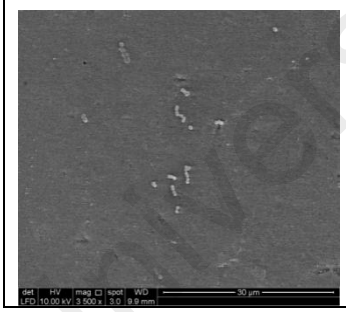
4.4.2.3.2. Bacterial Adherence in Dual-species Biofilm pre-treated with 50% concentration of respective oral rinses

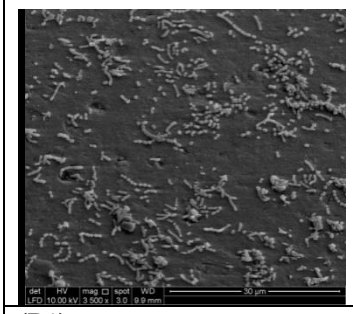
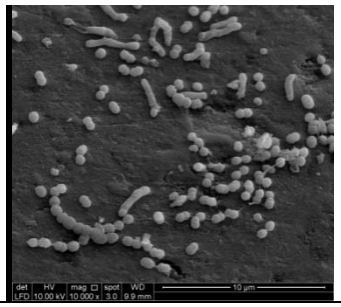
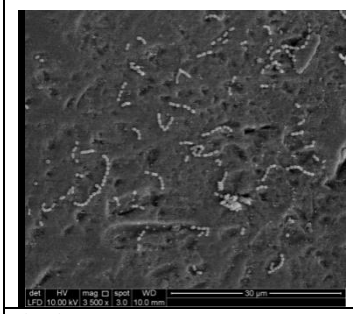
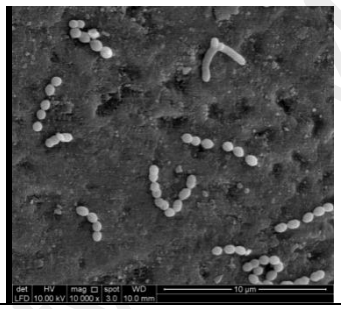
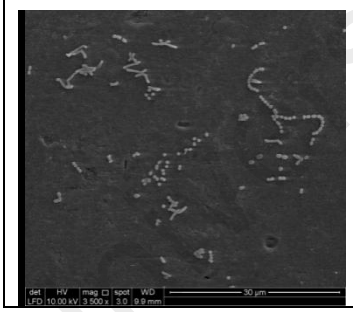
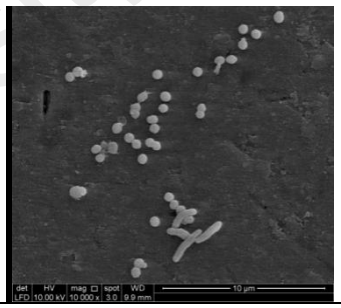
It was observed similar to that of single-species biofilm that the bacteria (dual-species) adhered to the experimental pellicle pre-treated with the respective oral rinse showed less adherence as compared to the negative control (Table 4.6). Micrographs of all dual-species in 24 hours biofilms showed high numbers of bacterial cells existing on the glass beads (experimental pellicle) with negative control (ddH2O). After the negative control, CHX-treated experimental pellicle showed moderately high adherence of bacterial species and this was followed by CPC.

Table 0.11: Population and characteristics of *A. viscosus* + *S. mitis* adhering to the saliva-coated glass beads pre-treated with respective oral rinses (50% of their original concentration) observed under SEM in dual-species biofilm.

A. viscosus* + *S. mitis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Rods and cocci cells were present in high numbers.
(B1) 	(B2) 	• Fewer adhered cells of cocci and rods were seen • The rods cells were in pairs and cocci maintained short chains
(C1) 	(C2) 	• Less attachment of cells was observed on the surface • Cocci in short chains while rods were least in number

SEM micrographs (3500X and 10,000X) showing population of *A. viscosus* (rods) and *S. mitis* (cocci) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated experimental pellicle (Micrographs B1 and B2) and CHX-treated experimental pellicle (Micrographs C1 and C2).

Table 0.12: Population and characteristics of *A. viscosus* + *S. sanguinis* adhering to the saliva-coated glass beads pre-treated with respective oral rinses (50% of their original concentration) observed under SEM in dual-species biofilm.

A. viscosus* + *S. sanguinis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Large numbers of cocci cells were observed with fewer rods. • Cocci cells were present very close to one another and present in the form of multiple long and short chains.
(B1) 	(B2) 	• Less number of cells adhered to the CPC-treated saliva coated glass beads was observed. • Cells were observed in single or in multiple broken chains (3-5 cells/chain)
(C1) 	(C2) 	• Cells were present in the form of broken chains. • Aggregation of rods and cocci observed**.**

SEM micrographs (3500X and 10,000X) showing population of *A. viscosus* (rods) and *S. sanguinis* (cocci) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated experimental pellicle (Micrographs B1 and B2) and CHX-treated experimental pellicle (Micrographs C1 and C2).

4.5. Effect of the commercially available Oral Rinses to the Bacterial Retention in Single- and Dual-species Biofilms

The effect of commercially available oral rinses at their 100% and 50% concentrations was determined towards the retention of bacterial cells in already developed (24 hours) single- and dual-species biofilms and will be conversed in the following sub-sections.

4.5.1. Effect of the 100% concentration of Oral Rinses With Respect To Bacterial Retention in Single- And Dual-Species Biofilms

4.5.1.1. Bacterial Retention in 24-hrs Single-species Biofilm upon treatment with 100 % concentration of respective oral rinses

The percentage of bacterial retention in single-species biofilm after treatment with the respective oral rinse will be described separately with respect to the bacterial species (retained/remained in the biofilm).

Figure 0.14 exemplifies the retention potential of *A.viscosus* to the treated biofilm. The percentage of bacterial retention was observed highest for the 24 hourly formed EO-treated biofilm and this was followed by CHX and CPC. On using One-way ANOVA, Bonferroni post-hoc test analysis a statistically significant difference was observed between the negative control (ddH2O) and the three respective treatment groups ($p<0.05$). Similarly, statistically significant difference was observed between EO and the other two treatment groups (CPC and CHX) ($p<0.05$).

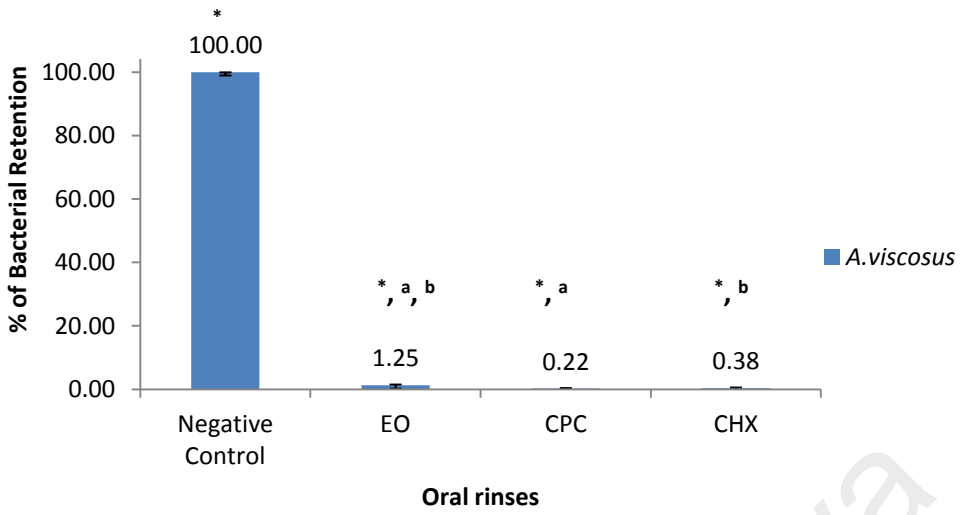


Figure 0.14: Percentage of *A.viscosus* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "b" represents significant difference between EO- and CHX-treatment groups (p<0.05); "c" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

Figure 0.15 shows the percentage of *S. mitis* retention in the treated biofilm. The percentage of *S. mitis* retention followed the similar trend to that of *A. viscosus*: EO > CHX > CPC. On statistical analysis using a One-way ANOVA, Bonferroni post-hoc test, significant difference in bacterial retention was observed between the negative control (ddH2O) and the treatment groups (p<0.05). The analysis between the treatment groups showed a statistically significant difference in the bacterial retention to the EO-treated biofilm compared to the biofilm treated with other two respective treatment groups (CPC and CHX). The difference between CPC and CHX was not statistically significant.

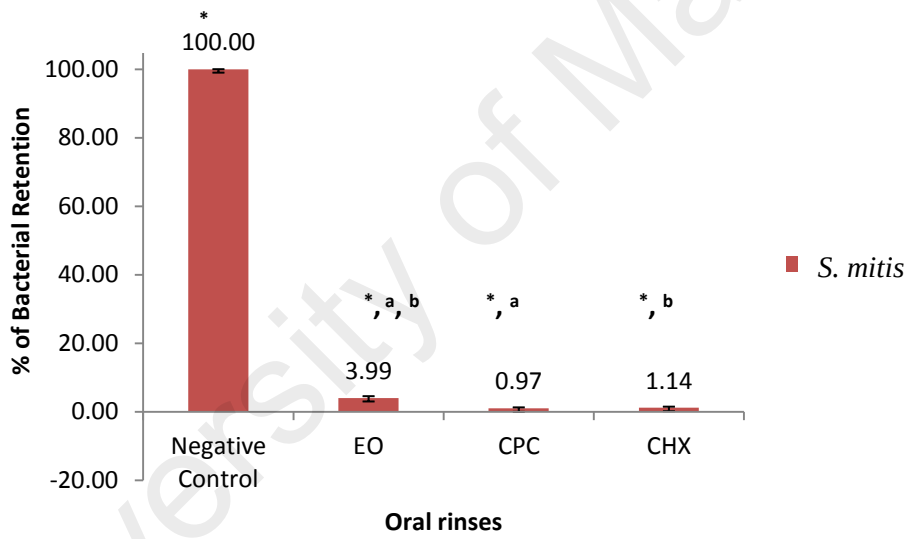


Figure 0.15: Percentage of *S. mitis* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "b" represents significant difference between EO- and CHX-treatment groups (p<0.05); "c" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

The retention potential of *S. sanguinis* to the 24 hours biofilm treated with EO showed the maximum (4.66%) (Figure 0.16). The percentage of retention was reduced in CHX-treated biofilm. A further reduction was observed when CPC oral rinse was used to treat the biofilm. Statistical analysis was performed using One-way ANOVA, Bonferroni post-hoc test, and statistically significant difference was observed in the retention of *S. sanguinis* between the negative control (ddH2O) and the three respective treatment groups (p<0.05). On statistical analysis between the three treatment groups, significant difference was observed between EO with other two treatment groups (p<0.05), but the difference between CHX and CPC was not significant (Figure 4.18).

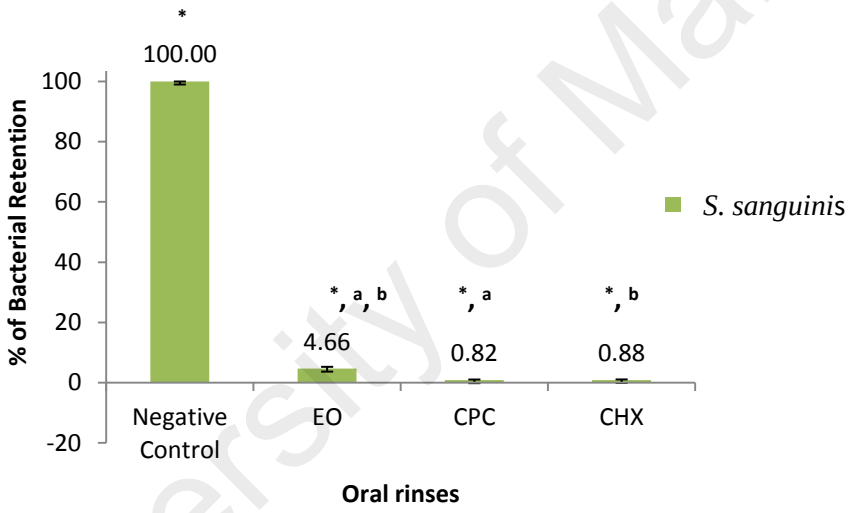


Figure 0.16: Percentage of *S. sanguinis* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "b" represents significant difference between EO- and CHX-treatment groups (p<0.05); "c" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

4.5.1.2. Bacterial Retention in 24-hrs Dual-species Biofilm upon treatment with 100 % concentration of respective oral rinses

The percentage of dual-species bacterial retention in 24 hours biofilm treated with the respective oral rinse (100% concentration) will be described separately with respect to the bacterial species mixture.

The percentage of retention of *A. viscosus* and *S. mitis* (dual-species) in the biofilm treated with three respective oral rinses is shown in Figure 0.17. The retention was found highest (2.27%) in the EO-treated biofilm and this was followed by CHX- and CPC-treated biofilms respectively. On statistical analysis using a One-way ANOVA, Bonferroni post-hoc test, significant difference was observed between the negative control and the respective treatment groups ($p<0.05$). On analyzing the difference in the bacterial retention between all the three treatment groups, statistically significant difference was observed between all the three treatment groups (EO, CPC and CHX) ($p<0.05$).

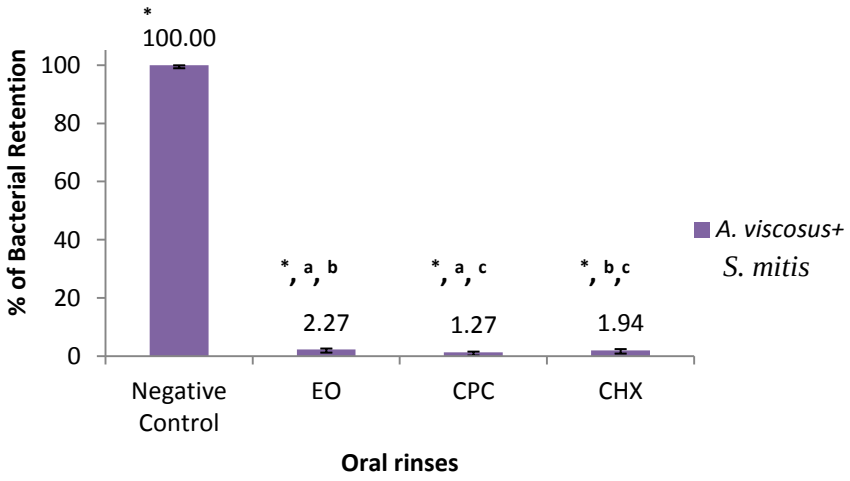


Figure 0.17: Percentage of *A.viscosus* + *S. mitis* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "[a]" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "[b]" represents significant difference between EO- and CHX-treatment groups (p<0.05); "[c]" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

The percentage retention of *A. viscosus* and *S. sanguinis* to biofilm treated with respective oral rinses followed the same order as *A. viscosus* and *S. mitis*. The biofilm treated with EO appeared to show the maximum retention of dual-species (1.89%) followed by CHX and CPC (Figure 0.18). On statistical analysis using One-way ANOVA, Bonferroni post-hoc test significant difference was observed between the negative control and the three respective treatment groups (p<0.05). On analyzing the difference between the three treatment groups, statistically significant difference was observed between CPC and the other two treatment groups (EO and CHX) (p<0.05). The difference between EO and CHX was not statistically significant.

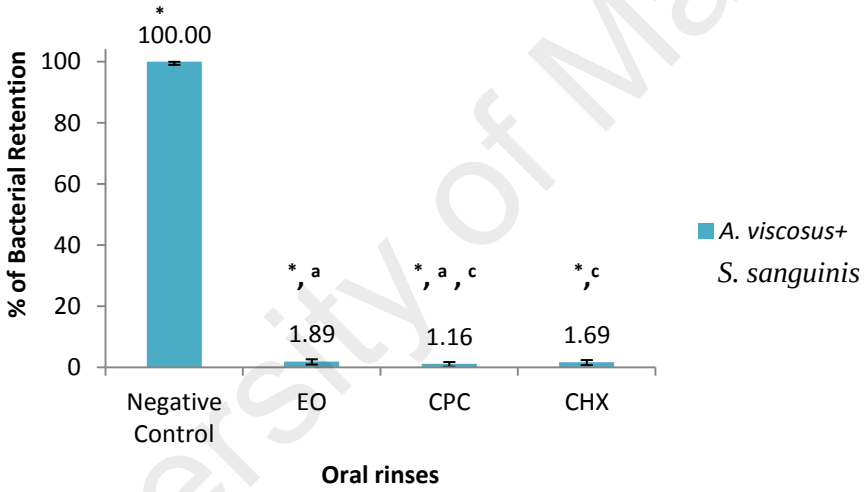


Figure 0.18: Percentage of *A.viscosus* + *S. sanguinis* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (100% concentration). The active ingredients were: EO (Essential oils), CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents statistical difference between EO- and CPC-treatment groups (p<0.05); "b" represents significant difference between EO- and CHX-treatment groups (p<0.05); "c" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

4.5.1.3. SEM Study for the Bacterial Population Retaining in the 24 Hours Biofilm (Single- And Dual-Species) Treated with Commercially Available Oral Rinses at 100% concentration

On SEM viewing of 24 hours single- and dual-species biofilms treated with the respective oral rinse at 100% concentration no bacterial cell retention was observed except for the negative control (ddH2O).

4.5.2. Effect of the 50% concentration of Oral Rinses With Respect To Bacterial Retention in Single- And Dual-Species Biofilms

The effect of respective active ingredient (CHX and CPC) in commercially available oral rinses towards the bacterial retention in the biofilm at 50% of the original concentration was determined and expressed as the percentage of bacterial retention in single- and dual-species biofilms. The analysis of the results is described in the following sub-sections.

4.5.2.1. Bacterial Retention in 24-hrs Single-species Biofilm upon treatment with the respective oral rinses at 50% of their original concentration

The percentage of retaining bacterial population in the 24 hours single-species biofilms treated with the respective oral rinses (50% concentration) will be described independently with respect to the individual bacteria.

Figure 0.19 shows the retention potential of *A.viscosus* in the biofilm after treatment with the respective oral rinse. The percentage of retention was observed highest in the biofilm treated with CHX and this was followed by CPC. On using One-way ANOVA, Bonferroni post-hoc test analysis, statistically significant difference was observed between the negative control and the two treatment groups ($p<0.05$). The difference between CPC- and CHX-treatment groups was not statistically significant.

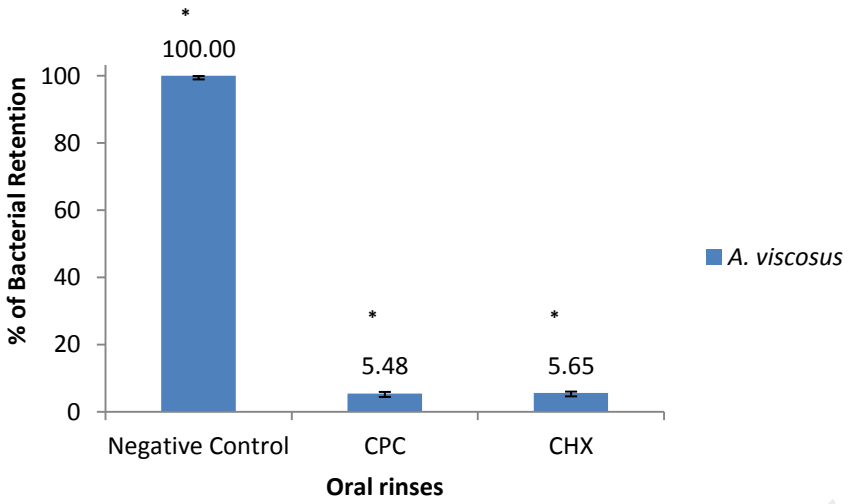


Figure 0.19: Percentage of *A.viscosus* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "[a]" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

The percentage of *S. mitis* retention in the biofilm treated with respective oral rinse is presented in Figure 0.20. The percentage of retention was observed for the respective oral rinse treated biofilm in the following decreasing sequence: CHX > CPC. On statistical analysis, a significant difference was observed in the *S. mitis* retention between the negative control (ddH2O) and treatment groups (p<0.05). Similarly a significant difference was observed among both of the treatment groups (p<0.05).

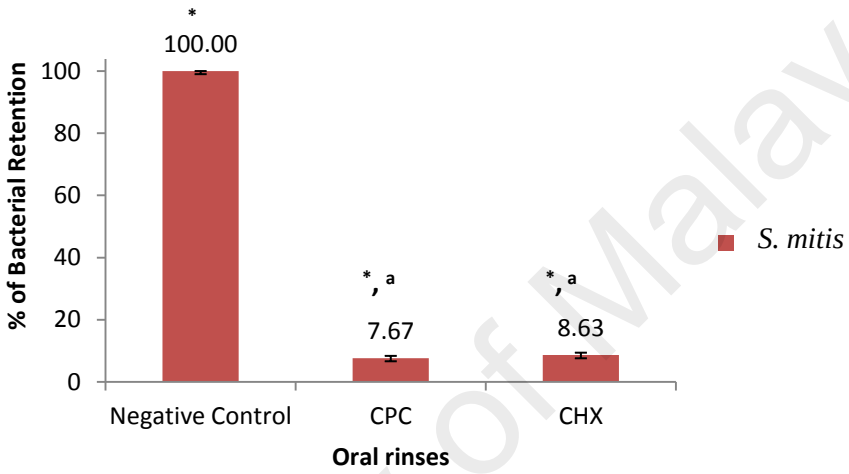


Figure 0.20: Percentage of *S. mitis* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "a" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

Figure 0.21 demonstrates the percentage of retention potential of *S. sanguinis* to the biofilm treated with oral rinses in the following decreasing sequence: CHX > CPC. On statistical analysis using One-way ANOVA, Bonferroni post-hoc test, a significant difference was observed between the negative control and the two respective treatment groups (p<0.05). Similarly statistically significant difference was observed between CPC and CHX treatment groups (p<0.05).

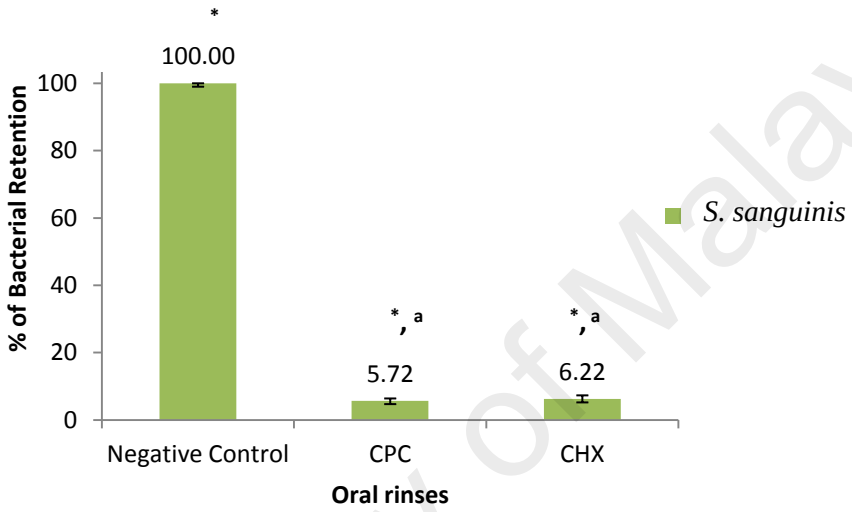


Figure 0.21: Percentage of *S. sanguinis* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "[a]" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

4.5.2.2. Bacterial Retention in 24-hrs Dual-Species Biofilm upon treatment with respective oral rinses at 50% of their original concentration

The percentage of retaining bacterial population in the 24 hours dual-species biofilm will be described individually for the bacterial mixture.

The retention of *A.viscosus* and *S. mitis* (dual-species) was determined in the biofilm treated with oral rinses at their 50% concentrations (Figure 0.22). Biofilm treated with oral rinses showed bacterial retention in the following decreasing order: CHX > CPC. Statistical analysis was performed using One-way ANOVA, Bonferroni post-hoc test, significant difference was observed between the negative control (ddH2O) and the two treatment groups (p<0.05). Similarly a significant difference was observed on statistical analysis between CPC and CHX treatment groups (p<0.05).

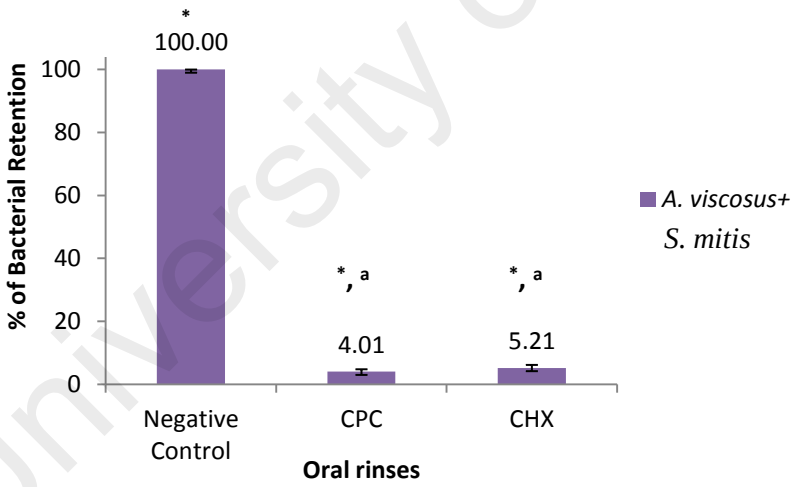


Figure 0.22: Percentage of *A. viscosus* + *S. mitis* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "[a]" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

Figure 0.23 clarifies the percentage of bacterial species retention: *A. viscosus* and *S. sanguinis* in the biofilm treated with the oral rinses at 50% (two fold dilution) of their original concentration. Dual-species retention showed the following decreasing manner: CHX > CPC. Statistically significant difference was observed between the negative control (ddH2O) and the two respective treatment groups (p<0.05), similarly a significant difference was observed in the percentage of bacterial retention in the biofilm treated with CPC and CHX treatment groups (p<0.05).

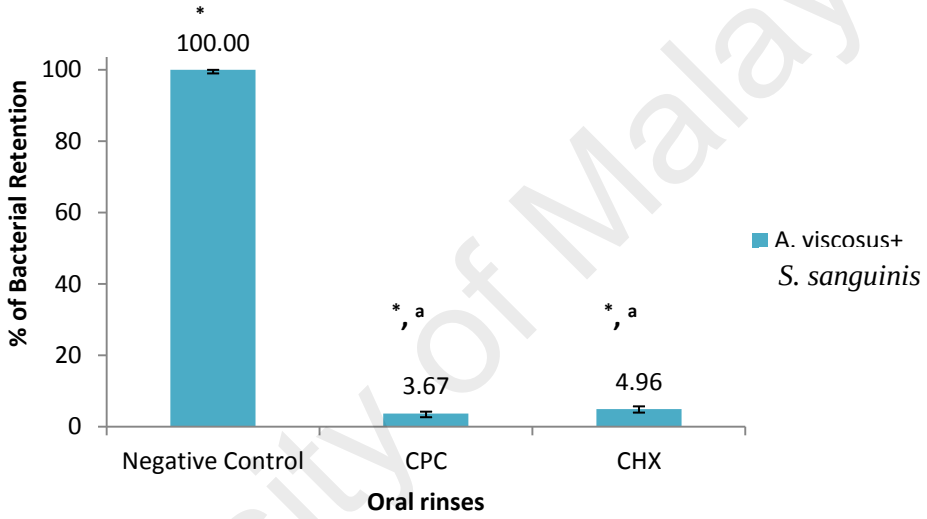


Figure 0.23: Percentage of *A. viscosus* +*S. sanguinis* retention expressed in Mean ± SD towards the 24-hrs biofilm treated with the respective oral rinses (50% concentration). The active ingredients were: CPC (Cetylpyridinium chloride) and CHX (Chlorohexidine gluconate). The percentage of retention for the negative control (ddH2O) was assumed to be 100 %. The individual experiment was performed in triplicate. Symbols " * " represents statistically significant difference observed between the negative control and the treatment groups (p<0.05); "[a]" represents significant difference between CPC- and CHX-treatment groups (p<0.05). The data was analyzed using One-way ANOVA, Bonferroni post-hoc test.

4.5.2.3. SEM Study for the Bacterial Population Retaining in the 24-hrs Biofilm (Single- And Dual-Species) Treated with Commercially Available Oral Rinses at 50% of their original concentration

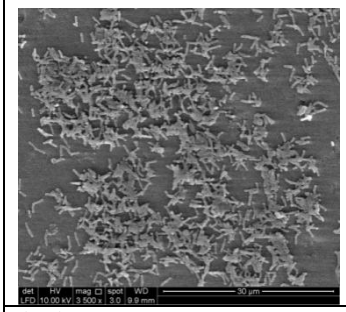
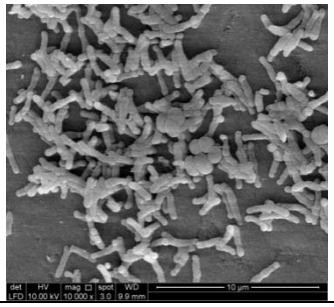
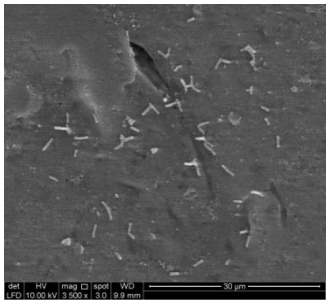
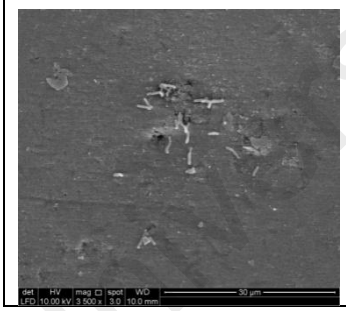
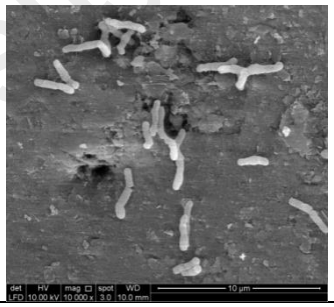
The observed characteristics of bacterial population in single- and dual-species biofilms are summarized in Tables 4.13, 4.14, 4.15, 4.16 and 4.17.

4.5.2.3.1. Bacterial Retention in Single-Species Biofilm treated with respective oral rinses at 50% of their original concentration

It was observed that the bacterial retention to the 24 hour biofilm treated with the respective oral rinse was observed to be less as compared to the negative control (Tables 4.13 - 4.15). Micrographs of all the single-species in 24 hours biofilms showed high numbers of bacterial cells existent on the glass beads with negative control (ddH2O). Compared to the negative control less retention of bacteria was allowed by CHX-treated biofilm and this was followed by CPC.

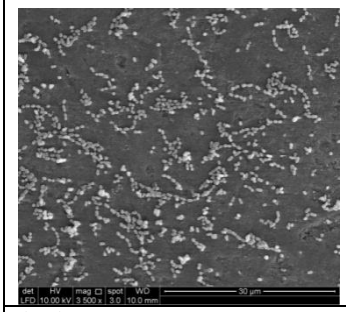
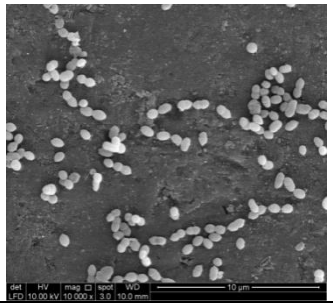
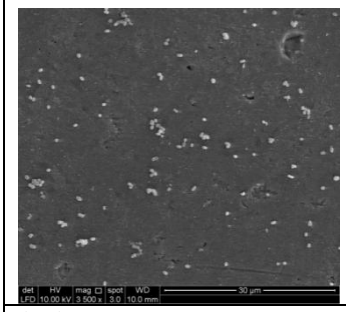
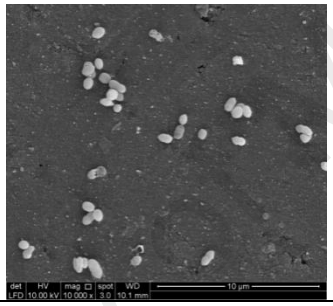
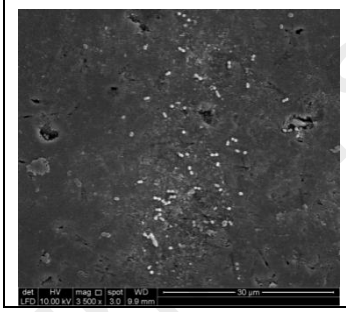
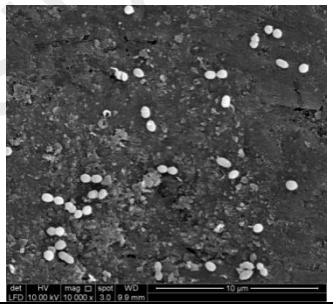
Bacterial population and characteristics of bacterial cells in single-species biofilms treated with various rinses are tabulated separately with respect to the bacterial species.

Table 0.13: Population and characteristics of *A. viscosus* retaining in Single-specie biofilms on the glass beads after treatment with the respective oral rinse (50% of their original concentration) observed under SEM.

A. viscosus		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Loads of rod cells were observed on the glass beads. • Cells were accumulated.
(B1) 	(B2) 	• More retention of rod cells was seen compared to CHX-treated beads. • Cells were present in dispersed pairs.
(C1) 	(C2) 	• Moderate number of rods was observed. • Cells were observed in pairs and scattered. • Individual cells were also present.

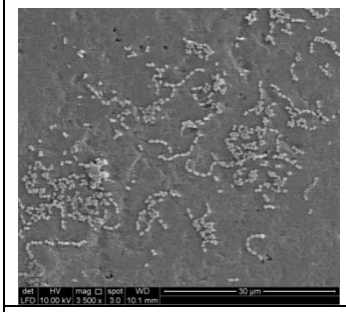
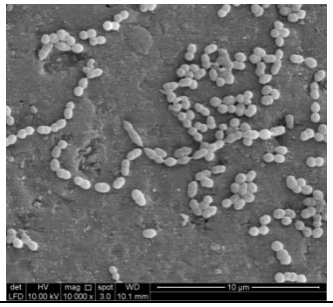
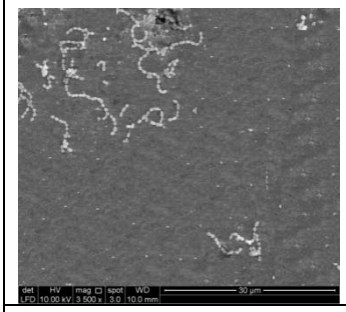
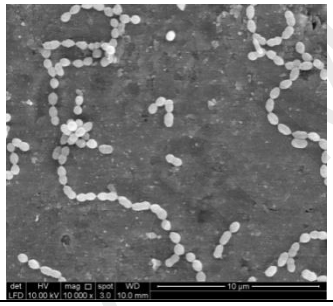
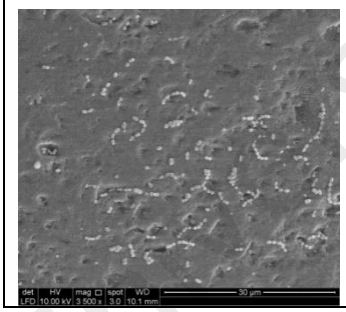
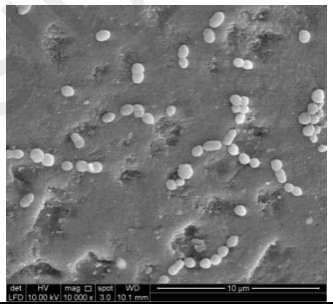
SEM micrographs (3500X and 10,000X) showing population of *A. viscosus* (rods) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated biofilm (Micrographs B1 and B2) and CHX-treated biofilm (Micrographs C1 and C2).

Table 0.14: Population and characteristics of *S. mitis* retaining in Single-species biofilms on the glass beads after treatment with the respective oral rinse (50% of their original concentration) observed under SEM.

S. mitis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Lots of round cocci cells were observed. • Cocci were dispersed in multiple small chains. • Many single cells were also seen.
(B1) 	(B2) 	• Cocci cells in reduced population was observed compared to negative control and CHX-treatment. • Cells were found in small groups of four to six cells. • Single cells were dispersed over the bead`s surface.
(C1) 	(C2) 	• More retention of cocci observed compared to CPC-treated surfaces. • Few short chains of cocci found. • Cells were discipated all over.

SEM micrographs (3500X and 10,000X) showing population of *S. mitis* (cocci) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated biofilm (Micrographs B1 and B2) and CHX-treated biofilm (Micrographs C1 and C2).

Table 0.15: Population and characteristics of *S. sanguinis* retaining in Single-species biofilms on the glass beads after treatment with the respective oral rinse (50% of their original concentration) observed under SEM

S. sanguinis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Round, small cocci were present in high number. • Cells were closely packed, forming long chains. • Few cells were found in groups.
(B1) 	(B2) 	• Moderate retention of cocci cells were observed compared to CHX. • Long chains were seen with closely packed round cells. • Individual cells were scattered.
(C1) 	(C2) 	• More retention of cocci was observed compared to CPC. • Cocci were found to be in short chains. • Cells were also making small groups and pairs.

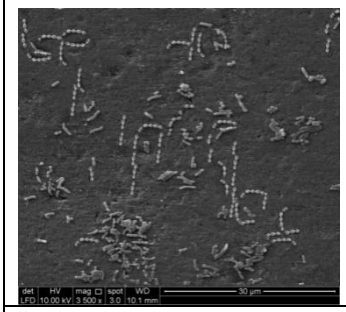
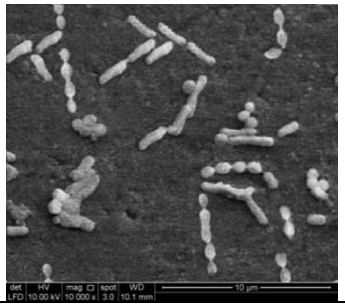
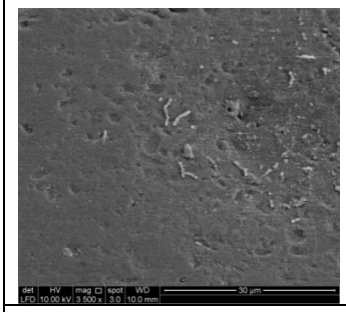
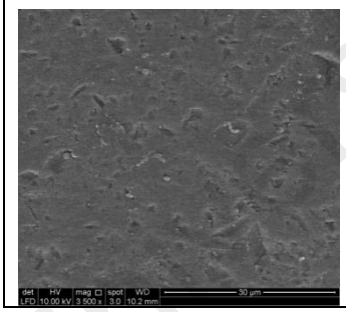
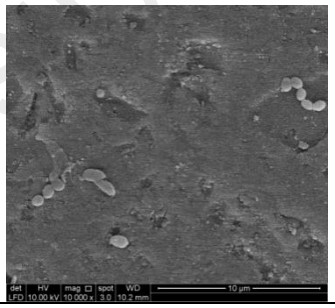
SEM micrographs (3500X and 10,000X) showing population of *S. sanguinis* (cocci) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated biofilm (Micrographs B1 and B2) and CHX-treated biofilm (Micrographs C1 and C2).

4.5.2.3.2. Bacterial Retention in Dual-Species Biofilm treated with the respective oral rinses at 50% of their original concentration

It was observed similar to that of single-species biofilm that the bacteria (dual-species) retaining to the biofilm treated with the respective oral rinse showed less retention compared to the negative control (Table 4.16 and 4.17). Micrographs of all dual-species in 24 hours biofilms showed high numbers of bacterial cells remaining on the glass beads (experimental pellicle) with negative control (ddH2O). After the negative control, CHX-treated biofilm showed moderately high retention of bacterial species and this was followed by CPC.

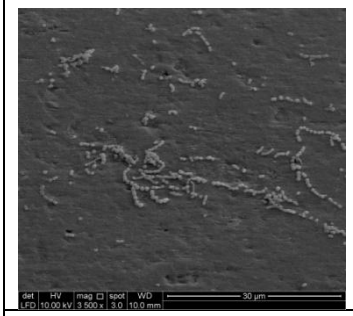
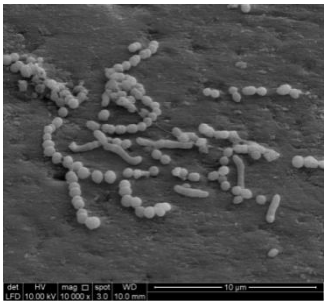
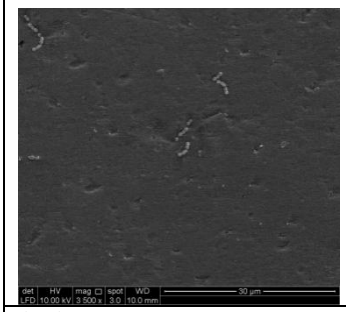
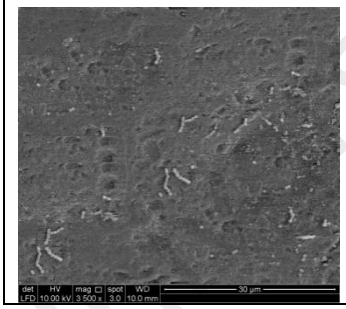
Bacterial population and characteristics of bacterial cells in the respective oral rinse-treated dual-species biofilms are summarized separately with respect to the bacterial species mixture.

Table 0.16: Population and characteristics of *A. viscosus* + *S. mitis* retaining in Dual-species biofilms on the glass beads after treatment with the respective oral rinse (50% of their original Concentration) observed under SEM.

A.viscosus* + *S. mitis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Huge number of rods and cocci cells observed. • Cocci were forming chains. • Rods were present in aggregated groups. • Groups of rod and round shaped cellswere also seen.
(B1) 	(B2) 	• Cooci cells were making chain like structures 4 to 7 cells/chain. • Fewer rod cells were seen with in the chains of cocci.
(C1) 	(C2) 	• Moderate retention of cells observed compared to CPC treated beads. • Cocci were seen in short chains, few were dispersed as single cells.. • Small debris of dead cells were also found.

SEM micrographs (3500X and 10,000X) showing population of *A. viscosus* (rods) and *S. mitis* (cocci) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated biofilm (Micrographs B1 and B2) and CHX-treated biofilm (Micrographs C1 and C2).

Table 0.17: Population and characteristics of *A. viscosus* + *S. sanguinis* retaining in Dual-species biofilms on the glass beads after treatment with the respective oral rinse (50% of their original concentration) observed under SEM.

A.viscosus + S. sanguinis		
Magnification		**Characteristics**
3500x	**10,000x**	
(A1) 	(A2) 	• Large population of rods and cocci observed. • Rods were dispersed all over the surface while cocci were making long, strangulated chains of multiple cells.
(B1) 	(B2) 	• Cooci cells were making short chains. • Few rod cells were aslso observed in 3500 x magnification.
(C1) 	(C2) 	• Moderate to high retention of cells observed compared to CPCtreated beads. • Cocci were seen in short chains, few were dispersed as single cells. • Rod cells were present with cocci in chains. • Small debris of dead cells were also found.

SEM micrographs (3500X and 10,000X) showing population of *A. viscosus* (rods) and *S. sanguinis* (cocci) in 24hour biofilms: Negative control (Micrographs A1 and A2), CPC-treated biofilm (Micrographs B1 and B2) and CHX-treated biofilm (Micrographs C1 and C2).

CHAPTER 5: DISCUSSION

The early plaque colonizers play an important role in the development of dental plaque. Therefore, if the bacterial load of dental plaque is not removed in early stage it can lead to various oral diseases. Even on brushing there are various sites of the tooth where bristles of toothbrushes cannot reach to clean the surfaces (interproximal surfaces, pits and fissures). If the bacterial load of the biofilm on such sites is not reduced, it will undergo maturation resulting in pathogenic bacterial complexes. These complexes will further lead to dental caries, gingivitis and periodontitis (Gurenlian, 2007). Thus in addition to mechanical, chemical (oral rinses) cleaning of the oral cavity is also required. At present there is an increasing interest to investigate the effect of commercially available oral rinses on the residents of the oral cavity. Many of the investigations have been focused on the ability of the active ingredients to either promote the growth of beneficial organisms or inhibit the growth and metabolism of oral bacteria associated with certain diseases. Oral rinses with active ingredients CPC, CHX and EO were selected for the study being most commonly used as they are regarded efficient products to reduce the dental plaque load (DePaola and Spolarich, 2007; Al-Bayaty *et al*., 2011; Osso & Kanani, 2013; Rahim *et al*., 2014).

5.1.Growth Curve Study

It was necessary to determine the growth phases of bacterial specie prior to their use in the study. As the bacterial cell size varies at different stages of growth. The bacterial cell is largest during the exponential growth phase whereas, during the lag and the death phase the bacterial cells are smaller in size (Clark & Ruehl, 1919; Fathilah *et al*., 2009). The bacterium is required to be in the exponential growth phase as it has a potential to perform normal metabolic function to grow and flourish (McCall., 2001; Fathilah *et al* ., 2009) Secondly during growth phase, the bacteria secrete a large amount of

extracellular polysaccharides (water-insoluble) which contribute to the plaque matrix and consolidates attachment of bacterial cells (Gurenlian, 2007). To form the oral biofilm of early plaque colonizers it was necessary to determine the dynamics of the bacterial growth as the time of onset of growth phase varies for the respective bacterial specie. The growth phase determined for *A. viscosus* was between 12^{th} -21^{st} hour whereas for *S. mitis* 7^{th} -15^{th} hour and 6^{th} -13^{th} hour for *S. sanguinis*. The possible reason for the variation in time is the size of the respective bacterial species. *S. sangunis* being the smallest bacterial specie was observed to show growth phase earliest when compared with the other bacterial species. It was followed by *S. mitis* then *A. viscosus.*

5.2.MIC and MBC of the Oral rinses

It was necessary to determine the MBC and MIC of the commercial oral rinses in order to use respective concentration to later study their effect on the adherence and retention of the early plaque colonizers in the simulated biofilm. The commercially available concentration of CHX and CPC rinses (100%) showed to be the MBC respectively where the 100% (available) concentration of EO was found to be the MIC for the single- and dual-bacterial species (EO did not show to have MBC value for any of the single- or dual-species). Therefore in further experiments EO was not used at 50% concentration to determine the bacterial adherence and retention. The two fold dilution of CHX and CPC (50%) were found to be their MIC respectively for both single- and dual-bacterial species. This is in an agreement with Al-Bayaty and co-workers (2011) who reported the MIC value of CHX for the various bacterial species including early plaque colonizers. They suggested 50% (two fold dilutions) of the original concentration of the commercially available CHX based oral rinse as the MIC value for the plaque bacteria (including early colonizers).

A number of *in vitro* mouth models have been established to study the oral biofilm and to apprehend the complexity of the oral environ**.** It offers various advantages (Greenman *et al.*, 2013) particularly making easier to study biofilm using modern microscope (SEM) and analyze the obtained data. In this study ultimate need was to use a validated artificial mouth system to develop the (*in vitro*) oral biofilm. Nordini`s artificial mouth (NAM) model is a bench scale *in vitro* mouth system which is suitable for the current study as it meets the parameters like consistent temperature, flow rate and the sterility of the artificial mouth system which ensure the precision and reproducibility of the outcomes. This has been validated in various studies determining the effect of plant extracts and oral rinses on the adhering and retaining properties of oral bacteria (single- and mixed-species) to the experimental pellicle (Rahim *et al*., 2014). In our study NAM model was used (a) to validate the bacterial population of biofilm formed on differently placed glass beads (near inlet, at the middle, near outlet) in the glass capillary tube of the Nordini`s artificial mouth system. b) To compare the effect of the different oral rinses on the adhering capacity (adherence) of selected oral bacteria to the experimental pellicle and c) To compare the effect of the different oral rinses on the retention of selected oral bacteria in the biofilm.

5.3.Difference in bacterial population of the biofilm developed on glass beads differently placed along the glass capillary tube (inlet, middle and outlet)

The bacterial adherence on the differently placed glass beads in the glass tube was determined to validate the bacterial population in the NAM model. The difference in the bacterial species adhered on differently placed glass beads for single- and dual-bacterial species (plaque colonizers) was determined to standardize the selection of glass beads for CFU determination. The difference between the bacterial adherence on the inlet, middle and outlet glass beads was observed to be less than 5% for all of the bacterial species harvested in the single- and dual- species biofilm. This suggests that the glass

beads at the 3 different locations should be used in the determination of bacterial population and the average of the 3 represent the bacterial population of the biofilm.

On comparing the CFU count of bacteria adhered in the single-species biofilm *S. mitis* showed to have maximum adherence potential. The adherence for the *S. sanguinis* was observed to decrease followed by the *A. viscosus*. This is in agreement with what has been reported by Hasnor *et al*., (2008) who determined the behaviors of the selected bacterial species adherence potential in single- and mixed-species biofilm. They used the same bacterial species of the early plaque colonizers as those of our study. The bacterial adherence reported for single species followed a similar adherence pattern to that of our results showing maximum bacterial adherence for *S. mitis,* followed by *S. sanguinis* and *A. viscosus*. However the pattern of bacterial adherence contradicted the results reported by Fathilah *et al.,*(1999), the possible reason could be the difference in conditions provided for bacterial cell growth. They used a system in which the cells are grown in a planktonic state (static), whereas in this study the bacterial cells are grown in a dynamic environment. Black *et al.,* (2004) and Ceri *et al.,* (1999) also reported difference on the bacterial adherence when grown under dynamic and planktonic conditions.

On comparing the CFU count for bacterial species adhered in dual-species biofilm *A.viscosus* and *S. mitis* exhibited higher adhering potential to the experimental pellicle as compared with *A.viscosus* and *S. sanguinis*. It is in agreement with a study reported by Hasnor (2008) that who observed similar pattern of bacterial adherence for the dual-species biofilm.

5.4.Effect of Oral Rinses on Adherence of Single- and Dual-Species in the Biofilm

The determination of bacterial adherence referred to the use of oral rinse before meals (before biofilm formation). Therefore the experimental pellicle was treated with respective oral rinses followed by pumping the respective bacterial specie inoculum for 24-hrs to develop simulated biofilm (Fatin-Majdina *et al*., 2014; Rahim *et al*., 2008).

5.4.1. Effect of Oral Rinses at 100% of the original Concentration on the Adherence of Single- and Dual-Species in the Biofilm

The adhering potential of *A. viscosus* was determined on pretreated biofilm with respective commercially available concentrations of oral rinses. The percentage adherence was found to be highest for biofilm pretreated with negative control. The percentage of adherence decreased for the biofilm pretreated with EO commercial available concentration (100%). Decrease in percentage was further observed for *A. viscosus* pretreated biofilm with commercially available concentrations of CHX followed by CPC.

Similar to *A. viscosus,* maximum *S. mitis* percentage of adherence was observed for biofilm pretreated with negative control. The percentage of adherence for *S. mitis* was observed to decrease for the biofilm pretreated with 100% concentration of EO, followed by (100%) CHX pretreated biofilm. The percentage of adherence further decreased for the biofilm pretreated with 100% concentration of oral rinse containing active ingredient CPC. The percentage of bacterial adherence observed for *S. mitis* biofilm pretreated with EO was approximately similar to that reported by Fatin-Majdina and co-workers (2014) (22.5%). They compared the effect of *Salvadora persica* (a medicinal plant which is recommended for use to clean teeth by the Muslims) on the adherence potential of *S. mitis* using NAM model. They used EO as a positive control

and ddH2O as the negative control (Fatin-Majdina *et al*., 2014). The percentage of adherence for *S. sanguinis* to pretreated biofilm was observed to be similar to that of *A. viscosus* and *S. mitis*. The maximum percentage was observed for the *S. sanguinis* biofilm pretreated with negative control followed by 100% concentration of EO. Decrease in percentage of adherence was observed for biofilm pretreated with 100% concentration of CHX. It was observed that the decrease in the percentage of adherence of *S. sanguinis* to the biofilm pretreated with 100% concentration of CPC is maximum. The percentage adherence for *S. sanguinis* was observed to be higher on pretreatment with EO as compared to results reported by Fatin-Majdina *et al*., (2014). There could be various reasons for the difference in the percentage of *S. sanguinis* adherence most importantly the bacterial species used in our study were from the American Type Culture Collection (ATCC) whereas they probably used the clinical isolates of *S. sanguinis* which can likely produce the difference. Secondly the saliva of individual varies from person to person in the amount of protein content which plays an important role for the adherence of the bacterial species to the experimental pellicle as saliva contains salivary amylase which produces dextrin; this amylase takes part in the formation of acquired pellicle on tooth, also binds with pili of *S. sanguinis* oral bacteria and promotes bacterial adhesion subsequently formation of plaque (Fábián *et al.*, 2012; Rogers *et al.*, 2001; Scannapieco *et al.*, 1995).

The dual-species biofilms pretreated with 100% concentration of various oral rinses showed a similar pattern of bacterial adherence to that of single-species, except the total number of bacterial cells adhered decreased for the dual-species biofilm pretreated with commercially available oral rinses. The dual species *A. viscosus* and *S. mitis* showed maximum bacterial percentage of adherence on pretreatment with negative control. The percentage of adherence was observed to decrease for the *A. viscosus* and *S. mitis* biofilm pretreated with 100% concentration of EO followed by pretreatment with 100%

concentration of CHX respectively. Minimal percentage of bacterial adherence was observed for the biofilm pretreated with 100% concentration of CPC.

The biofilm for dual-species *A. viscosus* and *S. sanguinis* pretreated with negative control showed maximum percentage of adherence. The percentage of adherence decreased for the biofilm pretreated with 100% concentration of EO, further decrease was observed on pretreatment with 100% concentration of CHX. The biofilm pretreated with CPC showed minimal percentage of bacterial adherence. It is in agreement with the study reported by Witt *et al.,* (2005). Witt and co-workers carried out study on a population of 20 individuals who were instructed to use oral rinse after brushing the teeth with a dentifrice. Three groups of individuals were compared given following regimes to rinse after brushing 1) placebo 2) CPC containing rinse 3) EO containing rinse. Significant reduction in the adherence was observed of dental plaque consisting of *A. viscosus*. They have also reported that the adherence reduction for individuals using CPC oral rinse was similar to that of individuals using EO. In this study it was shown that the EO containing oral rinse is not as potent as CPC containing oral rinse. The possible reason could be that we conducted an *in vitro* experiment, whereas they conducted an *in vivo* experiment. In an *in vivo* environment there could be various factors that could lead to reduction in the bacterial adherence in the biofilm such as the salivary clearance.

5.4.2. Effect of the Oral Rinses at 50 % of the original Concentration of on the Adherence of Single- and Dual-Species in the Biofilm

The CPC and CHX containing respective oral rinses were used to determine the bacterial adherence of single- and dual- species to the experimental pellicle on using two fold dilution (50%). The main reason to use CPC and CHX containing respective oral rinse on two fold dilution (50%) was they were observed to be the MIC

concentration for the single and dual bacterial species. Therefore, EO was excluded from the study of 50% concentration of oral rinses, as the commercially available concentration was observed to be the MIC value.

The percentage of *A. viscosus* adherence was found to be highest for biofilm pretreated with negative control. The percentage of adherence decreased for the biofilm pretreated with CHX two fold dilution of the commercially available concentration (50%). Decrease in percentage was further observed for *A. viscosus* pretreated biofilm with 50% concentration of CPC commercially available oral rinse.

Similarly maximum percentage of adherence was observed for *S. mitis* pretreated biofilm with negative control. The percentage of adherence for *S. mitis* was observed to decrease for the biofilm pretreated with 50% concentration of CHX, followed by (50%) CPC pretreated biofilm.

The percentage of adherence for *S. sanguinis* in the pretreated biofilm was observed to be similar for *A. viscosus* and *S. mitis*. The maximum percentage was observed for the *S. sanguinis* biofilm pretreated with negative control followed by 50% concentration of CHX. The percentage of adherence of *S. sanguinis* was observed to maximum decrease for the biofilm pretreated with 50% concentration of CPC.

The dual-species *A. viscosus* and *S. mitis* showed maximum bacterial percentage of adherence on pretreatment with negative control. The percentage of adherence was observed to decrease for the *A. viscosus* and *S. mitis* biofilm pretreated with CHX (50%) followed by CPC at 50% of the commercially available concentrations respectively.

The biofilm for dual-species *A. viscosus* and *S. sanguinis* pretreated with negative control showed maximum percentage of adherence. The percentage of adherence decreased for the biofilm pretreated with 50% concentration of CHX. The biofilm

pretreated with 50% concentration of commercially available CPC showed minimal percentage of bacterial adherence.

5.5.Effect of Oral Rinses on Retention of Single- and Dual-Species in the Biofilm

To determine the effect of oral rinses if used after having food, the methodology was designed accordingly and was regarded as bacterial retention. The biofilm of single- and dual-bacterial species was allowed to develop for 24 hrs followed by treatment with the respective oral rinse (Fatin-Majdina *et al*., 2014).

5.5.1. Effect of 100% Concentration of Oral Rinses on Retention of Single- and Dual-Species in the Biofilm

The percentage of *A. viscosus* retention was determined on biofilm treated with respective commercially available concentrations (100%) of oral rinses. The percentage of *A. viscosus* retained was observed to be highest for biofilm treated with ddH2O (negative control). The percentage of retention was found to decrease for the biofilm treated with 100% concentration of EO commercial available. The percentage of retention was observed to decrease for *A. viscosus* biofilm treated with commercially available concentrations of CHX. There was a further decrease in the retention of *A. viscosus* in biofilm treated with CPC. In this study the pattern of reduction of *A. viscosus* retention is in agreement with a study reported by Witt and co-workers in which they tested various plaque colonizers using a kill kinetic model. They reported that CPC has a potent effect of killing *A. viscosus* up to 99.5%. Therefore it has been suggested that CPC containing oral rinse has a capability to reduce the bacterial retention (Witt *et al*., 2005).

Percentage of bacterial retention of *S. mitis*, similar to *A. viscosus* was observed to be maximum on the biofilm treated with negative control. The percentage of retention for

S. mitis was observed to decrease for the biofilm on treated with 100% concentration of EO, followed by (100%) CHX treated biofilm. The percentage further decreased for the biofilm treated with 100% concentration of oral rinse containing active ingredient CPC. The percentage of bacterial retention on EO treated *S. mitis* biofilm was observed to be extremely low in our study (3.99 %) as compared to the results reported by Majidina and co-workers (2014) (31.0 %). Secondly the percentage of bacterial retention was higher in their study as compared to the percentage of bacterial adherence. It is in disagreement with the results obtained in this study, as the percentage of bacterial retention in this study was lower than the percentage of bacterial adherence in the biofilm for *S. mitis*. Though it is indicated by the manufacturer that the bacterial count is decreased on use of oral rinse after dental plaque formation, which is in agreement with our results. Similarly it has been reported EO has a potential to penetrate dental plaque (after biofilm formation) and plays an active role against bacteria embedded in the plaque and kills a wide range of aerobic and anaerobic bacterial species (DePaola and Spolarich, 2007; Fine *et al.*, 2001; Foster *et al.*, 2004; Ouhayoun, 2003; Pan *et al.*, 2000).

Similar to *A. viscosus* and *S. mitis*, the percentage of *S. sanguinis* retention on the biofilm was observed to be maximum for the negative control followed by 100% concentration of EO. Decrease in percentage of retention was observed for biofilm treated with 100% concentration of CHX. Maximum decrease in the percentage of *S. sanguinis* retention was observed for the biofilm treated with 100% concentration of CPC. Witt and co-workers tested effect of CPC on *S. sanguinis* using a kill kinetic model and reported its potential to kill up to 99.9% bacterial population. It is in agreement with our results that CPC containing oral rinse has a capability to reduce the retention potential of bacteria (Witt *et al.*, 2005). Fatin-Majdina *et al* (2014) reported higher percentage of *S. sanguinis* retention (after meals) on treatment of biofilm with

EO containing oral rinse as compared to treatment of experimental pellicle with EO based oral rinse (before meals). It is in disagreement with our results where the percentage of bacterial retention was observed to decrease for EO treated biofilm as compared to percentage of adherence (treatment of experimental pellicle before the development of biofilm). However, results are comparable with studies reported by various researchers suggesting the potential of EO based oral rinse to kill *S. sanguinis* containing biofilm (Ross *et al.*, 1989).

The dual-species biofilm of *A. viscosus* and *S. mitis* showed maximum percentage of retention with negative control. The percentage was observed to decrease dual-species (*A.viscosus* and *S.mitis*) retention in the biofilm on treatment with EO(100%) followed by biofilm treated with 100% concentration of CHX. Low bacterial retention was observed on the biofilm treated with 100% concentration of CPC. The percentage of dual-species retention (*A.viscosus* and *S.mitis*) in the biofilm was observed to be lower as compared to the percentages of adherence on treatment with 100% concentration of the respective oral rinses.

The *A. viscosus* and *S. sanguinis* biofilm on treatment with negative control showed maximum percentage of species retention. The percentage was observed to decrease for the biofilm treated with 100% concentration of EO; further decrease in the percentage of bacterial retention was observed for the biofilm treated with 100% concentration of CHX. Minimal percentage of bacterial retention was observed for the *A. viscosus* and *S. sanguinis* biofilm treated with 100% concentration of CPC. The retention percentage of *A. viscosus* and *S. sanguinis* in the biofilm on treatment with 100% concentration of respective oral rinses was observed to be lower in comparison to percentage of bacterial adherence (treated with 100% concentration of oral rinses).

5.5.2. Effect of 50 % Concentration of Oral Rinses on Retention of Single- and Dual-Species in the Biofilm

The oral rinse used to determine the bacterial retention on two fold dilution (50%) included CPC and CHX. EO was not included in the study to determine bacterial retention at 50% concentration because the concentration available commercially was observed to be the MIC value.

Maximum percentage of *A. viscosus* retention was observed for the biofilm treated with negative control. Decreased in the percentage retention on the biofilm was observed on treatment with two fold dilution of CHX (50% of the commercially available concentration). Maximum reduction in percentage of retention was observed for the *A. viscosus* biofilm treated with 50% concentration of CPC commercially available oral rinse.

Similarly maximum percentage of *S. mitis* retention was observed on the biofilm treated with negative control. The percentage of retention was observed to decrease on treating the biofilm with 50% concentration of CHX, followed by (50%) CPC treated biofilm.

The percentage of *S. sanguinis* retention was highest on biofilm treated with negative control similar to that for *A. viscosus* and *S. mitis*. The percentage was observed to decrease for the biofilm treated with 50% concentration of CHX, maximum reduction in percentage of retention was observed on the biofilm treated with 50% of the CPC commercially available concentration.

The maximum percentage of retention was observed on the dual-species *A. viscosus* and *S. mitis* biofilm treated with negative control. The percentage retention was

decreased for the biofilm treated with CHX (50%) followed by CPC 50% of the commercially available concentrations respectively.

The maximum percentage of *A. viscosus* and *S. sanguinis* retention was observed to be on the biofilm treated with negative control. The percentage of retention decreased on the biofilm treated with 50% concentration of CHX. The maximum reduction in percentage of retention was observed for the biofilm treated with 50% concentration of CPC.

CHAPTER 6: CONCLUSION

The adhering capacity of early plaque colonizers bacteria to the experimental pellicle and subsequently their population in the oral biofilm formed is proficiently reduced by using oral rinse in effective concentration and at the right time to avoid any disturbance in the normal ecology of florae. This capacity of bacteria to adhere/retain is also influenced by the growing situation as well as the other bacterial species in the mixture. CPC rinse and CHX rinse when used at their commercially available concentration (100%) respectively were found to be very effective formulations against the bacterial adhesion (referred to the usage before meal) and at 50% (half of their original concentration) respectively were effective against the bacterial retention (referred to the usage after meal). For the maintenance of oral ecology 50% concentration of (CPC and CHX) is preferable to be used after meal. For EO, it is recommended to be used after meal at the commercially available concentration. This was because at that concentration (100% of the original concentration), the effect of EO on bacterial population in the biofilm at that concentration was only satisfactory when compared with the other two oral rinses.

Future Studies

Future studies are required with these commercial oral rinses in different scenarios:-

i) Determining the effect of oral rinses in NAM model with a modification (using human enamel rather than glass beads) treating them with respective oral rinse and bacterial inoculum.

ii) Determining the effect of oral rinses on the oral biofilm of other oral bacterial species should be tested using NAM model.

iii) Comparing the use of these three oral rinses in clinical trials by dividing different groups using the treatment after meals at different concentrations respectively.

iv) Comparing the use of these three oral rinses in clinical trials by dividing different groups using the treatment before meals at different concentrations respectively.

Limitations of Study

The limitation of the study was due to financial constrain with respect to research grant.

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