IN VITRO STUDY ON THE EFFECT OF PLANT EXTRACT MIXTURE AND ITS INDIVIDUAL CONSTITUENTS TOWARDS SINGLE- AND DUAL-SPECIES BIOFILMS

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FACULTY OF DENTISTRY
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Name of Candidate: Zaleha Binti Shafiei

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Field of Study: Oral Biology

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ABSTRACT

The plant extract mixture (PEM) and its individual constituents (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.) were investigated *in vitro* for their anti-plaque and anti-caries potential towards *Streptococcus sanguinis* ATCC BAA-1455 and *Streptococcus mutans* ATCC 25175 in single- and dual-species biofilms. The ability of these plant extracts as antibacterial, anti-adherence agents and in reducing bacterial population in biofilms, cell-surface hydrophobicity and acid production activities were determined. The reduction in expression of adhesins genes of *S. mutans* (*gtfB, gtfC, gbpB* and *spaP*) due to treatment with PEM in planktonic and biofilms conditions was also determined using Real-time RT-PCR analysis. The anti-adherence and anti-biofilm assays were determined based on bacterial population retained in biofilms using the percentage colony forming unit per ml (CFU/ml) and visualised under Scanning Electron Microscope (SEM). The phenolic compounds of the plant extracts were assessed by Total Phenolic Content (TPC) and UPHLCMS/MS analysis. Fold change in the expression of *S. mutans* adhesins-genes (*gtfB, gtfC, gbpB* and *spaP* genes) relative to the internal control in the PEM-treated samples compared to the untreated control was calculated using relative quantification of $2^{-\Delta\Delta Ct}$ method. All the plant extracts exhibited antibacterial activities with the PEM (MIC value of 1.96 mg/ml) was the highest and exhibiting synergistic interaction. At sub-MIC value of 0.5 mg/ml, the plant extracts highly reduced the bacterial adherence towards experimental pellicle and bacterial populations of *S. sanguinis* and *S. mutans* in single- and dual-species biofilms. PEM significantly reduced adherence capacity by 48-49% and creating equilibrium communities of *S. sanguinis* and *S. mutans,* and was reflected in the SEM analysis. In normal condition, sucrose enhanced the growth of *S. mutans* (by 10^7 CFU/ml) and restricted *S. sanguinis* (10^5 CFU/ml) growth. However, the equal proportion (10^6 CFU/ml) of the two bacteria was observed in dual-species biofilms grown in the absence and presence of sucrose. PEM and *Mangifera* sp. effectively reduced *S. mutans*

population in single- and dual-species biofilms grown in the absence of sucrose. In the presence of sucrose, PEM effectively reduced both the two types of bacterial population in single-species biofilm but *S. mutans* was hard to remove compared to *S. sanguinis* in dual-species biofilms. PEM exhibited the highest potential in reducing cell-surface hydrophobicity and acid production of the two types of bacteria (in single- and dual-species). PEM as a potential anti-plaque and anti-caries agent correlates with the synergistic interaction of different active compounds present in the extract. PEM strongly reduced the expression of *gtfB* genes of *S. mutans* by 2.04-fold (in singly, planktonic condition) and by 2.08-fold (in dual-species, biofilm condition). The expression of *gtfC*, *gbpB* and spaP genes were upregulated in both planktonic and biofilm conditions allowing the *S. mutans* to adhere and form biofilms in the presence of sucrose. In conclusion, the results strongly supported that the PEM is a better candidate as the anti-plaque and anti-caries agent and could be incorporated in oral healthcare product with further validation of the product *in vivo*.

ABSTRAK

Campuran ekstrak tumbuhan (PEM) dan ekstrak tumbuhan individu masing-masing (*Psidium* sp., *Mangifera* sp. dan *Mentha* sp.) telah dikaji secara *in vitro* untuk potensi sebagai agen anti-plak dan anti-karies terhadap *Streptococcus sanguinis* ATCC BAA-1455 dan *Streptococcus mutans* ATCC 25175 dalam biofilem spesies tunggal dan dwi-spesies. Keupayaan ekstrak tumbuhan ini sebagai agen antibakteria, anti-pelekatan dan dalam mengurangkan populasi bakteria dalam biofilem, hidrofobisiti permukaan sel dan aktiviti pengeluaran asid telah dikaji. Pengurangan ekspresi gen-gen adhesins *S. mutans* (gen-gen *gtfB, gtfC, gbpB* dan *spaP*) dengan rawatan PEM secara planktonik dan biofilem juga ditentukan menggunakan analisis kuantitatif RT-PCR. Ujian anti-pelekatan dan anti-biofilem ditentukan berdasarkan populasi bakteria dalam biofilem menggunakan unit bilangan koloni yang terbentuk per ml (CFU/ml) dan diperhatikan di bawah Mikroskop Pengimbasan Elektron (SEM). Komponen kimia ekstrak tumbuhan ditentukan menggunakan analisis Total Phenolic Content (TPC) dan UPHLCMS/MS. Perubahan berganda dalam ungkapan gen-gen mutasi adhesins (gen-gen *gtfB, gtfC, gbpB* dan *spaP*) berbanding dengan kawalan dalaman bagi sampel yang dirawat PEM berbanding dengan kawalan yang tidak dirawat dikira menggunakan kaedah kuantifikasi relatif $2^{-\Delta\Delta Ct}$. Semua ekstrak tumbuhan yang mempamerkan aktiviti antibakteria dengan PEM (nilai MIC 1.96 mg/ml) mempamerkan interaksi sinergistik tertinggi. Pada nilai sub-MIC 0.5 mg/ml, ekstrak tumbuhan mengurangkan pelekatan bakteria terhadap pelikel experimen dan populasi bakteria *S. sanguinis* dan *S. mutans* dalam biofilem species tunggal dan biofilem dwi-spesies. PEM mengurangkan kapasiti pelekatan sebanyak 48-49% dan mewujudkan populasi seimbang di antara *S. sanguinis* dan *S. mutans* dalam biofilem dwi-spesies dan dipamerkan melalui analisis SEM. Pada keadaan normal, sukrosa meningkatkan pertumbuhan *S. mutans* (oleh 10^7 CFU/ml) dan merencat pertumbuhan *S. sanguinis* (10^5 CFU/ml). Walau bagaimanapun, bilangan koloni yang sama (10^6 CFU/ml) bagi kedua-dua

bakteria terbentuk dalam biofilem dwi-spesies yang tumbuh dalam media tanpa dan dengan kehadiran sukrosa. PEM dan *Mangifera* sp. berkesan mengurangkan populasi *S. mutans* dalam biofilem species tunggal dan dwi-spesies dalam media tanpa sukrosa. Dalam media mengandungi sukrosa, PEM berkesan mengurangkan kedua-dua jenis populasi bakteria dalam biofilem spesies tunggal tetapi *S. mutans* sukar disingkirkan berbanding dengan *S. sanguinis* dalam biofilem dwi-spesies. PEM mempamerkan potensi tertinggi dalam mengurangkan hidrofobisiti permukaan sel dan pengeluaran asid dari kedua-dua jenis bakteria (dalam spesies tunggal dan dwi-species). PEM sebagai agen anti-plak dan anti-karies yang berpotensi berkorelasi dengan interaksi sinergistik sebatian-sebatian aktif yang berbeza dalam ekstrak. PEM mengurangkan ekspresi gen *gtfB S. mutans* sebanyak 2.04 kali ganda (dalam species tunggal, planktonik) dan 2.08 kali ganda (dalam dwi-spesies, biofilem). Pengekspresan gen-gen *GtfC*, *gbpB* dan *spaP* meningkat dalam keadaan planktonik dan biofilem membolehkan *S. mutans* melekat dan membentuk biofilem dengan kehadiran sukrosa. Kesimpulannya, hasil kajian ini sangat menyokong bahawa PEM adalah calon yang terbaik sebagai agen anti-plak dan anti-karies dan boleh diintegrasikan dalam produk penjagaan kesihatan mulut selepas kajian lanjut secara *in vivo.*

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LIST OF SYMBOLS AND ABBREVIATIONS

g	:	Gram
mg	:	Milligram
µg	:	Microgram
µl	:	Microliter
L	:	Litre
%	:	Percentage
µM	:	Micromolar
M	:	Molar
NSPH	:	Non-specific Plaque Hypothesis
SPH	:	Specific Plaque Hypothesis
EPH	:	Ecological Plaque Hypothesis
KPH	:	Keystone-Pathogen Hypothesis
BHI	:	Brain heart infusion
EPS	:	Extracellular polysaccharide
IPS	:	Intracellular polysaccharide
PUM	:	Phosphate urea magnesium
MATH	:	microbial adherence test to hydrocarbon
NAM	:	Nordini Artificial Mouth
CFU/ml	:	Colony Forming Unit per millilitre
^{o}C	:	Degree Celsius
nm	:	Nanometre
OD	:	Optical density

w/v	:	Weight per volume
v/v	:	Volume per volume
w/w	:	Weight per weight
SD	:	Standard deviation
min	:	Minute
bp	:	base pair
kb	:	Kilobase
DTT	:	1,4-Dithio-DL-threitol
TAE	:	Tannic acid equivalent
UPHLCMS/MS	:	ultra-high-performance liquid chromatography system coupled with a tandem mass spectrometer
NTC	:	No template control
PBS	:	Phosphate Buffered Saline
CHX	:	Chlorhexidine
SEM	:	Scanning electron microscope
PCR	:	Polymerase chain reaction
qRT-PCR	:	Quantitative reverse transcriptase-polymerase chain reaction or (Real-Time Polymerase chain reaction)
Ct	:	Threshold cycle
ΔΔCt	:	Delta-delta Ct

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Dental caries is the most prevalent and costly oral disease worldwide. Dental caries results from the interactions of specific bacteria and their metabolic/virulence products with salivary constituents and dietary carbohydrates, that occurs on susceptible tooth surface. The formation of the extracellular polysaccharide (EPS)-rich biofilm matrix, acidification of the milieu and the maintenance of a low-pH environment at the tooth-biofilm interface are major controlling virulence factors that modulate dental caries pathogenesis. Biofilms formed *in vivo* are comprised of mixed flora, although Mutans group streptococci are recognised as the primary producers of the EPS-rich matrix. *Streptococcus mutan*s (*S. mutans*) plays a key role in the development of virulent biofilms, although additional microorganisms may be also involved in the pathogenesis of the disease (Beighton, 2005; Moraes *et al.*, 2002; Wang *et al.*, 2017). This bacterium (i) effectively utilises dietary sucrose (and possibly starch) to rapidly synthesise EPS through the activity of glucosyltransferases (Gtfs) and a fructosyltransferase that are adsorbed to saliva-coated tooth enamel surfaces, (ii) adheres tenaciously to glucan-coated surfaces, and (iii) is both acidogenic and acid tolerant (Bowen & Koo, 2011; Quivey *et al.*, 2000). Thus, *S. mutans* thrives in the complex oral microbiota and effectively modulates the transition from nonpathogenic to cariogenic biofilms.

Besides *S. mutans*, *Streptococcus sanguinis* (*S. sanguinis*) was chosen in this study due to its ability to have antagonistic role in dental caries and periodontal disease. The *S. sanguinis*, being an early plaque coloniser of biofilm and health-compatible oral commensal, attaches directly to the acquired pellicle and serves as a tether for adherence of late coloniser such as *S. mutans* in dental plaque (Becker *et al.*, 2002). Previous studies showed early colonisation of *S. sanguinis* in dental plaque that significantly correlated

with a delay in colonisation of *S. mutans* and thus, could result in a reduction in dental caries (Caufield *et al.,* 2000; Kreth *et al.,* 2005; Ge *et al.,* 2008). *In vitro* study showed that bacteriocin (mutacins) produced by *S. mutans* and hydrogen peroxide produced by *S. sanguinis* are used as chemical weapons for interspecies competition (Kreth *et al.,* 2005). However, caution is warranted here because the S. *sanguinis* has been implicated in infective endocarditis. Stingu *et al.* (2008) reported that aggresive periodontitis is associated with a loss of colonisation of *S. sanguinis*. Pre-colonisation with *S. sanguinis* reduced the level of *Porphyromonas gingivalis* (*P. gingivalis*) in experimental rats (Zhang *et al.,* 2000). Therefore, reducing the level of *S. mutans* and maintaining the population of *S. sanguinis* may create a healthy oral cavity.

Although fluoride and chlorhexidine remain the mainstay for the prevention of caries, natural products as a rich source of structurally diverse substances with a wide range of biological activities could be useful for the development of alternative or adjunctive anticaries therapies. Natural products have been used for thousands of years in traditional medicine for many purposes. Many herbal remedies have been used because of their antibacterial, anti-inflammatory, antifungal and antiviral activities. Cloves, which contain eugenol, have been used for a thousand years to alleviate the pain of toothache, disinfect root canals in temporary fillings and as an oral anaesthesia. It also inhibits the growth of bacteria with broad antimicrobial activities against Gram-positive, Gram-negative, acid-fast bacteria as well as fungi (Chaieb *et al.,* 2007). *Piper betle* L. has been reported to exhibit antibacterial activities towards *Streptococcus mitis* (*S. mitis*), *S. sanguinis* and *Actinomyces viscosus* (*A. viscosus*), some of the early colonisers of dental plaque and inhibits the adherence of these bacteria to the saliva-coated glass surface (Razak & Rahim, 2003). *Mangifera indica* L. (*M. indica* L.) (Anacardiaceae) also known as the mango plant is one of the most important tropical plants; its bark, roots and leaves have many traditional medicinal uses throughout the globe. Mangiferin isolated from *M. indica*

are shown to have antioxidant, radioprotective, antitumour, immunomodulation, anti-allergic, anti-inflammatory, anti-diabetic, lipolytic, anti-bone resorption, anti-ulcerogenic action on rodents, antiviral, antifungal, antibacterial and antiparasitic properties (Derese *et al.*, 2017; Severi *et al.*, 2009). It has also been reported based on *in vivo* study that *M. indica* leaf can cause a significant reduction in the proportion of *Prevotella intermedia* (*P. intermedia*) and *P. gingivalis* compared with toothbrush users when used as home care hygiene device (Bairy *et al.*, 2002).

Psidium guajava L. (*P. guajava* L.) (Myrtaceae), also known as the guava plant, is used in ethnomedicine as an antimalarial treatment. Infusions of the leaves are used for treating fevers, for diarrhoea and as a tonic in psychiatry. The leaves of *P. guajava* contain pentacyclic triterpenoid guajanoic acid, beta-sitosterol, uvaol, oleanolic acid and ursolic acid (Begum *et al.*, 2004). The ethyl acetate extract of *P. guajava* leaf extract exhibits antimicrobial activities towards *S. mutans* and *Candida albicans* (Jebashree *et al.*, 2011). However, no study on the effect of its leaf extract against multi-species biofilms either *in vitro* or *in vivo* study was reported.

The methanolic extract of peppermint leaves (*Mentha piperita*: Lamiaceae) was found to contain tannin and flavonoids with antibacterial and antifungal activities against selected oral pathogens. Regular intake of mint leaves are presumed to ward off the initial colonisation of pathogenic microbes (Pramila *et al.*, 2012). No study had reported the effect of these plant extracts against multi-species biofilms so far.

It has been shown *in vitro* that a mixture of aqueous extracts of three plant species (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.) which is referred to as Plant Extract Mixture (PEM) in this study has an anti-adherence effect towards early plaque colonisers as well as towards early and late colonisers in single-species biofilm (Rahim *et al.*, 2014; Wan Nordini Hasnor *et al.*, 2013). To date, there is no study on the effect of PEM and its

individual extracts on dual-species biofilms in a dynamic environment. Thus, this study was conducted to investigate the effects of PEM and its respective plant extracts towards single- and dual-species biofilms with respect to antibacterial, anti-adherence activities, reduction of bacterial population in biofilm and other virulence factors (such as acid production and cell-surface hydrophobicity) to determine whether the selected plant extracts have the potential as anti-plaque and anti-caries agents.

1.2 Objectives

The aim of this study is to investigate the anti-plaque and anti-caries agents of the plant extracts using microbiological and molecular approaches.

Specific objectives of this study are:

1. To screen antibacterial activity of the crude aqueous plant extracts using broth microdilution (MIC, MBC and FIC assays).
2. To determine the adhering capacities of oral bacteria (single- and dual-species) towards crude aqueous plant extracts (individual and mixture of plants)-treated experimental pellicle in a dynamic environment and its effect on the morphology of bacteria using Scanning Electron Microscope.
3. To determine the reduction of bacterial population in the crude aqueous plant extracts (individual and mixture of plants)-treated biofilms (single- and dual-species) with and without sucrose in a dynamic environment.
4. To determine the effect of the crude aqueous plant extracts on cell-surface hydrophobicity and acid production of *S. mutans* individually and co-culture with *S. sanguinis*.

5. To identify the phenolic compounds of the plant extracts which may have possible role as anti-adherence agents towards treated-experimental pellicle and reduction of bacterial population in biofilm (anti-biofilm).
6. To determine the regulatory expression of adhesins genes of *S. mutans* (*gtfB*, *gtfC*, *gbpB* and *spaP*) due to treatment with a plant extract mixture (PEM) using planktonic and biofilm conditions in the presence of sucrose using Real-Time-PCR analysis.

CHAPTER 2: LITERATURE REVIEW

2.1 Microbial diversity of human oral cavity

The mouth or human oral cavity is that part of the body bound by the cheeks, lips, palate, and the floor of the mouth (the area beneath the tongue). The nasal cavity is located above the mouth and is separated from the mouth by the hard and soft palate. Within the oral cavity is the tongue, teeth, tissue supporting the teeth (gums and bone) and salivary glands. The pharynx or throat is the region located behind the oral cavity.

The oral cavity is considered as an ideal habitat for numerous microorganisms because of its warm and moist in nature with a constant supply of nutrients from saliva and food intake. This condition makes oral cavity as a favourable site for growth of normal microbiota which lives in both healthy and non-healthy people. The oral cavity is an open growth system with an uninterrupted introduction and removal of microbes and their nutrients. It offers diverse habitats including the teeth, gingival sulcus, tongue, cheeks, hard and soft palates, and tonsils, wherein different species of microorganisms can prosper (Batabyal *et al.*, 2012; Marsh *et al.*, 2009) (Figure 2.1). Microorganisms must attach to a surface and form biofilms to persist. Once organisms have colonised, nutrients must be acquired for growth and cell division, and species must compete with other resident microbes to become established (Dewhirst *et al.*, 2010; Zaura *et al.*, 2009). Furthermore, the microbial inhabitants must develop strategies to cope with the innate and adaptive arms of the host defences (Marsh *et al.*, 2009).

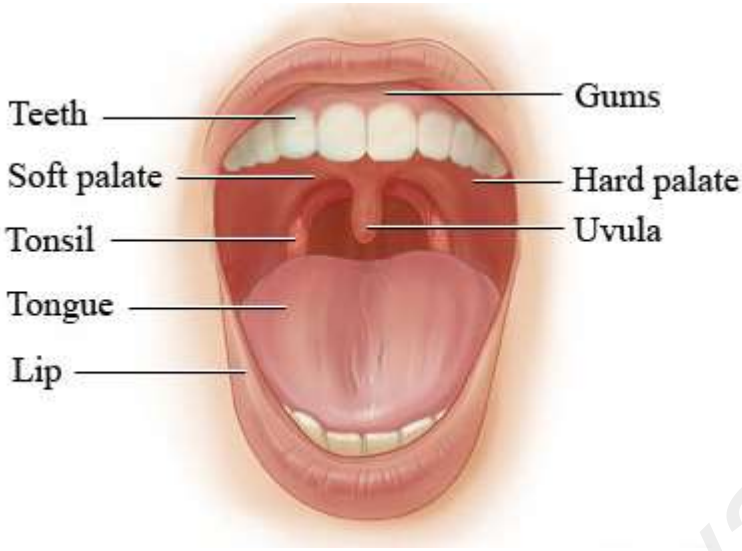


Figure 2.1: Anatomy of the human mouth (adapted from: http://www.webmd.com/oral-health/anatomy-of-the-mouth)

Table 2.1: Diverse microbial habitat in the mouth (Marsh *et al.*, 2009)

Habitat	Comment
Lips, cheek, palate	Biomass limited by desquamation. Some surface has specialised host cell types. E.g. *Streptococcus* sp. predominant.
Tongue	Highly papillated surface. Act as a reservoir for obligate anaerobes. E.g. *Streptococcus, Actinomyces, Rothia, Neisseria*, some Gram-negative anaerobes [e.g. *P. gingivalis, Fusobacterium nucleatum (F. nucleatum)* and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*)].
Teeth	Non-shedding surface enabling large masses of microbes to accumulate (dental plaque biofilms). Teeth have distinct surfaces for microbial colonisation (e.g, fissures, smooth surface, approximal, gingival crevice) will support a distinct microbiota because of their biological properties. E.g. *Streptococcus, Actinomyces, Veillonella, Fusobacterium, Prevotella, Treponema*, unculturable organisms.
Gingival crevice/ pocket	Health (aerobic), disease (anaerobic)

Molecular analysis of oral microbial communities by cloning and sequencing the bacterial 16S ribosomal RNA (16S rRNA) gene present has indicated that the human

mouth provides a habitat for more than 700 species of bacteria and that between 100 and 200 different species are present in the healthy mouth of any individual (Aas *et al.*, 2008; Kilian *et al.,* 2016). This oral microbiota normally maintains an ecological balance through antagonistic as well as mutualistic interspecies interactions (Qi & Kreth, 2017).

2.2 Healthy human oral microbiota

The microbial colonisation in the human oral cavity (also called human oral microbiota) is diverse due to the mouth is composed of several varied habitats supplied with diverse nutrients. Among diverse oral microbiota, bacteria become predominant species. The composition of bacterial genera colonised healthy dentate mouth was shown in Table 2.2.

Individuals are born with a sterile oral cavity but are colonised by maternal microbiota via passive transfer from the mother, from organisms' present in the milk, water or birth canal that can colonise mucosal tissues of the lips, cheeks, soft palate, tongue and the saliva. *Streptococcus salivarius* (*S. salivarius*) (98%) and *S. mitis* are the early microbiota which colonises an individual shortly after birth. The eruption of dentition at 6 months of age provides colonisation sites for *S. sanguinis* (formerly *S. sanguis*) and mutans group streptococci (*S. mutans* and *S. sobrinus*), as well as other tooth-inhabiting microorganisms. Marsh *et al.,* (2009) reported that oral streptococci including *S. oralis*, *S. anginosus*, *S. gordonii* and *S. sanguinis* can be detected in most children by the end of the first year of life. Previous study reported that *S. oralis* and *S. sanguinis* are abundantly present on the surfaces of the teeth, whereas *S. mutans* form only a minority of the supragingival plaque (Macpherson *et al.*, 1991).

Table 2.2: The principal bacterial genera of healthy dentate mouth (Marsh *et al.*, 2009)

	Gram-positive		**Gram-negative**
Cocci	**Rods**	**Cocci**	**rods**
Abiotrophia	*Actinobaculum*	*Anaeroglobus*	*Aggregatibacter*
Enterococcus	*Actinomyces*	*Mega sphaera*	*Campylobacter*
Finegoldia	*Alloscardovia*	*Moraxella*	*Cantonella*
Gemella	*Arcanobacterium*	*Neisseria*	*Capnocytophaga*
Granulicatella	*Atopobium*	*Veillonella*	*Centipeda*
Peptostreptococcus	*Bifidobacterium*		*Desulfomicrobium*
Streptococcus	*Corynebacterium*		*Desulfovibrio*
	Cryptobacterium		*Dialister*
	Eubacterium		*Eikenella*
	Filifactor		*Flavobacterium*
	Lactobacillus		*Fusobacterium*
	Mogibacterium		*Haemophilus*
	Olsenella		*Johnsonii*
	Parascardovia		*Kingella*
	Propionibacterium		*Leptotrichia*
	Pseudoramibacter		*Methanobrevibacter*
	Rothia		*Porphyromonas*
	Scardovia		*Prevotella*
	Shuttleworthia		*Selenomonas*
	Slackia		*Simonsiella*
	Solobacterium		*Tannerella*
			Treponema
			Wolinella

- Mycoplasma are also isolated from the mouth
- There are also unculturable bacteria that have yet to be placed in a genus.

Initial colonisation of the mouth of a child with *S. sanguinis* and mutans streptococci usually occurs during a "window of infectivity" around 9 months and 18-36 months of age, respectively (Carletto Korber *et al.*, 2005; Caufield *et al.*, 2000). In addition, the early colonisation of *S. sanguinis* and its elevated levels in the oral cavity were correlated to a significant delay in the colonisation of mutans streptococci (Caufield *et al.*, 2000). As a child grows the proportion of anaerobic bacteria such as *Fusobacterium* sp. and *Prevotella* sp. also increase (Tanner *et al.*, 2002). Changes in the microbiota can occur as a direct and indirect effect of ageing. Direct effects include waning of cell-mediated immunity leading to increased populations of non-oral bacteria (e.g. staphylococci and

enterobacteria). Indirect effects include the increased wearing of dentures among the elderly, which promotes colonisation by yeasts. Older people are also more likely to be taking long-term medication, a common side-effect of which is a reduced salivary flow rate promoting colonisation by lactobacilli (Marsh *et al.*, 1992).

Table 2.3 summarises the predominant normal microbiota which is frequently found in the mouth and it will change with the age. Age-related changes in the oral cavity result from tooth eruption, dietary changes, hormonal fluctuations and salivary flow.

Table 2.3: Oral microbiota changes with age (Marsh *et al.*, 2009)

Time during a lifetime	Major components & changes in oral microbiota
Newborn	Oral cavity sterile. Soon colonised by facultative and aerobic organisms; especially *S. salivarius.*
6 months	Flora becomes more complex and includes anaerobic organisms e.g. *Veillonella* sp. and Fusobacteria.
Tooth eruption	Increase in complexity. *S. sanguinis*, *S. mutans* and *A. viscosus* appear. New habitats include hard surfaces and gingival crevice.
Child to adult	Various anaerobes frequently found include members of the Bacteroidaceae. Spirochaetes isolated more frequently.
Loss of teeth	The disappearance of *S. mutans*, *S. sanguinis*, spirochaetes and many anaerobes.
Dentures	The reappearance of bacteria able to grow on hard surfaces.

2.3 Factors influencing microbial growth in the oral cavity

Several factors contribute to the optimal growth of bacteria in the mouth. These factors regulate the number, composition, development, coexistence and distribution of oral microorganisms on surfaces of the oral cavity. The four factors include (a) physicochemical such as water (humidity), salivary pH, temperature and redox potential; (b) adhesion, aggregation and coaggregation; (c) nutritional and (d) host protection.

Water is an important factor for microbial development whereby water promotes the exchange of nutrients, metabolic reactions and the elimination of waste products. The mouth is continuously bathed with saliva which has a role in regulating the growth and metabolic activity of the oral microbiota. Saliva (i) helps to maintain the pH at values around 6.75-7.25 which is optimal for the growth of many organisms, (ii) contains glycoproteins, peptides and amino acids for microbial growth, and (iii) delivers a spectrum of innate and specific immune host defence factors (Marsh *et al.*, 2016). Most oral microorganisms require a pH around neutrality for optimal growth and are sensitive to extremes of acid or alkali. A decrease in pH occurs due to acid production from bacterial metabolism of sucrose or sugar. Acidic pH promotes tooth demineralisation while basic pH promotes the accumulation of calculus. Changes in environmental pH can cause major shifts in the proportions of bacteria within dental plaque biofilms. Prolonged acidic pH due to frequent consumption of carbohydrate after meal enhanced accumulation of acid-tolerant (aciduric) organisms such as mutans streptococci and lactobacilli, which normally absent of only minor components in a dental plaque at healthy sites (Marsh & Devine, 2011).

The temperature in the oral cavity ranges between 35-36^{o}C. Microorganisms have a capability to withstand the most unfavourable conditions of temperature by altering their physiology and activating the expression of certain virulent-related genes (fimbriae formation, protease production, synthesis of superoxidase dismutase) affecting its pathogenicity (Amano *et al.*, 1994; Percival *et al.*, 1999). Most oral microorganisms are strict anaerobes or facultative anaerobes and many species (e.g., *Neisseria*) use oxygen, creating low local redox potential in the oral cavity.

Microorganisms in the oral cavity are always exposed to salivary flow, chewing, swallowing, oral hygiene practice and epithelial cell desquamation which influence the removal of bacteria from the oral surfaces. Thus, to protect themselves to overcome the

forces of removal by using adhesion (occur between the microorganism and host tissues), aggregation and coaggregation mechanism (occur between different species).

Endogenous nutritional supply comes from saliva and crevicular fluids while exogenous supply comes from diet normally carbohydrate. Saliva contains amino acids, peptides, proteins, and glycoproteins (which also act as a source of sugars and amino-sugars), vitamins and gases. It also provides the main buffering capacity for the mouth (Marsh *et al.*, 2009). Some bacteria metabolised dietary sucrose to produce acid and decrease the pH, thus limiting the growth of sensitive microorganisms. The integrity of teeth and mucosa, cell desquamation, lymphoid tissues, saliva and chewing, swallowing and suction are host factors that limit the establishment, multiplication and penetration of microorganisms in the oral cavity. Saliva is the first line of host defence for maintaining oral health because of its buffering capacity, its role in tooth remineralisation and its antibacterial effect (Lagerlöf, 1994). It contains proteins which function to retain moisture and act as a protective barrier for tooth structures and structures surrounding teeth, by forming an acquired pellicle. Proteins such as lysozyme and β-defensins have antibacterial properties. Lactoferrin binds tightly to iron, thus preventing the use of this element by many microorganisms. Saliva contains immune proteins of which immunoglobulin A is predominant, being secreted by plasma cells of connective tissue. These antibacterial and immune proteins delay bacterial growth and tooth decay (Marsh *et al.*, 2009).

2.4 Dental plaque as an oral biofilm

Dental plaque is a microbial biofilm with a high species diversity found on the tooth surface, embedded in a matrix of extracellular polymeric substances of bacterial and host origin (Pleszczynska *et al.*, 2017). Plaque that becomes calcified is referred to as calculus

or tartar. The production of extracellular polymers forms a functional matrix. Biofilm formation is a dynamic process, whereby the attachment, growth, removal and reattachment of bacteria are continuous processes. The formation of dental plaque involves several stages (Table 2.3; Figure 2.2) before the diverse climax communities are derived (Figure 2.3).

Table 2.4: Stages of plaque biofilm formation (Adapted from Marsh *et al.*, 2009)

	Stage	Plaque biofilm formation
1.	Pellicle formation	Host and bacterial molecules, salivary glycoproteins are adsorbed onto the tooth surface leading to acquired pellicle formation.
2.	Transport	Transport of bacteria to the pellicle occurs via natural salivary flow, Brownian movement or chemotaxis. Adsorption of coccal bacteria onto pellicle occur within 2 hours – pioneer species include *Neisseria, S. sanguinis, S. oralis* and *S. mitis,* also gram-positive rods, mainly *Actinomyces.*
3.	Long-range interactions (reversible attachment)	Long-range (> 50 nm) physiochemical interactions leading to reversible adhesion between the microbial cell surfaces and the acquired pellicle involving van der Waal's attractive forces and electrostatic repulsion produces a weak area of attraction.
4.	Short-range interactions (irreversible attachment)	Short-range stereochemical interactions (covalent and hydrogen bonds) between adhesins on the microbial cell surface and receptors on acquired pellicle lead to irreversible adhesion.
5.	Co-aggregation/ co-adhesion	The primary colonisers form a biofilm by autoaggregation (attraction between same species) and coaggregation (attraction between different species). Coaggregation (coadhesion) of new bacteria to the already attached bacteria results in increasingly diverse microbiota which forms Corncobs and Rosettes morphologic structures.
6.	Multiplication and maturation	Multiplication of attached organisms leads to confluent growth and biofilm formation. Adherent bacteria synthesise extracellular polymers. Plaque becomes matured when the increased in microbial diversity and quantity occur.
7.	Detachment	A detachment of bacteria allows colonisation at new sites.

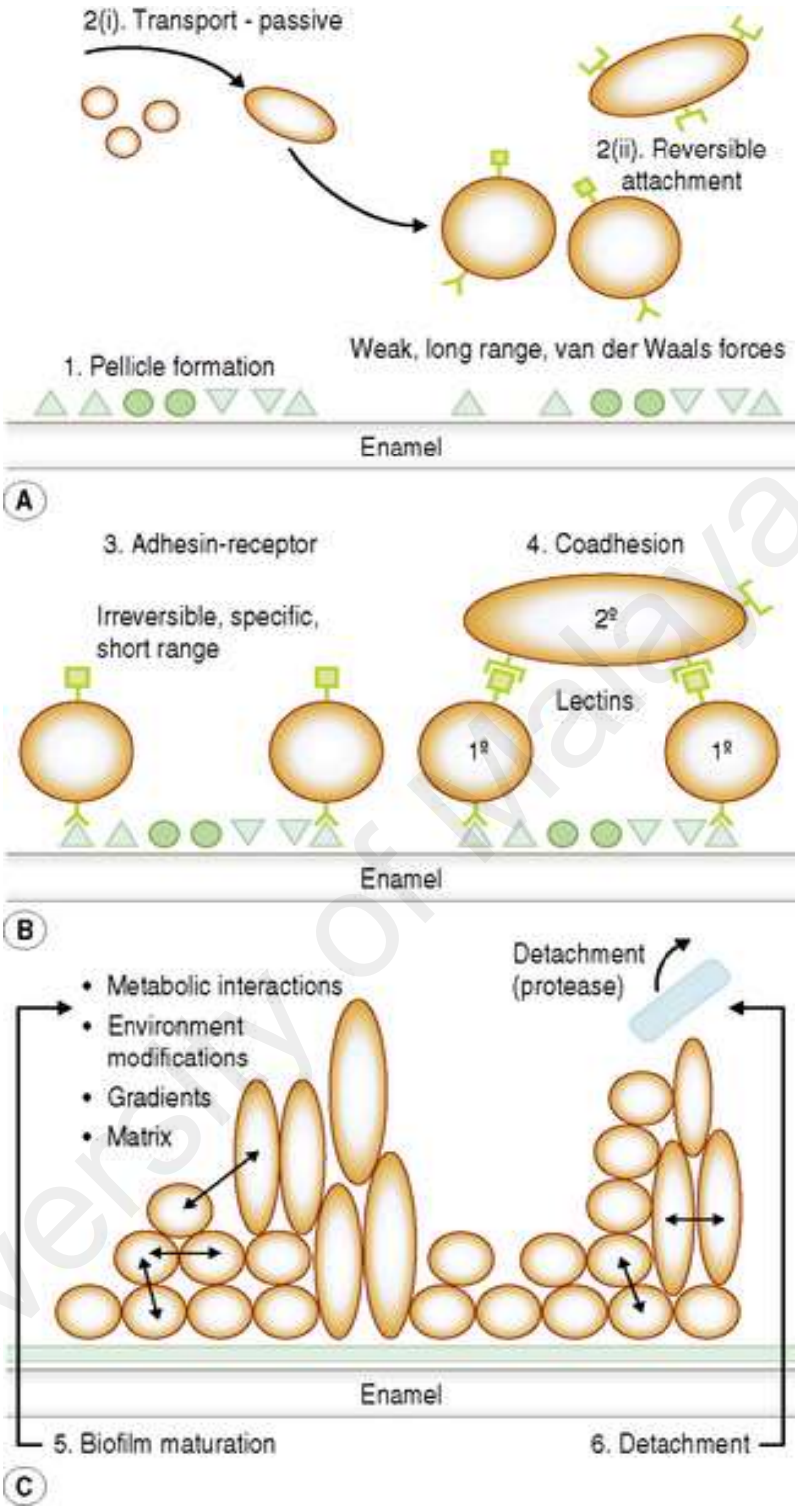


Figure 2.2: Stages in the dental plaque development. (A) 1. Pellicle forms on a clean tooth surface. 2(i) Bacteria are transported passively to the tooth surface where they 2(ii) may be held reversibly by weak electrostatic forces of attraction. (B) 3. Attachment becomes irreversible by specific stereochemical molecular interactions between adhesins on the bacterium and receptors in the acquired pellicle, and 4. secondary colonisers attach to primary colonisers, often by lectin-like interactions (coadhesion). (C) 5. growth results in biofilm maturation, facilitating interbacterial interactions. 6. Eventually, detachment can occur, sometimes because of the degradation by bacteria of their adhesins. {Marsh *et al.*, (2009)}.

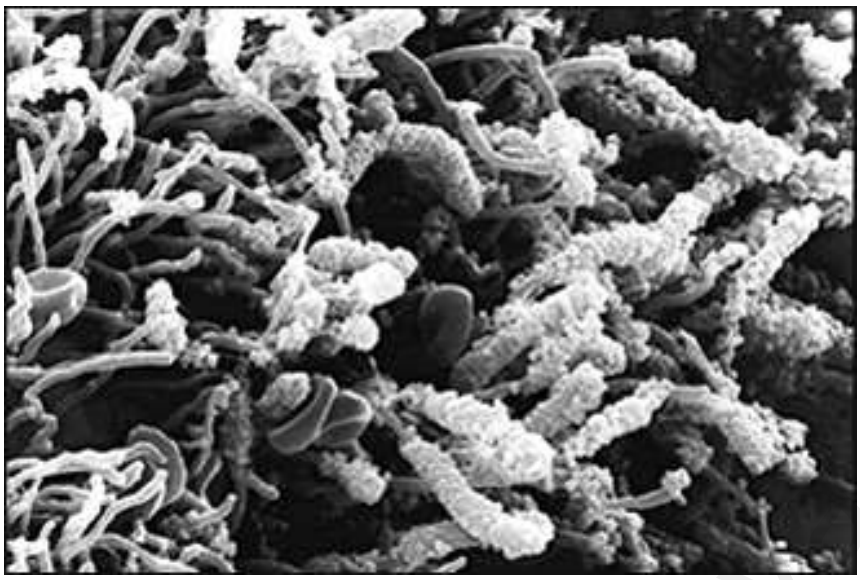


Figure 2.3: SEM viewing of mature dental biofilm showing corn cob morphology
(Overman, 2000)

2.4.1 Characteristics of biofilms

Dental plaque is an example of a biofilm. More than 90% of all bacteria in dental plaque exist as multi-species biofilms. The biofilm formed on the surface of teeth consist of glucans (10-20% of dry weight), fructans (1-2% of dry weight) and proteins (40% dry matter). The biofilm formed differs from the surrounding saliva in terms of the levels of lipids, calcium, magnesium, fluorine, and phosphorus. *In situ,* 80 % of the biofilm consists of water (Bowen & Koo, 2011). They are more resistant to biocides and antibiotics, show different gene- and protein expression patterns (e.g., an increase in exopolyphosphatase expression and a decrease of lactate dehydrogenase or pyruvate kinase expression) are metabolically different and have different growth rates (Svensater *et al.*, 2001). Therefore, the *in vitro* study should be carried out in biofilm conditions to be able to extrapolate results obtained in the laboratory to real life.

Biofilms served as protection from host defence mechanisms and potentially toxic substances like antibiotics. Organisms in biofilms are 1000-1500 times more resistant to

antibiotics than in their planktonic state. The mechanisms of this increased resistance differ from species to species, antibiotic to antibiotic and for biofilms growing in different habitats. This antibiotic resistance in bacteria is thought to be affected by their nutritional status, growth rate, temperature, pH and prior exposure to sub-effective concentrations of antimicrobial agents (Socransky & Haffajee, 2002).

Biofilm also facilitates processing and uptake of nutrients, cross feeding and removal of potentially harmful metabolic products through the voids or water channels between the micro-colonies.

2.5 Plaque hypotheses

There are four plaque hypotheses proposed to explain the role of plaque bacteria in the aetiology of dental caries and periodontal disease (Rosier *et al.*, 2014). The "Non-specific Plaque Hypothesis" (NSPH) was proposed by Loesche (1976) and was based on work of researchers such as Black (1884) and Miller (1890). The NSPH hypotheses proposed that dental infections were caused by the non-specific overgrowth of all bacteria in dental plaque. Any plaque has an equal potential to cause disease, the best way of disease prevention would be non-specific mechanical removal of as much plaque as possible by for example, tooth brushing or tooth picking (Theilade, 1986).

Due to development of culture-based techniques and microscopy, Loesche (1976) announced the "Specific Plaque Hypothesis" (SPH), postulating that dental caries was an infection with specific bacteria in the dental plaque such as mutans group streptococci, (main species: *S. mutans* and *S. sobrinus*) and lactobacilli. This hypothesis proposed that the use of antibiotics kanamycin against specific bacterial species could cure and prevent caries (Loesche & Nafe, 1973; Loesche, 1976, 1986; Loesche *et al.*, 1977). However, even though the use of antibiotic kanamycin reduced the abundance of cariogenic bacteria

but failed to eliminate them as soon as the treatment was stopped and abundance increased, while a long period of treatment leads to antibiotic resistance (Loesche & Nafe, 1973; Loesche *et al.,* 1977; Kornman & Karl, 1982). These suggested "specific-pathogens" are part of the indigenous microbiota and unlike foreign pathogens cannot be eliminated from the oral cavity (van Palenstein Helderman, 1984).

The development of the anaerobic hood in the 1970s for the first time allowed cultivation of the strict anaerobic species. This extended SPH to periodontal diseases which were proposed to be inflammations caused by specific periopathogens and antibiotic treatment would be effective (Loesche, 1986). However, some limitations involve such as (a) the use of chlorhexidine after scaling and root planing in patients with chronic periodontitis had only a modest positive effect and concluded that the extensive use of chlorhexidine may be questioned (Eberhard *et al.,* 2008); (b) almost 50% of periopathogens were uncultivable species and the bias towards easily cultivable species (Handelsman, 2004; Siqueira & Rôças, 2013). The finding of different species related to periodontal disease led to the idea that oral disease could be initiated by a number of specific pathogens (Socransky, 1977; Theilade, 1986). This idea was further investigated over the next decades and led to the famous Socransky-complexes which include bacterial clusters based on their association with periodontal disease (Socransky *et al.,* 1998).

Theilade (1986) noticed that the "specific-pathogens" from the SPH were indigenous bacteria and sometimes common bacteria in health, which led to an updated NSPH in 1986 focusing on periodontal disease. At this time, most researchers seemed to agree that gingivitis was a non-specific inflammatory reaction to a complex indigenous microbiota. The updated NSPH took into consideration that some indigenous subgingival bacteria can be more virulent than others and that plaque composition changes from healthy to disease condition. It also was considered that some people have gingivitis for a lifetime without tissue and bone destruction, while others encounter rapid progression into periodontitis.

Unlike the classic NSPH, the updated NSPH could explain that differences in the plaque microbial composition could lead to differences in pathogenic potential.

The "Ecological Plaque Hypothesis" (EPH) by Marsh (1994; 2003) focused on the dynamic and reversible processes of demineralisation and remineralization in the plaque by linking between sugar supply, pH change and microbiota shift. For example, frequent exposure to a low pH, for instance as the result of sugar fermentation, leads to a relative increase of acid-tolerant species (Marsh, 1994, 2003). Marsh also considered that early colonisers of supragingival dental surfaces, are usually facultative anaerobic bacteria use the oxygen, producing carbon dioxide and hydrogen in the environment (Marsh, 2003). This lowers the redox potential giving strict anaerobes a chance to settle and multiply in the biofilm.

The Extended EPH proposed by Takahashi and Nyvad (2008; 2011) revealed that acidogenic non-mutans streptococci (such as *S. gordonii, S. oralis, S. mitis* and *S. anginosus*) and *Actinomyces* play the key roles in maintaining dynamic stability. These bacteria produce acid from sugary foods and demineralise the enamel. However, the temporary decreases in pH are easily returned to neutral level by homeostatic mechanisms in the plaque (Marsh *et al.,* 2009). On the other hand, when the sugar is supplied frequently or salivary secretion is too scarce to neutralise acids produced, the pH decreases in the plaque may enhance the acidogenicity and acidurance of the non-mutans streptococci adaptively. The low-pH non-mutans streptococci and *Actinomyces* then increase via acid selection, leading to a microbial shift to a more acidogenic microbiota. These changes in the phenotype and genotype of the microbiota may shift the demineralization/remineralization balance from 'net mineral gain' to 'net mineral loss' and initiate lesion development (acidogenic stage). If prolonged acidic environment prevail, lesion development ('net mineral loss') is likely to progress and more aciduric bacteria such as mutans streptococci and lactobacilli may replace the 'low-pH' non-

mutans streptococci and further accelerate the caries process (aciduric stage). At this stage, mutans streptococci and lactobacilli as well as aciduric strains of non-mutans streptococci, A*ctinomyces*, bifidobacteria and yeasts may become dominant. Many acidogenic and aciduric bacteria are involved in caries. Environmental acidification is the main determinant of the phenotypic and genotypic changes that occur in the microbiota during caries.

Finally, the recent "Keystone-Pathogen Hypothesis" (KPH) proposes that certain low-abundance microbial pathogens can cause inflammatory disease by interfering with the host immune system and remodelling the microbiota (Hajishengallis *et al.*, 2012). For example, *P. gingivalis* manipulates the native immune system of the host and was hypothesised that *P. gingivalis* does not only facilitates its own survival and multiplication, but of the entire microbial community (Darveau, 2010). In contrast, the dominant species that influence inflammation by their abundant presence, keystone pathogens can trigger inflammation in low numbers (Hajishengallis et al., 2012).

2.6 Oral Streptococci

Streptococcus is a dominant genus in the human oral cavity, making up about 20% of the more than 700 species of bacteria that have been identified and about 80% of the early biofilm colonisers (Kilian *et al.*, 2016; Rosan & Lamont, 2000). The genus human streptococci are taxonomically divided into six major clusters. These are (i) the pyogenic group, which includes *S. agalactiae, S. canis*, *S. dysgalactiae, S. equi*, *S. iniae*, *S. porcinus*, and *S. pyogenes*; (ii) the bovis group, which includes *S. bovis, S. equinus*, and *S. alactolyticus*; (iii) the salivarius group, which includes *S. salivarius, S. thermophilus*, and *S. vestibularis*; (iv) the mutans group, which includes *S. criceti, S. ratti*, *S. downei*, *S. macacae, S. ferus. S. mutans*, and *S. sobrinus*; (v) the anginosus group (also referred to

as the milleri group), which includes *S. anginosus*, *S. constellatus*, and *S. intermedius*; and (vi) the mitis group, which includes *S. mitis*, *S. oralis*, *S. pneumoniae*, *S. sanguinis*, *S. parasanguis*, *S. gordonii*, *S. cristatus*, *S. oligofermentas*, *S. sinensis*, *S. australis*, *S. peroris* and *S. infantis*). These classifications based on a combination of features including patterns of haemolysis observed on blood agar plates, antigenic composition, growth characteristics, biochemical reactions, and more recently, 16S rRNA gene sequencing (Ergin, 2010; Kawamura *et al.*, 1995; Marsh *et al.*, 2009).

S. mutans and *S. sobrinus* are frequently isolated from dental caries and have been implicated as cariogenic bacteria in the oral cavity because they have biological properties such as high productivity of dental caries-causing lactic acid and extracellular polysaccharides (Singla *et al.*, 2016). In addition, *S. mutans* also cause systemic diseases such as endocarditis and septicaemia (Kojima *et al.*, 2012; Nobbs, 2017). In this study, the commensal species of *S. sanguinis* and cariogenic species of *S. mutans* were selected to investigate the effect of a plant extract mixture (PEM) and its individual constituents plant extract (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.) towards the two bacteria in single- and dual-species biofilms. *S. sanguinis* and *S. mutans* were investigated due to their nature as normal oral flora but in other hands can bring harm if their numbers are increased in form of biofilm. The detailed characteristics of the two bacteria are described below.

2.6.1 *Streptococcus sanguinis* (*S. sanguinis*)

S. sanguinis is a Gram-positive facultative anaerobe (Xu *et al.*, 2007). Like most oral streptococci, it produces greenish colour or alpha-haemolysis on blood agar and is therefore categorised as one of the viridans group streptococci. In one current classification scheme, the viridans group streptococci are divided into five or six groups,

with *S. sanguinis*, *S. australis*, *S. cristatus* (formerly *S. crista*), *S. gordonii*, *S. mitis*, *S. oralis*, *S. infantis*, *S. oligofermentans*, *S. parasanguinis* (formerly *S. parasanguis*), *S. pseudopneumoniae*, *S. pneumoniae*, *S. peroris* and the latest grouped species, *S. tigurinus* belong to Mitis group. Currently, the complete genome sequences of 7 species of this mitis group such as *S. pneumoniae, S. pseudopneumoniae, S. mitis, S, oralis, S. gordonii, S. sanguinis* and *S. parasanguinis*) are stored on the National Center for Biotechnology Information (NCBI)'s FTP site (Zheng *et al.*, 2016).

S. sanguinis is a health-compatible species and normal inhabitant of the human oral cavity. The cells of *S. sanguinis* grow in chains and clusters that directly bind to the salivary pellicle of the tooth surface and serves as a tether for the attachment of a variety of other oral microorganisms which colonise the tooth surface, form dental plaque and contribute to the aetiology of both caries and periodontal disease (Kolenbrander, 2000; Xu *et al.*, 2007).

S. sanguinis is also often found in the bloodstream which allows it to inhabit the heart valves causing bacterial endocarditis, a serious heart disease that can possibly lead to death. Endocarditis is an infection of heart valves or the endocardium. As a key agent to infective endocarditis, *S. sanguinis* adhere and colonise the bloodstream and damage heart valves via fibrin and platelets are deposited at the site of endothelial cell trauma, forming a sterile vegetation (Loukachevitch *et al.*, 2016). Hydrogen peroxide (H_2O_2) produced by *S. sanguinis* through the action of *spxB*, a pyruvate oxidase is known to be involved in survival of *S. sanguinis* in human bloodstream and the bacterial H_2O_2 exhibits cytotoxicity against human neutrophils (Sumioka *et al.*, 2017). Endocarditis can proceed through the entrance of oral streptococci to the bloodstream during dental procedures or even during normal daily activities such as eating and tooth brushing (Que & Moreillon, 2011; Thornhill *et al.*, 2016).

2.6.1.1 Cell Structure and Metabolism of *Streptococcus sanguinis*

S. sanguinis is a coccus shaped Gram-positive, facultative anaerobe bacterium that is normal inhabitant of the human oral cavity. As a primary coloniser of the oral cavity, *S. sanguinis* serves as a tether for the attachment of other oral pathogens (Evans *et al.*, 2014). This organism has a very well-built system for energy production despite an incomplete TCA cycle (Kreb Cycle or Citric acid cycle). It contains many enzymes that enhance metabolic pathways including biosynthesis, pentose phosphate pathway, gluconeogenesis, fermentation of sugars and carbohydrates, and so on. Such enzymes used for gluconeogenesis allow the bacterium to convert amino acids into fructose-6-phosphate, an important metabolic precursor used to make peptidoglycan (cell wall) and an initial substrate required for pentose phosphate pathway. Many of these enzymes were found in other streptococci and some are not present (Xu *et al.*, 2007).

Sortase A (SrtA), which cleaves LPXTG-containing proteins and anchors them to the bacterial cell wall, as a possible virulence factor of *S. sanguinis*. A study shows that *srtA* gene in *S. sanguinis* participates in anchoring adhesin proteins on the bacterial surface, especially the surface of teeth using hydrophobic interactions. They suggest that the decreases in hydrophobicity are due to a lack of *srtA* gene, indicated that *srtA* gene may have a role of "adhesion to teeth, restorable dental materials and epithelial cells in the oral cavity". They also reported that *srtA* contributes to anti-opsonisation in streptococci (Yamaguchi *et al.*, 2006).

2.6.1.2 Interaction of *S. sanguinis* with other commensal Streptococci

After a freshly cleaned tooth surface is colonised by pioneer oral streptococci, *S. sanguinis* and *S. gordonii* are among the early colonisers that colonise the tooth surface express specific cell surface adhesin molecules with similar binding capacities. Hence,

they compete for binding to the same receptors provided by the host (Nobbs *et al.*, 2007; Zheng *et al.*, 2017). *S. sanguinis* has a greater natural prevalence in plaque and saliva but is surprisingly unable to eliminate *S. gordonii*. Further, *S. gordonii* competes more effectively with *S. sanguinis* than any other tested oral streptococci for adherence to saliva-coated hydroxyapatite, a model system to simulate the natural tooth environment. Mutational analysis revealed that a specific surface adhesin, the sialic acid-binding protein (Hsa) was responsible for conferring a competitive advantage to *S. gordonii* (Nobbs *et al.*, 2007). They also reported that the competition is influenced not only by sheer abundance but also by the efficiency of adherence to certain salivary components. This enables *S. gordonii* to establish itself in a competitive environment, even with the genetically similar competitor *S. sanguinis*. Because the early colonisers provide the attachment sites for the later colonisers, this competition has direct implications for the spatial and temporal composition of the developing dental biofilm.

Furthermore, the higher colonisation of *S. sanguinis* leads to the reduction of aggressive periodontitis caused by *A. actinomycetemcomitans* (Stingu *et al.*, 2008).

2.6.1.3 Interaction of commensal *S. sanguinis* with cariogenic *S. mutans*

S. sanguinis presence significantly higher numbers in healthy subjects, while subjects with caries possessed almost no detectable levels of *S. sanguinis* (Becker *et al.*, 2002). Likewise, high levels of *S. sanguinis* correlated with delayed acquisition of *S. mutans* (Caufield *et al.*, 2000).

The *in vitro* study indicated that *S. sanguinis* and *S. gordonii* engage in chemical warfare with *S. mutans* for dominance over a given niche. The oral streptococci utilise different weapons to face one another. All three streptococci can produce small chromosomally encoded antimicrobial peptides called bacteriocins. *S. mutans* produces

three bacteriocins (lantibiotic family) called mutacins, I, II and III, and nonlantibiotic mutacin IV (Nakahara *et al.*, 2017). *S. gordonii* produces bacteriocins called streptocins and *S. sanguinis*, the bacteriocin called sanguicin (Deng *et al.*, 2004). Bacteriocin is a protein/peptide produced by bacteria to inhibit the growth of related species. When *S. sanguinis* was co-cultivated with *S. mutans*, the two bacteria exhibit antagonistic and competitive reactions. A previous study reported that when the cell has enough energy to compete for the limited nutrients, *S. mutans* produce mutacin to inhibit the growth of *S. sanguinis*, while hydrogen peroxide produced by *S. sanguinis* able to inhibit the growth of *S. mutans*. However, their finding also reported that in media rich nutrient, production of hydrogen peroxide is shut off. The energy that uses to produce hydrogen peroxide was used for cell metabolism to compete for a nutrient with *S. mutans*. While in depleting nutrient, or pH environment is low, hydrogen peroxide production is turned on allowing competition against *S. mutans* (Kreth *et al.*, 2005). The release of extracellular DNA (eDNA) into the environment by *S. sanguinis* correlated to the hydrogen peroxide production (Itzek *et al.*, 2011; Kreth *et al.*, 2008). A pyruvate oxidase (*spxB*) gene are responsible for the release of hydrogen peroxide. The SpxB is responsible for the generation of growth-inhibiting amounts of H_2O_2 and able to antagonise cariogenic *S. mutans*. This interaction promotes the two bacteria occur in homoeostasis reaction (Zheng *et al.*, 2011).

The hydrogen peroxide (H_2O_2) production is oxygen dependent and the oxygen tension in saliva is sufficient to allow for aerobic respiration and H_2O_2 production during initial colonisation (Kreth *et al.*, 2008). The oxygen tension declines once the biofilm reaches a certain thickness and cell density due to diffusion limitations. This could result in a decrease of H_2O_2 production to a non-inhibiting level. Under those biofilm conditions, *S. mutans* has the advantage by using bacteriocins to inhibit *S. sanguinis* and *S. gordonii*. Bacteriocin production is not influenced by oxygen

availability and expression is optimised for growth under the high cell density conditions of a mature biofilm, making *S. mutans* an aggressive competitor for *S. sanguinis* and *S. gordonii* (Kreth *et al.*, 2008).

2.6.1.4 Interaction of oral streptococci with the host

S. sobrinus is an oral streptococcus with cariogenic potential which has developed a mechanism to suppress an immune response. The immune suppression occurs due to the presence of the cytoplasmic glycolytic enzyme enolase localised on the bacterial cell surface. Enolase triggers the release of the anti-inflammatory cytokine IL-10 (Veiga-Malta *et al.*, 2004). Enolase as a surface component of *S. mutans* which bind to human plasminogen and salivary mucin MG2 and has a role in *S. mutans* attachment, clearance or breach of the bloodstream barrier (Ge *et al.*, 2004).

The initial adherence of oral streptococci to epithelial cells is likely to involve multiple surface adhesins. The surface proteins of the antigen I/II adhesin family, *sspA* and *sspB* of *S. gordonii* are crucial for adherence to epithelial cells (Forsgren *et al.*, 2009). *S. mutans* attached to the host salivary pellicle via sucrose-dependent and sucrose independent mechanisms. The surface-associated protein P1 (*spaP*), also known as antigen I/II or PAc (sucrose-independent adherence), was one of the first gene products linked to adherence of *S. mutans* to salivary agglutinin which coats the teeth surfaces (Matsumoto-Nakano, 2017).

2.6.2 *Streptococcus mutans* (*S. mutans*)

S. mutans is classified under mutans group of Streptococci along with *S. sobrinus, S. criceti, S. ratti, S. macacae, S. downei* and *S. ferus*. They are Gram-positive cocci,

facultative anaerobes that inhabit the human oral cavity and are first acquired by infants soon after their first tooth emerges, with the mother being identified as the major source (Caufield *et al.*, 1993). *S. mutans* is mesophilic and grow at temperatures between 18-40^{o}C. *S. mutans* strains are classified into four serotypes, *c*, *e*, *f,* and *k,* based on the chemical composition of their cell surface rhamnose-glucose polymers (Marsh *et al.*, 2009). Serotype *c* is the major type in oral isolates from healthy subjects, with a distribution frequency of approximately 70 to 75%, followed by serotype *e* (frequency of approximately 20%). The distribution frequencies for serotypes *f* and *k* are lower than 5% (Nakano & Ooshima, 2009). *S. mutans* (serotypes c, e, f) and *S. sobrinus* (serotypes d, g) participate in the formation of dental biofilm and significantly associated with human dental caries (Marsh *et al.*, 2009). The risk factors for dental caries include individual behavioural risk factors such as snacking, poor oral hygiene, lack of fluoride exposure and non-dental attendance, tooth brushing and dental attendance are also associated with caries in pre-school children (World Health Organisation, 2017).

2.6.2.1 Virulence properties of *Streptococcus mutans* in the initiation of dental caries

Dental caries is a biofilm-related oral disease, which affects most individuals worldwide. The disease results from the interaction of specific bacteria with constituents of the diet within a biofilm formed on the tooth surface which was known as dental plaque. Although other microorganisms also involved, *S. mutans* plays a key role in the development of dental caries in humans (Agnello *et al.*, 2017; Valdez *et al.*, 2017). Several virulence factors contribute to the cariogenicity of *S. mutans* (Krzysciak *et al.*, 2014; Kuramitsu & Wang, 2006). These include the ability to

(i) rapidly transport sugar when in competition with other plaque bacteria and rapid conversion of such sugar to produce acid (acidogenicity);

(ii) tolerate, grow and continue to produce acid at low-pH environment (aciduric);

(iii) synthesis of extracellular polymers (especially glucan and mutan) from metabolism sucrose to consolidate attachment;

(iv) adhere tenaciously to acquired pellicle on tooth surfaces; and

(v) produce intracellular polysaccharides (IPS) for storage nutrients which can be converted to acid when dietary sugars are not available (Banas, 2004; Busuioc *et al.*, 2009; Lemos *et al.*, 2005; Takahashi *et al.*, 1991).

In addition, a bacteriocin produced by *S. mutans* and cell to cell communication (quorum sensing has been reported also involved in the biofilm formation (Shanker & Federle, 2017; Zhang *et al.*, 2009).

The combination of these virulence properties allows *S. mutans* to effectively colonise tooth surfaces and modulate the transition of non-pathogenic to highly cariogenic dental biofilms, which leads to the initiation of caries. Therefore, approaches aimed at inhibiting the viability and virulence properties of *S. mutans* could be precise and selective for the prevention of dental caries. The virulence factors of *S. mutans* are described in detail as follows.

2.6.2.1.1 Acidogenicity and aciduricity

Plaque bacteria ferment sucrose to produce acids, which *in vitro* lower the pH value to below 5.0. *S. mutans* as an aciduric or acid tolerant organism can survive at low pH, while other streptococci which are acid-sensitive organisms (*S. sanguinis, S. mitis and S. gordonii*) are killed in lower pH. The enzyme, invertase of *S. mutans* splits sucrose into glucose and fructose molecules for energy production, which are then converted to lactic acid by the glycolytic pathway (Marsh *et al.*, 2009).

S. mutans can tolerate the typically low pH of dental plaque and continue to produce acids contributing to its cariogenicity. Acid tolerance is one of the microorganism's most

important virulence factors. These include the membrane-bound F_1F_0-ATPase system, lactate dehydrogenase and enolase. The membrane-bound F_1F_0-ATPase system protects *S. mutans*, which pumps out extra protons to maintain intracellular pH at 7.5 (Quivey *et al.*, 2001). Lactate dehydrogenase (LDH) responsible for producing lactic acid and enolase (a glycolytic enzyme) produces phosphoenolpyruvate (PEP), which is a key component of the PEP: carbohydrate phosphotransferase system (PTS), further contributes to the promotion of cariogenicity by *S. mutans* (Marsh *et al.*, 2009).

2.6.2.1.2 Sucrose-dependent adhesion

S. mutans produces three glucosyltransferases (Gtfs) and one frucosyltransferase (Ftf) enzymes, are used to metabolise sucrose to produce glucans and fructans, respectively. Specifically, *S. mutans* produces GTF-I (GtfB), GTF-SI (GtfC), and GTF-S (GtfD), which are encoded by *gtfB, gtfC, and gtfD* genes, respectively. Cell-associated GTF-I (GtfB) mainly synthesising water-insoluble glucans (rich in α-1,3-linkages; called as mutan). The GtfB avidly adhere to the surfaces of other oral microorganisms and converting them to glucan producer when exposed to sucrose. GTF-S (also known as GtfD) is produced extracellularly, synthesising water-soluble glucans, absorbs into enamel-coated salivary pellicle and act as a primer for GtfB. While GTF-SI (GtfC) synthesises both water-soluble (with mostly α-1,6-linkages; called as dextran) and water-insoluble glucans. GtfC has the greatest affinity to adhere to saliva-coated enamel. These adhesive glucans make adhesion to the tooth surface tighter and irreversible (Banas & Vickerman, 2003; Bowen & Koo, 2011). While fructans act as extracellular nutrient storage compounds. Excess polysaccharide is stored by some species as intracellular storage compounds, which allows continuing acid production in the absence of dietary sugar (Marsh *et al.*, 2009).

Glucan-binding proteins (Gbps) encoded by *gbpA*, *gbpB* and *gbpC* genes at the bacterial surface mediate cell to cell attachment via glucan. GbpC is involved in glucan-dependent aggregation of cells, while GbpA is essential for biofilm structure integrity and GbpD for biofilm rigidity (Banas & Vickerman, 2003).

Wall-associated protein A (WapA, originally antigen A or antigen III) is a 29 kDa surface protein was previously used as a vaccine in animal studies for immunization against dental caries (Zhu *et al.*, 2006). The *wapA* contributed to sucrose-dependent adherence indirectly. This is because the inactivation of the *wapA* gene resulted in a reduction in aggregation and sucrose-dependent adherence to a smooth, glass surface (Qian & Dao, 1993). However, Zhu and coworkers (2006) revealed that sucrose (0.5%) strongly repressed *wapA* gene expression in both planktonic and biofilm cells. Their result suggested that WapA protein plays an important structural role on the cell-surface, which ultimately affects sucrose-independent cell-cell aggregation and biofilm architecture.

2.6.2.1.3 Sucrose-independent adhesion

Antigen I/II (known as antigen B, *spaP* or PAc or P1 adhesin) of *S. mutans* is anchored in the bacterial cell wall, interacts with salivary pellicle (glycoprotein-340) (serve as sucrose-independent adhesion to tooth surface) and is encoded by the *spaP* gene (Wen *et al.*, 2010). The cell-surface protein antigen c (PAc) is composed of several domains, including an N-terminal signal sequence, an alanine-rich repeat region (A-region), a proline-rich repeat region (P-region) and an anchor region (Matsumoto-Nakano *et al.*, 2008). PAc adhesins are played by salivary agglutinin. The initial stage of adhesion and biofilm formation of *S. mutans* is stimulated by salivary agglutinins and other salivary protein (e.g., mucin and acidic proline-rich proteins). Interactions between salivary agglutinin and the adhesin PAc of *Streptococcus mutans* contribute to bacterial

aggregation and mediate sucrose-independent adherence to tooth surfaces (Ahn *et al.*, 2008). Availability of sucrose in the media also play a role to the *spaP* gene expression (Shemesh *et al.*, 2007a)

2.6.2.1.4 Bacteriocin

Bacteriocin is a ribosomally synthesised antibacterial peptide that either kill or inhibit the growth of similar or closely related bacterial strain. One such bacteriocin is mutacin that is the potential virulence factor associated with *S. mutans* (Hamada & Ooshima, 1975). This allows *S. mutans* to compete with other streptococci in early dental biofilms (e.g., *S. sanguinis*) and allows it to sustain colonisation on the tooth surface (Kuramitsu *et al.*, 2007; Loyola-Rodriguez *et al.*, 1992). *S. mutans* UA159 can synthesise mutacin V and IV (Hossain & Biswas, 2011). Synthesis of mutacin can slow down the growth of bacteria. Thus, in the carbohydrate-rich environment, *S. mutans* use energy for growth instead of microbial antagonism (Kreth *et al.*, 2005). While in a multispecies community, *S. sanguinis* inhibited *S. mutans* by producing hydrogen peroxide (Kreth *et al.*, 2005). *S. mutans* produces *gshAB* gene to detoxify hydrogen peroxide and continue biofilm formation (Zheng *et al.*, 2013).

2.6.2.1.5 Quorum sensing

Quorum sensing or cell density-mediated gene expression involves the regulation of expression of specific genes through the accumulation of signalling compounds that mediate intercellular communication. Quorum sensing functions through the secretion and detection of autoinducer (AI) molecules which accumulate in a cell density-dependent manner. Quorum sensing may give biofilms their distinct properties. Example expression of genes for antibiotic resistance at high cell densities has potential to

influence community structure by encouraging the growth of commensal or beneficial species and inhibiting competitor's species to the biofilm (via bacteriocin production) (Slippers *et al.*, 2005; Zhang *et al.*, 2009). In *S. mutans*, quorum sensing is mediated by competence stimulating peptide (CSP), whose precursor is encoded by *comC* wherein genes are responsible for multiple functions (biofilm formation, competence and acid tolerance) (Wang *et al.*, 2013). Increased cell density detected by quorum sensing proteins during the log phase of growth further induces *S. mutans* to produce acid tolerance responses (Li *et al.*, 2001).

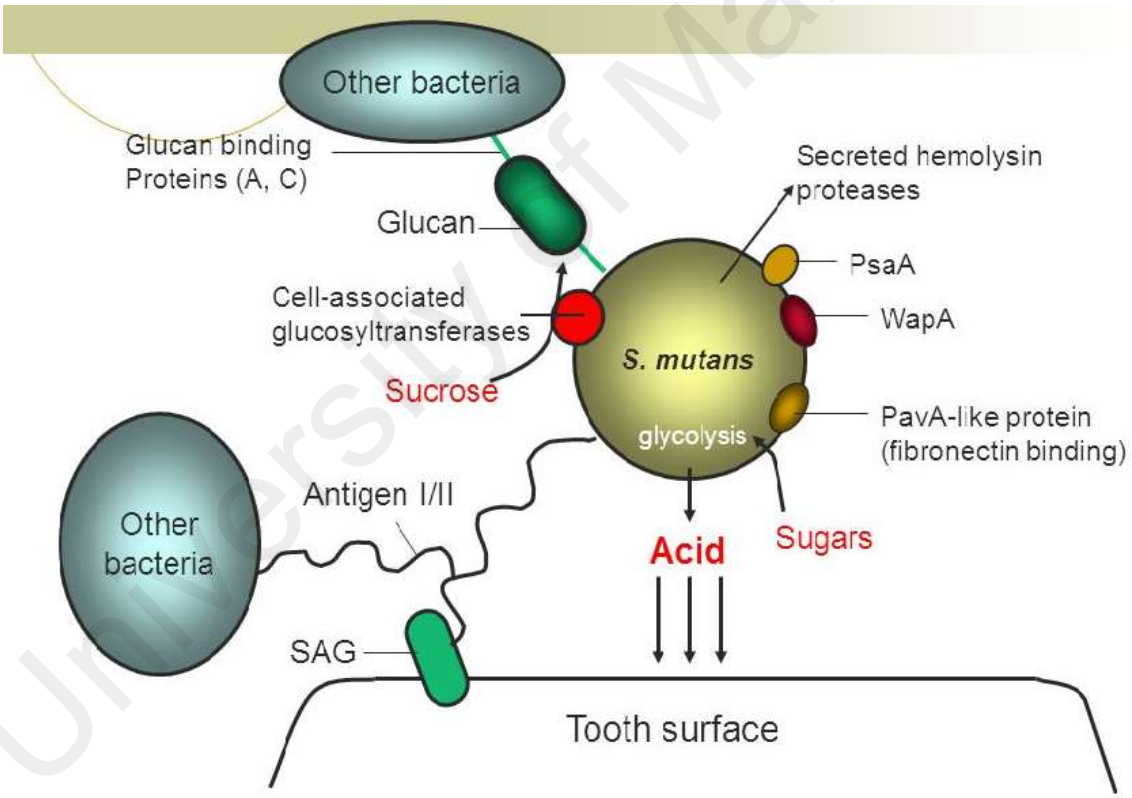


Figure 2.4: Virulence factors of *S. mutans*. *S. mutans* as a main cariogenic species is acid production through metabolisms of sugar, adhere firmly to the tooth surface via glucan production, glucan-binding protein and surface structure (Antigen I/I and secreted haemolysin {Adapted from Krzysciak *et al.*, 2014; Mitchell, 2003}. Photo citation: http://images.slideplayer.com/15/4830258/slides/slide 18.jpg

2.6.2.2 Expression of biofilm-associated genes of *Streptococcus mutans*

S. mutans is a bacterial species that exhibits a biofilm lifestyle for survival and persistence in its natural ecosystem. It is assembled as communities attached to tooth surfaces and forms matrix-embedded biofilms (Marsh, 2005). Adhesion is the initial step in the formation of biofilm communities. As a cariogenic species, the mechanisms by which *S. mutans* adheres to tooth surfaces are important potential targets for anti-cariogenic intervention. Sucrose-dependent mechanisms of adherence, as mediated by extracellular enzymes [three glucosyltransferases (Gtfs) and one fructosyltransferase (Ftf)] and glucan-binding proteins (Gbps) have well-established roles in the virulence of *S. mutans* (Banas & Vickerman, 2003; Inagaki *et al.*, 2013). Sucrose-independent mechanisms (Antigen I/II or PAc adhesin) can also foster microbial colonisation by providing binding sites for bacteria (Tang *et al.*, 2016). Beyond initial adherence, a variety of genes is required for the adaptation of *S. mutans* and other oral streptococci in biofilms. These include genes associated with intercellular communication systems and environmental sensing systems, regulators of carbohydrate metabolism and adhesion-promoting genes (Lemos & Burne, 2002; Senadheera *et al.*, 2005; Shemesh *et al.*, 2007a). The genes include *brpA* (lytR) and *vicR* encoding regulatory proteins, the adhesion promoting genes such as *gbpB* and *spaP*, and the genes encoding polysaccharide-synthesising enzymes, including *gtfB, gtfC* and *ftf* (Banas & Vickerman, 2003; Chatfield *et al.*, 2005; Jakubovics *et al.*, 2005; Li *et al.*, 2002; Senadheera *et al.*, 2005; Shemesh *et al.*, 2006; Wen & Burne, 2002). Moreover, *relA* gene is required by *S. mutans* to form stable biofilms and tolerate acid stress (Lemos *et al.*, 2004). While *smu0630* is important in both sucrose-dependent and sucrose-independent biofilm formation (Brown *et al.*, 2005). Numerous studies have indicated that expression of the genes responsible for biofilm formation is dependent on environmental conditions and is also genetically

regulated (Hudson & Curtiss, 1990; Kiska & Macrina, 1994; Lee *et al.*, 2004; Li & Burne, 2001).

2.7 Role of microorganisms in plaque formation and disease

The oral cavity contains complex, multispecies microbial communities. The residents in this community should display extensive interactions while forming biofilm structures, carrying out physiological functions and inducing microbial pathogenesis. This interaction includes (1) competition between bacteria for nutrients, (2) synergistic interactions which may stimulate the growth or survival of one or more residents (Takahashi & Nyvad, 2008), (3) production of an antagonist by one resident which inhibits the growth of another (Kreth *et al.*, 2005), (4) neutralisation of a virulence factor produced by one organism by another resident (Takahashi, 2003, Takahashi 2005; Takahashi *et al.*, 1997) and (5) interference in the growth-dependent signalling mechanisms of one organism by another (Periasamy & Kolenbrander, 2010).

2.8 Bacterial metabolism in supragingival plaque and dental caries

Saliva contains glycoproteins such as mucins, peptides and amino acids and becomes the main source of nutrient for the supragingival plaque. Glycoproteins can be degraded into sugar molecules and proteins by bacterial and human glycosidases. Proteins can be broken down into peptides and amino acids by bacterial and human proteases. *Streptococcus* sp. and *Actinomyces* sp. are predominant in the supragingival area. They adhere to the saliva-coated tooth surface by attachment between adhesins (located on bacterial cell surfaces) and receptors (contained mainly in the salivary coating on tooth surfaces and are known to utilise salivary components as nutrients (Marsh *et al.*, 2016). These saccharolytic bacteria degrade carbohydrates derived from foods through the

Embden–Meyerhof–Parnas pathway to form lactic, formic, acetic, succinic and other organic acids, and concomitantly consume oxygen by NADH oxidase. Taken together, these activities create acidic and anaerobic conditions. The acidification is rapid and the supragingival pH can reach around 4 within several minutes (Takahashi, 2005).

Generally, sugars are metabolised to acids by supragingival saccharolytic bacteria, including *Streptococcus*, *Actinomyces* and *Lactobacillus*, while amino acids can also be metabolised by these bacteria, mainly into acids and ammonia. Overall, the production of acidic and alkaline molecules, together with the continuous flow of saliva, maintains an almost neutral supragingival pH. When dietary sugars are supplied, these bacteria initiate efficient acid production, resulting in the demineralisation of tooth surfaces. The tooth surfaces are subsequently remineralised mainly by salivary washing, acid neutralisation, and the supply of calcium and phosphate ions. In addition, bacterial alkali production and acid neutralisation can contribute to remineralization. For example, lactate can be converted into weaker acids, such as acetate and propionate by *Veillonella*, *Lactobacillus*, and *Actinomyces*, which contributes to acid neutralisation (Takahashi & Yamada, 1999). However, when demineralisation occurs at a greater rate and frequency than that of remineralisation, dental caries can develop.

2.9 Establishment of cariogenic microbial ecosystem in the supragingival area

In the supragingival area, non-mutans streptococci (like *S. sanguinis*) and *Actinomyces* sp. can acidify the environment, although they are not as acidogenic or aciduric as mutans streptococci (*S. mutans* and *S. sobrinus*). Further colonisation of *S. mutans* which, being cariogenic and aciduric, and through resistance to acid can increase their cariogenic potential. *S. sanguinis* convert oxygen into hydrogen peroxide (H_2O_2) which oxidises the thiocyanate (SCN-) in saliva to hypothiocyanite (OSCN-) by the catalysis of salivary

peroxidase. OSCN inhibits the glycolytic activity of *S. mutans* efficiently and possibly represses their growth (Ashby, 2008).

As supragingival plaque becomes thickened and mature, in addition to causing acidification, the oxygen concentration decreases and results in anaerobic conditions. These acidic and anaerobic conditions can then facilitate colonisation of the supragingival plaque by more aciduric and oxygen-labile bacteria like *S. mutans*. In addition, *S. mutans* produces water-insoluble extracellular glucan from sucrose by glucosyltransferase (Gtf), further increasing their colonisation potential. *S. mutans* as well as *S. sanguinis* have the capacity to increase their aciduric and acidogenicity by acid adaptation (Takahashi & Yamada, 1999).

2.10 Periodontal diseases: definition and distribution

Inflammation of the gingival and periodontal tissues is manifested by swelling of the gums and bleeding on brushing. Severe recession of the gums occurs with inevitable loss of dental alveolar bone resulting in tooth mobility and eventually tooth loss. Gingival bleeding has been reported to be highly prevalent among adult populations in all regions of the world, whereas advanced periodontal diseases (with deep periodontal pockets of ≥ 6 mm) affect some 10% to 15% of adults worldwide (Petersen & Ogawa, 2005). In Malaysia, only 26% of 15-19-year-olds have been reported to have healthy periodontal tissues. In those aged 35-44 years, only 5% was reported to have healthy periodontal tissues. Overall, 7.2% were reported to have deep pockets of ≥ 6 mm ("National oral health plan for Malaysia 2011-2020," 2012).

Periodontal diseases have been linked to anaerobic Gram-negative bacteria (*P. gingivalis*, *A. actiomycetemcomitans*, *P. intermedia* and *F. nucleatum*). The risk factors for periodontal diseases related to poor oral hygiene, tobacco use, excessive

alcohol consumption, obesity, blood pressure, stress, and diabetes mellitus (Amano & Inaba, 2012). However, there are fundamental knowledge gaps in the mechanisms of disease initiation and progression. There is also a lack of ability to identify high-risk forms of gingivitis that progress to periodontitis and lack of evidence on how to prevent the diseases effectively (Dentino *et al.*, 2013).

2.10.1 Bacterial metabolism in subgingival plaque and periodontal disease

Gingival crevice and desquamated epithelia are continuously bathed by the efflux of gingival crevicular fluid (GCF) derived from blood plasma. Thus, GCF is nutritionally rich in nitrogenous compounds such as amino acids, peptides and proteins (Takahashi, 2005). Nitrogenous compounds can be degraded into short chain fatty acids, ammonia, sulphur compounds and indole/skatole by subgingival bacteria, including *Fusobacterium*, *Prevotella*, and *Porphyromonas* also known as saccharolytic and anaerobic and/or proteolytic bacteria (Takahashi, 2015). Proteolytic bacteria can degrade nitrogenous compounds into small peptides and amino acids by cell membrane-bound and/or extracellularly secreted proteases for subsequent use as metabolic substrates. For instance, *P. gingivalis* has gingipains (trypsin-like cysteine proteases) and dipeptidylpeptidases, while *P. intermedia* has several proteases that degrade albumin and immunoglobulins (Takahashi, 2005). Overall, the production of acids and alkalis, with the continuous flow of gingival crevicular fluid, results in the maintenance of an almost neutral/weakly alkaline subgingival pH. Short-chain fatty acids, ammonia, and sulphur compounds are known to be cytotoxic to induce tissue inflammation by modulating immune responses (Niederman *et al.*, 1997) and to promote apoptosis (Kurita-Ochiai *et al.*, 2008). These processes contribute to the initiation and promotion of periodontal diseases.

2.10.2 Establishment of periodontopathogens ecosystem in the subgingival area

Host responses such as inflammation and immunoreactions can be induced by bacterial proteases, metabolic end products and bacterial cell components like lipopolysaccharide. Proteases degrade host tissues directly and disturb the host defence indirectly through the degradation of host defence proteins such as complements, immunoglobulins and the blood coagulation system. Metabolic end products such as short-chain fatty acids (propionic, butyric, isobutyric and isovaleric acids), ammonia and sulphur compounds (hydrogen sulphide and methyl mercaptan) impair host cell functions and subsequently disturb the host defence (Niederman *et al.*, 1997).

Subgingival plaque bacterium such as *P. gingivalis* prefers neutral pH and anaerobic conditions (Takahashi *et al.*, 1997; Takahashi & Schachtele, 1990). This bacterium rarely detected in supragingival areas where the environmental pH has become acidic. Conversely, *P. intermedia* and *F. nucleatum* are capable of growth at acidic and neutral pH and are frequently found in supragingival plaque (Mayanagi *et al.*, 2004). In addition, *P. intermedia* and *F. nucleatum* can neutralise the acidic environmental pH by changing the acid-base balance through amino acid metabolism (Takahashi, 2003, Takahashi 2005; Takahashi *et al.*, 1997). Furthermore, *P. intermedia* can utilise glucose as well as nitrogenous compounds, and alter its metabolic characteristics depending upon the nutrients available. In the absence of glucose, this bacterium increases its proteolytic activity and formation of cytotoxic metabolic end products (Saito *et al.*, 2001).

These findings suggest the following succession in the establishment of a pathogenic microbial ecosystem in the subgingival area. Firstly, *F. nucleatum* and *P. intermedia* colonise a shallow gingival pocket (where the pH is variable and sometimes becomes acidic) and then promote the establishment of a neutral pH environment. This bacterial colonisation induces the host defence, such as inflammation of the subgingival area, and

results in the increase of GCF rich in host proteins. This situation may induce the colonisation of a more proteolytic, but acid-intolerant bacterium, *P. gingivalis*, and enhance the pathogenicity of *P. intermedia* though the increase in proteolytic activity and cytotoxic end products. The increase of GCF efflux is obviously effective against the bacterial attack since GCF is rich in various host defence proteins. However, it should be noted that it might supply metabolic substrates to proteolytic and asaccharolytic bacteria living in the periodontal pocket. The activity of subgingival bacteria to degrade host defence proteins, as well as direct destruction of host periodontal tissue, is central to their metabolic adaptation for survival in the subgingival area (Takahashi, 2005).

2.10.3 The role of host susceptibility in periodontal disease

Plaque bacteria are necessary but not sufficient for the development of periodontal diseases, a susceptible host is necessary. In gingivitis, plaque accumulation appears to be a universal finding but the rate of development and the degree of the clinical inflammatory response varies between individuals even under similar plaque accumulation conditions (Tatakis & Trombelli, 2004). Several factors have been shown to modulate the clinical expression of gingival inflammation in response to plaque accumulation. These factors include metabolic factors such as puberty and pregnancy, genetic factors such as Down syndrome, vitamin C deficiency, the intake of drugs, systemic diseases such as leukaemia, immune deficiencies and diabetes mellitus, and other conditions such as stress (Tatakis & Trombelli, 2004).

The difference in susceptibility to periodontitis has largely been attributed to genetic factors (Wankhede *et al.,* 2017). Genetic diseases associated with periodontitis, such as Down syndrome (Michalowicz *et al*., 2000) and Papillon-Lefevre syndrome (Hart *et al*., 1999), highlight the importance of hereditary factors in determining susceptibility to

periodontal disease. Most genes known to be associated with periodontitis in humans are also linked to the immune response. These include genes that affect the expression of interleukin-1, interleukin-6, tumor necrosis factor, interleukin-10, E-selectins, Fc-gamma receptor, CD14, Toll-like receptors, caspase recruitment domain 15 and vitamin D receptor (Laine *et al.,* 2012). However, the understanding the genetic basis of chronic periodontitis or defining the specific loci and genes involved in host susceptibility to the disease in humans has not been determined yet (Kinane & Hart, 2003; Wankhede *et al.*, 2017).

2.11 Treatment of dental caries and periodontal disease

The most common procedures to treat dental caries and periodontal disease are as follows:

a) Mechanical removal of plaque by tooth-brushing and flossing are routine use.
b) Plaque control is effective in the prevention of gingivitis and maintenance of oral health. In chronic periodontitis, debridement of the root surfaces is the most effective routine approach for plaque control.
c) The use of antimicrobial mouthwashes (containing chlorhexidine) or toothpaste containing fluoride or triclosan) is the most commonly use in controlling dental caries.

However, some studies reported that the use of chemical antimicrobial mouthwashes (containing 0.12% chlorhexidine) had common side-effects such as change in taste, increase tartar, dry mouth, mouth/throat irritation or decreased taste sensation (Ciancio, 1995; Slot *et al.*, 2014). In addition, some cases of ototoxicity, deafness, conjunctivitis and colitis have also been reported (Moghadam *et al.*, 1991). Previous studies also reported that microorganisms may develop resistance due to continuous use of 0.2% chlorhexidine up to two years (O'Reilly, 2003; Solís *et al.*, 2011). Hence, the search for

alternative products that are safe, effective and economical continues and natural phytochemicals isolated from plants used in traditional medicine are considered as good alternatives to synthetic chemicals (Palombo, 2011; Prabu *et al.*, 2006; Torwane *et al.*, 2014).

Currently, many studies have been focusing on medicinal plant/ plant extract/ chemotherapeutic which has that have biological activities against *S. mutans* would be potential candidates to be used as an adjunct to prevent/reduce dental caries. These agents may be preventing dental caries through one or more of the following mechanisms (i) antimicrobial approaches which include inhibition of bacterial growth through bactericidal or bacteriostatic mechanisms and (ii) physiological approaches, which involve inhibition of bacterial virulence properties, such as inhibition of adherence ability, biofilm formation, acid production, cell-surface hydrophobicity and gene expression. Inhibition the bacterial virulence properties such as bacterial enzyme and proton permeability also involved (Ban *et al.*, 2012). Hence, the search for alternative yet safe products continues, and natural phytochemicals isolated from plants used as traditional medicines come as good alternatives.

2.12 Medicinal plant as anti-cariogenic agent

2.12.1 *Psidium* guajava Linn

Psidium guajava Linn. also known as guava (Figure 2.5) is classified under Kingdom: Plantae, Division: Magnoliophyta, Class: Magnoliopsida, Subclass: Rosidae, Order: Myrtales, Family: Myrtaceae, Subfamily: Myrtoideae, Genus: *Psidium* and species: *guajava*, Binomial name: *P. guajava* (Esimone *et al.*, 2007; Gupta *et al.*, 2011; Joseph & Priya, 2011). It is a small tree which grows up to 20 feet in height. Leaves are opposite, oblong, three to seven inches in length, with prominent veins below. Flowers

are of white colour and about one inch in diameter. Fruits of *P. guajava* (hereafter referred to as guava) are round ovoid to a pear shape. It is thin shelled with many seeds embedded in a firm pulp or thick shelled with few seeds (Rishika & Sharma, 2012).

This plant is available in the South America, European, Africa and Asia. The tree is also known as *guayabo* (Spanish), *guofa* (Yoruba) and *goba* (Nigeria). In Malaysia, it is generally known as *jambu batu* besides other local names given such as *jambu burung*, *jambu pelawas*, *jambu biji*, *jambu berasu* and *jambu melukut* (Zakaria & Mohd, 1994).

P. guajava is one which has an enormous wealth of medicinal values. Guava is rich in antioxidants compounds and contains a high level of ascorbic acid. Several studies reported that *P. guajava* are effective as agent with anti-diarrhoeal, antimicrobial, anti-mutagenic, antioxidant, anti-parasitic, anti-tussive, hepatoprotective, anti-genotoxic, anti-allergic, anti-cancer and anti-hyperglycemic effects (Gupta *et al.*, 2011; Ravi & Divyashree, 2014a). It is also has been generally used in the treatment of dysentery, menstrual disorders, vertigo, anorexia, digestive problems, gastric insufficiency, inflamed mucous membrane, laryngitis, skin problems, ulcers, vaginal discharge, cold, cough, cerebral ailments, nephritis, jaundice, diabetes, malaria and rheumatism (Daswani *et al.*, 2017; Payal *et al.*, 2010).

The important constituents of *P. guajava* are vitamins, tannins, phenolic compounds, flavonoids, essential oils, sesquiterpene alcohols and triterpenoid acids (Barbalho *et al.*, 2012). Leaves contain phenolic compounds, isoflavonoids, gallic acid, catechin, epicatechin, rutin, naringenin, kaempferol having hepatoprotective, antioxidant, anti-inflammatory, anti-spasmodic, anti-cancer, antimicrobial, anti-hyperglycemic and analgesic actions (Barbalho *et al.*, 2012). The leaf contains two important flavonoids quercetin known for its spasmolytic, antioxidant, antimicrobial and anti-inflammatory

actions, and guaijaverin known for its antibacterial action (Joseph & Priya, 2011; Payal *et al.*, 2010; Rishika & Sharma, 2012).

(a) (b) (c)

Figure 2.5: *Psidium guajava* Linn tree (a), leaves (b) and freeze-dried powdered leaves extract (c)

Several studies demonstrated *P. guajava* or guava leaves effective as anti-plaque agent by reducing bacterial adherence and biofilm formation on the tooth surface, thus prevent dental caries and periodontal disease (Fathilah, 2011). The paste of tender leaves of *P. guajava* has been traditionally used to maintain oral hygiene (Prabu *et al.*, 2006). Guava has shown antibacterial activity against both Gram-positive and Gram-negative bacteria (Gashe *et al.*, 2010; Joseph *et al.*, 2010). The antimicrobial activity of guava is mainly attributed to flavonoids: guaijaverin and quercetin (Arima & Danno, 2002; Kamath *et al.*, 2008; Metwally *et al.*, 2010; Prabu *et al.*, 2006). Quercetin derived from *P. guajava* leaves had shown excellent antibacterial actions against periodontal pathogens (*P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *F. nucleatum*) and caries-

related bacteria (*S. mutans*, *S. sanguinis* and *Actinomyces* sp.) (Geoghegan *et al.*, 2010; Shu *et al.*, 2011). The antibacterial action of quercetin is probably due to the disruption of membrane and inactivation of extracellular proteins by forming irreversible complexes (Shu *et al.*, 2011). The active flavonoid compound, quercetin-3-*O*-alpha-l-arabinopyranoside (guaijaverin) extracted from leaves has high potential anti-plaque activity by inhibiting the growth of *S. mutans* and *Staphylococcus aureus* (*S. aureus*) (Abdelrahim *et al.*, 2002; Limsong *et al.*, 2004; Prabu *et al.*, 2006). Guaijaverin decreases the hydrophobicity, one of the most important initial factors for the oral pathogenic bacteria to adhere to the tooth surface. This action is possible because guava extracts bind to the cell surface proteins reducing the overall cell hydrophobicity and hence guava may have the potential for development as a natural anti-plaque agent (Prabu *et al.*, 2006). Guava and *Piper betle* extracts have shown the bacteriostatic effect on the primary colonizers of teeth during plaque formation *S. sanguinis*, *Streptococcus mitis* (*S. mitis*) and *Actinomyces* sp. (Fathilah, 2011; Fathilah *et al.*, 2009). *S. mitis* and *S. sanguinis* showed lesser adherence to the experimental pellicle treated with guava extracts when compared with untreated pellicle due to the ability of guava to disrupt the pellicle preventing adherence of bacteria (Wan Nordini Hasnor *et al.*, 2013).

Mouthrinse containing guava leaf extract had a profound effect on gingivitis (Kraivaphan *et al.*, 1991). The aqueous extracts of *P. betle* and guava were found to have anti-plaque activity by their effect on ultrastructure of plaque bacteria by interfering with normal growth cycle and development, reducing the adhering capacity of the acquired pellicle which forms on the surface of tooth during early plaque formation, diminishing the cell-surface hydrophobicity of bacteria which are required to assist the adherence process (Fathilah *et al.*, 2009; Razak *et al.*, 2006; Razak & Rahim, 2003). The cytotoxic property of guava would be of added value for the use of guava as adjuncts in the development of oral healthcare products (Fathilah *et al.*, 2010). Guava extract has

demonstrated *in vitro* anti-plaque actions by inhibiting growth, adherence and co-aggregation of dental plaque bacteria. Guava extracts may inhibit plaque development without disrupting homeostasis of the oral cavity (John *et al.*, 2013b). Thus, guava is said to be an excellent antibacterial and anti-plaque agent. It also has potential in treating periodontal disease (Ravi & Divyashree, 2014).

2.12.2 *Mangifera indica* Linn.

Mangifera indica L. (mango or locally known as *Mangga* in Malaysia and Singapore), (Figure 2.6) belongs to the family Anacardiaceae, is native from tropical Asia with the highest diversity occurs in Malaysia, particularly in peninsular Malaya, Borneo and Sumatra (Bompard, 2009). It is a large evergreen tree that grows to a height of 10-45 m, dome shaped with dense foliage, typically heavy branched from a stout trunk. The leaves are spirally arranged on branches, linear-oblong, lanceolate, elliptical, pointed at both ends, the leaf blades mostly about 25-cm long and 8-cm wide, sometimes much larger, reddish and thinly flaccid when first formed and release an aromatic odour when crushed. The inflorescence occurs in panicles consisting of about 3000 tiny whitish-red or yellowish-green flowers. The fruit is a well-known large drupe, but shows a great variation in shape and size. It contains a thick yellow pulp, single seed and thick yellowish-red skin when ripe. The seed is solitary, ovoid or oblong, encased in a hard, compressed fibrous endocarp (Shah *et al.*, 2010).

According to Ayurveda, varied medicinal properties are attributed to different parts of a mango tree. *M. indica* L. is used medicinally to treat ailments such as asthma, cough, diarrhoea, dysentery, pains, malaria, dentifrice, antiseptic, astringent, diaphoretic, stomachic, vermifuge, tonic, laxative and diuretic (Shah *et al.*, 2010).

(a)

(b)

(c)

Figure 2.6: *Mangifera indica* Linn tree with flowers (a), fresh leaves (b) and freeze-dried powdered of leaves extract (c)

The qualitative phytochemical analysis of the aqueous extract of *M. indica* revealed the presence of tannins, flavonoid, steroid, cardiac glycoside, alkaloids, carbohydrate and protein. These secondary metabolites may be responsible for the anti-diarrhoeal properties of the crude extract (De & Pal, 2014). The bark is reported to contain protocatechic acid, catechin, mangiferin, alanine, glycine, γ-aminobutyric acid, kinic acid, shikimic acid and the tetracyclic triterpenoids cycloart-24-en-3β,26-diol, 3-ketodammar-24 (E)-en-20S,26-diol, C-24 epimers of cycloart-25 en 3β,24,27-triol and cycloartan-3β,24,27-triol (Scartezzini & Speroni, 2000). Mangiferin, (a natural C-glucoside-xanthone) 2-C-β-D-gluco-pyranosyl-1,3,6,7-tetrahydroxyxanthone; $C_{19}H_{18}O_{11}$; MW: 422.35g/mol; melting point, anhydrous 271°C has been reported in various parts of *M. indica* leaves, fruits, stem bark, heartwood and roots. Mangiferin is extracted from mango at high concentration from young leaves (172 g/kg) and old leaves (94 g/kg) (Kalita, 2014; Muruganandan *et al.*, 2002). Mangiferin, being a polyphenolic antioxidant

and a glucosyl xanthone, it has strong antioxidant, anti-lipid peroxidation, immunomodulation, cardiotonic, hypotensive, wound healing, anti-degenerative and anti-diabetic activities (De & Pal, 2014).

2.12.3 *Mentha piperita* Linn

Peppermint (*Mentha piperita* L.) also known as mint (Figure 2.7) is a medicinally important plant belongs to the Family Lamiaceae (Labiatae) (Sujana *et al.*, 2013). *M. piperita* is a hybrid of spearmint (*M. spicata* L.) and water mint (*M. aquatica* L.), it grows particularly well in areas with high water-holding capacity soil (McKay & Blumberg, 2006). Peppermint originally came from Europe and Middle East and nowadays it is found in various countries of the world both as cultivated or wild. The medicinal parts are the essential oil extracted from the aerial parts of the flowering plant, the dried leaves, the fresh flowering plant and the whole plant. It has been documented in the literature that *M. piperita* leaves (fresh and dried) and the essential oil extracts from the leaves are used in many food, cosmetic, dental preparations, mouthwashes, soaps, alcoholic liquors and pharmaceutical products for both its flavouring and fragrance properties (McKay & Blumberg, 2006; Sujana *et al.*, 2013).

The chemical components of peppermint leaf and oil vary with plant maturity, variety, geographical region and processing conditions (McKay & Blumberg, 2006; Park *et al.*, 2016). The fatty acid composition of the non-polar lipid fraction of peppermint leaves is dominated by palmitic (16:0), linoleic (18:2) and linolenic (18:3) acids (Maffei & Scannerini, 1992). The main volatile components identified in the essential oil of peppermint are menthol (33-60%), menthone (15-32%), isomenthone (2-8%), 1,8-cineole (eucalyptol) (5-13%), menthyl acetate (2-11%), menthofuran (1-10%), limonene (1-7%), β-myrcene (0.1-1.7%), β-caryophyllene (2-4%), pulegone (0.5-1.6%) and carvone (1%)

(Dimandja *et al.*, 2000; Nair, 2001; Skalicka-Woźniak & Walasek, 2014). The leaves contain 1.2-3.9% (v/w) essential oil (0.38% yield from fresh leaves), while an infusion of dried leaves is reported to contain 21% of the original oil (25 mg/L) (Kaul *et al.*, 2001; Picuric-Jovanovic *et al.*, 1997). The phenolic constituents of the leaves include rosmarinic acid and several flavonoids, primarily eriocitrin, luteolin and hesperidin and smaller quantities of 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone, pebrellin, gardenin B and apigenin (Areias *et al.*, 2001; Hoffmann & Lunder, 1984; McKay & Blumberg, 2006; Riachi & De Maria, 2015).

(a) (b)

Figure 2.7: *Mentha piperita* fresh leaves (a) and freeze-dried powdered leaves extract (b)

It is well documented *in vitro* studies that the essential oil or extracts of *M. piperita* possess antimicrobial, fungicidal, antiviral, insecticidal, strong antioxidant and antitumor actions (McKay & Blumberg, 2006; Tsai *et al.*, 2013). The antibacterial and antifungal activity of methanolic leaf extract of *M. piperita* is attributable to the presence of tannins and flavonoids (Kapp *et al.*, 2013; Pramila *et al.*, 2012). The aqueous extract of

M. piperita has considerable antibacterial activity against *Helicobacter pylori,* the main etiological agent of chronic gastritis and peptic ulcer disease (Castillo-Juárez *et al.*, 2009). Peppermint oil possesses a fresh sharp menthol odour and a pungent taste followed by a cooling sensation. Nowadays, menthol is added in commercial tooth pastes to offer protection against oral microbial infections. Animal model studies demonstrate a relaxation effect on gastrointestinal (GI) tissue, antiallergenic and anti-inflammatory actions, analgesic and anaesthetic effects in the central and peripheral nervous system, immunomodulating actions and chemopreventive potential. Human studies on the gastrointestinal tract, respiratory tract and analgesic effects of peppermint oil and its constituents have been reported. Several clinical trials examining the effects of peppermint oil on irritable bowel syndrome (IBS) symptoms have been conducted (Ford *et al.*, 2008; Kline *et al.*, 2001). However, human studies of peppermint leaf are limited, and clinical trials of peppermint tea are absent. Adverse reactions to peppermint tea have not been reported, although caution has been urged for peppermint oil therapy in patients with GI reflux, hiatal hernia or kidney stones (McKay & Blumberg, 2006).

CHAPTER 3: MATERIALS AND METHODS

3.1 Preparation of solution and media

3.1.1 Preparation of 0.1% hydrochloric acid (HCl) solution

This was done by dissolving 2.7 ml of 37% (v/v) of hydrochloric acid (HCl) (Merck, Germany) in 997.3 ml deionised water to prepare 0.1% (v/v) of HCl solution and kept at room temperature in 1 L Schott bottle. The solution was used for acid washes of glass beads and rubber tubing by running through the solution to remove the contaminants using a peristaltic pump which set 5 ml/min.

3.1.2 Preparation of 70% ethanol for disinfection purposes

This was carried out by dissolving 3.9 ml of 95% (v/v) ethanol (Merck, Germany) in 496.1 ml deionised water to prepare 70% (v/v) ethanol in 500 ml, mixed homogeneously and kept in sprayer bottle for further use. The 70% (v/v) ethanol was effective as an antibacterial agent to kill the Gram-positive and Gram-negative bacteria on the inanimate surface such as table-tops and laminar flow.

3.1.3 Washing and sterilisation of apparatus used

All the bottles, bicars, conical flasks (2 L) and any other items used that withstand at higher temperature and pressure were washed, dried at 50^{o}C overnight and sterilised by autoclaving at 121^{o}C for 20 min with pressure at 15 ppm (Tomy SX-500, Japan). The inoculum loop, forceps and glass rods were sterilised by direct flaming using Bunsen burner. Glass beads (1 mm and 3 mm in diameter) were washed with mild hydrochloric acid (HCl; 0.1% v/v) and rinsed with tap water followed by rinsing with deionised water,

then were dried at 60°C for 2 days before being sterilised by autoclaving at 121°C for 20 min in universal bottle and kept at 50°C for further use. The used rubber tubing was cleaned by running through the deionised water for 30 min followed by acid washed (0.1% v/v of HCl) for another 30 min and continued with flow in the deionised water using peristaltic pump at flow rate 5 ml/min by 2 times changing the deionised water for 1 hour. After that, the outer layer of the rubber tubing was immersed using deionised water and dried at 60°C for 1 day before being sterilised by autoclave. All the sterilised items were dried and kept at 50°C for further use.

3.1.4 Preparation of Phosphate Buffer Saline

Two tablets of Phosphate Buffer Saline (PBS) (Oxoid, UK) were dissolved in 200 ml of deionised water, stirred and autoclaved at 121°C for 20 min with pressure at 15 ppm. The cold 0.1 M PBS solution (pH 7.4) was kept at 4°C for further use.

3.1.5 Preparation of Brain Heart Infusion (BHI) broth

This step involved weighing 18.5 g of Brain Heart Infusion (BHI) powder (Oxoid, UK) to mix homogeneously with 500 ml of deionised water using hot plate stirrer in 1 L Schott bottle and was sterilised by autoclaving at 121°C for 20 min with 15 ppm pressure. The cool sterile broth was kept at 4°C for further use.

3.1.6 Preparation of Brain Heart Infusion (BHI) agar

A weight of 42.3 g of BHI powder (Oxoid, UK) was added to 900 ml of deionised water in 1 L Schott bottle, stirred using hot plate stirrer and autoclaved at 121°C for 20 min with 15 ppm pressure. The warm sterile BHI agar at 60°C was poured onto 25 mm

diameter sterile Petri dishes in laminar flow and the cooled agar was kept at 4^{o}C in their plastic bag for further use.

3.1.7 Preparation of Brain Heart Infusion-containing sucrose (BHI+1% (w/v) or 2% (w/v) sucrose) broth

A weight of 8.5 g of BHI powder (Oxoid, UK) and 5 g of D(+)-sucrose (1% w/v) was added into 500 ml of deionised water (in 500 ml Schott bottle), stirred using hot plate stirrer and autoclaved at 121^{o}C for 20 min with 15 ppm pressure (Tomy SX-500, Japan). The cool sterile BHI-containing 1% (w/v) sucrose broth was then kept at 4^{o}C for further use. Similar procedure was carried out to prepare BHI-containing 2% (w/v) sucrose broth by adding 10 g sucrose into 500 ml of deionised water.

3.2 Experimental Assays:

3.2.1 Ethical approval

The study protocol was reviewed and approved by the Ethics committee of Faculty of Dentistry University of Malaya, Kuala Lumpur, Malaysia (The Ethic committee/IRB reference number: DF OB1506/0070(P) (Please see Appendix: The Ethic Approval) Written consent was obtained from one donor for saliva collection.

3.2.2 Plant collection and authenticated

Fresh leaves of *Psidium* sp. (voucher no. 48126) and *Mangifera* sp. (voucher no. 48124) were obtained from Balai Ungku Aziz of the University of Malaya, Kuala Lumpur and the UPM Agriculture Park, Selangor, respectively. Leaves of *Mentha* sp. (voucher

no. 48127) grown in Cameron Highlands, Pahang, were obtained from a local market in Selangor, Malaysia. The plant leaves were identified by Dr. Sugumaran a/l Manikum and deposited under the stated voucher numbers in Rimba Ilmu Herbarium, University of Malaya.

3.2.3 Extraction of crude aqueous plant extract

The fresh plant leaves were washed with tap water, followed by deionised water, dried using tissue paper, weighed and cut into small pieces before boiling process (Nalina & Rahim, 2006). One hundred g of fresh leaves were boiled in 1 L of deionised water for several hours until the final volume was one-third of the initial volume. The decoction was then filtered using a muslin cloth to remove any debris and the clear filtrate was further centrifuged at 1500 *g*, 4^{o}C for 15 min to eliminate any sediment. The supernatants were filtered using Whatman No. 1 paper with a diameter of 150 cm and boiled again until the final volume of 100 ml. After being cooled at room temperature, the supernatants were then frozen overnight at -80^{o}C followed by freeze-dried for 2 days using freeze dryer (Eyela FDV-1200, China) in sterile environment. The sterile dried powdered crude aqueous extracts were weighed and stored at -20^{o}C for further use in 50 ml sterile polystyrene test tubes and the desired working concentration (w/v) were prepared accordingly prior to the experiments. The yield of powdered crude aqueous extract was calculated as percentage yield of crude aqueous extract (X):

$$X = \frac{\text{yield of powdered crude aqueous extract (g)}}{\text{Fresh leaves extract (g)}} \times 100$$

3.2.4 Preparation of bacterial stock

The frozen stock of bacteria (*S. sanguinis* and *S. mutans*) kept at -80^{o}C freezer were thawed at room temperature, suspended into 20 ml sterilised BHI broth and incubated for 15 hours at 37^{o}C. The bacterial suspensions were streaked onto the surface of BHI agar followed by incubation at 37^{o}C for 24 hours. The three pure colonies formed were selected and suspended into 20 ml sterilised BHI broth and further incubated 37^{o}C until the mid-log phase of growth (OD550 nm of 0.7-1.2) which was 6 hours for *S. mutans* and 8 hours for *S. sanguinis*. The bacterial suspensions were mixed with sterilised glycerol to give a suspension of 20% (v/v) glycerol. About 1 ml of the bacterial suspensions were then aliquoted into 1.5 ml microcentrifuge tubes and kept at -80^{o}C for further use.

3.2.5 Preparation and standardisation of bacterial suspension

S. sanguinis ATCC BAA-1455 (strain SK36) and *S. mutans* ATCC 25175 used in this study were obtained from American Type Culture Collection (ATCC, USA). Prior to the experiment, the respective 20% (v/v) glycerol stocks of each bacterium at -80^{o}C was thawed at room temperature and (1% v/v) of the bacterial suspensions was inoculated into 20 ml of sterile fresh Brain Heart Infusion (BHI) broth (Oxoid, UK), incubated aerobically at 37^{o}C with shaking at 150 rpm until mid-log phase of growth, which was 6 hours for *S. mutans* and 8 hours for *S. sanguinis*. The bacterial suspensions were centrifuged at 5800 *g*, 4^{o}C for 10 min and the pellets were washed three times with ice-cold sterilised deionised water, suspended in fresh BHI broth and incubated at 37^{o}C for 15 min to reactivate their growth phase. The turbidity of each bacterial species was standardised by adjusting the absorbance to 0.144 (equivalent to 1.00 x 10^{8} CFU/ml and 1.53 x 10^{7} CFU/ml for *S. sanguinis* and *S. mutans,* respectively) at 550 nm using a spectrophotometer (Shimadzu UV-1700, Japan) (Shafiei *et al.*, 2016). For the preparation

of dual-species bacteria, the standardised suspensions of the two bacteria were mixed in equal volumes (based on CFU/ml as stated above) prior to the experiment. The purity of the bacterial cultures was checked each time prior to every experiment by streaking the culture broth onto BHI agar and incubated at 37ºC for 24-48 hours.

3.2.6 Determination of growth curve of bacteria

Two ml of the standardised bacterial suspension (e.g. *S. sanguinis*) at OD 550 nm of 0.144 was inoculated in 200 ml of fresh BHI broth in 500 ml Schott bottles in triplicate. The bacterial suspensions were then incubated in an orbital shaker at 37ºC, shaking at 150 rpm for 24 hours. At every 1-hour intervals, 1 ml of the homogenous bacterial suspension from each bottle was measured the optical density at OD 550 nm in triplicate using a spectrophotometer. Then, the bacterial suspensions were reincubated for a subsequent hour until 24 hours' measurement was taken. The similar experiments were carried out for *S. mutans* and a mixture of *S. sanguinis* and *S. mutans*. The growth profile during incubation period was plotted to determine mid-log phase growth of the bacteria strains. The results were expressed as mean ± SD of 9 determinations.

3.2.7 Determination of minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and the sum of the fractional inhibitory concentration (ΣFIC) index

Broth microdilution method in accordance with standard protocol of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2012) and a modified method of Kuete *et al.* (2009) was used to determine the MIC of PEM and its individual constituent plant extracts (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.). One g of sterile dried extract (*Psidium* sp.) was reconstituted in 7.81 ml sterile deionised water giving a final

concentration of extract at 128 mg/ml. After sonication for 30 seconds and vortexing for 30 min, the reconstituted extract was centrifuged at 1500 *g*, 4^{o}C for 15 min. The supernatant of the reconstituted extract was serially diluted two-fold in BHI broth to obtain a range of concentration (0.25-64 mg/ml) in respective test tubes labelled as T1 to T9. The diluted extracts were transferred into the corresponding wells of a 96-well microtiter plate (NUNC™ Brand products) as follows. To the first three rows of the 96-well, the microtitre plate labelled as A1 to A9; B1 to B9; C1 to C9 (carried out in triplicate to ensure reproducibility), 100 µl of the respective serially diluted plant extract in BHI broth (T1 to T9) was added. To wells labelled as A11, B11, C11, 100 µl of BHI broth was added to ensure bacterial growth. To all of the wells above, 5 µl of *S. sanguinis* suspension (standardised to 10^6 CFU/ml) was added. This gave the final concentration of the extract in the respective wells (A1 to A9; B1 to B9; C1 to C9) ranging from 0.24-60.95 mg/ml. In addition to these, wells labelled as A12, B12, C12 were filled with 105 µl of BHI broth just to ensure the sterility of the broth. To another row of wells, one row apart from the others (E1 to E9), 105 µl of the two-fold serially diluted plant extract only was added which served as blank control. The microtitre plate was sealed and covered with aluminium foil, incubated aerobically at 37^{o}C with shaking at 150 rpm for 24 hours. Following incubation, the MIC was determined at 550 nm using ELISA reader based on turbidity determined by comparing the absorbance of the suspension in wells of test extract (A1 to A9; B1 to B9; C1 to C9) with that of the corresponding blank control. The MIC of each compound was defined as the lowest concentration that inhibited the bacterial growth. Similar procedure was repeated with *S. mutans*. The similar experiment was repeated for the other plant extracts (*Mangifera* sp., *Mentha* sp. and PEM). For PEM, an equal volume of the stock solution of extracts (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.) each with a concentration of 128 mg/ml was mixed followed by two-fold serial dilution.

For the MBC determination, 20 µl of the bacterial suspension from selected wells (A1 to A9; B1 to B9; C1 to C9) which showed almost no turbidity was inoculated on BHI agar (Oxoid, UK) and incubated for 48 hours at 37°C. The lowest concentration of the extracts that showed no growth corresponded to the MBC value.

The sum of the fractional inhibitory concentration (ΣFIC) determines the synergy of the plant extracts, calculated using the following equation:

$\Sigma FIC = 1/3(FIC_I + FIC_{II} + FIC_{III})$ and the FICs were calculated as follows:

$$FIC_I \text{ (FIC of } \textit{Psidium} \text{ sp.)} = \frac{\text{MIC (PEM)}}{\text{MIC (}\textit{Psidium}\text{ sp.)}}$$

$$FIC_{II} \text{ (FIC of } \textit{Mangifera} \text{ sp.)} = \frac{\text{MIC (PEM)}}{\text{MIC (}\textit{Mangifera}\text{ sp.)}}$$

$$FIC_{III} \text{ (FIC of } \textit{Mentha} \text{ sp.)} = \frac{\text{MIC (PEM)}}{\text{MIC (}\textit{Mentha}\text{ sp.)}}$$

The ΣFIC index determines the interaction between the different plant extracts in the mixture where the interaction is interpreted according to a range of values; value less than 0.5 as synergistic, value greater than 0.5 to 1 as additive, value greater than 1 to 4 as indifferent and value greater than 4 as antagonistic (van Vuuren & Viljoen, 2011).

3.2.8 Preparation of sterilised saliva

Undiluted sterile saliva was prepared according to the method as described in previous studies (De Jong & Van der Hoeven, 1987; Wan Nordini Hasnor *et al.*, 2013). To minimise variation, a single healthy volunteer (43 years old, female) was used to collect 100 ml of stimulated whole saliva (SWS). The SWS collection was done by expectoration after chewing sugar-free gum (before eating or at least 2 hours after eating) in ice-chilled test tubes. Exclusion criteria were younger than 20 years old or those having received

antibiotic treatment within the previous 4 weeks. The aggregation of the protein in the SWS was minimised by adding 1,4-Dithio-DL-threitol (DTT) (GE Healthcare, Sweden) to a final concentration of 2.5 mM. The mixture was stirred slowly for 10 min followed by centrifugation at 800 *g*, 4°C for 30 min. The supernatant was then filter sterilised through a disposable 0.22 µm (pore size) low-protein-binding filter (Cellulose acetate syringe filters Sartorius, USA) into sterile centrifuge tubes and stored at -20°C until further use. Prior to the experiment, the sterile SWS was thawed and centrifuged to remove any precipitate. It was later used to coat glass beads (3 mm in diameter) (Merck, Germany) in an artificial mouth (NAM) model forming a layer that mimics the acquired pellicle on the tooth surface and thus referred as the Experimental Pellicle.

3.2.9 Effect of plant extracts on adherence capacity of bacteria

Prior to use, the dried aqueous extracts (15 mg) were reconstituted in sterile deionised water (30 ml) to a final concentration of 0.5 mg/ml. The reconstituted extracts were further sterilised by filtration using 0.22 µm (pore size) nylon syringe filters (Millipore Billerica, USA). For PEM, the same concentrations of the sterile reconstituted individual extracts were mixed (1:1:1 v/v/v) to give a mixture of extracts of the same concentration (0.5 mg/ml).

Nordini's Artificial Mouth (NAM) model developed by Rahim *et al.* (2008) was used in the *in vitro* study of the anti-adherence effect of PEM and its individual constituent plant extracts towards the early and late plaque colonisers in single- and dual-species biofilms. The NAM model was used in this study to represent oral cavity. The model consists of a glass chamber (6 cm length), glass beads (3 mm diameter), water bath system, saliva reservoir, **bacterial** reservoir and a peristaltic pump (Rahim *et al.*, 2008). In this study, nine glass beads were placed in the glass chamber of the NAM model. The glass beads represent the tooth surface. The glass chamber which was immersed in the

water bath system, set at 37°C represents the oral cavity. The peristaltic pump (Masterflex L/S, USA) of the NAM model was used to flow the saliva/test extract/bacteria from the reservoir into the system via rubber tubing at a constant flow rate (0.3 ml/min for resting saliva). The experiment was carried out as follows. Saliva was first flowed into the NAM model for 2 min at a constant rate to form an experimental pellicle on the glass beads. Excess saliva was rinsed off by allowing sterile deionised water to flow in for 2 min. This allowed the experimental pellicle to be pre-treated with sterile deionised water. Following this, *S. sanguinis* which were standardised to 0.144 absorbances at 550 nm wavelength was allowed to flow into the NAM model for 24 hours to form a 24-hour biofilm on the experimental pellicle-coated glass beads (Shafiei *et al.*, 2016).

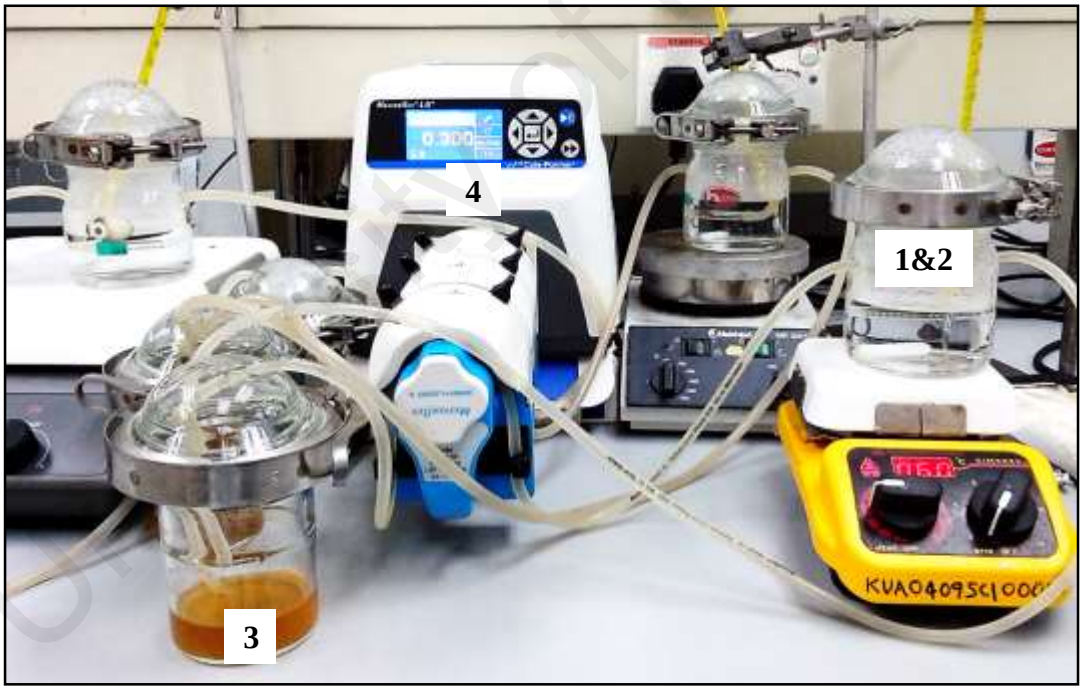


Figure 3.1: Photograph of NAM model of three different sets of the experiment running together. The NAM model consists of (1) glass beads in the glass chamber, represent tooth surface; (2) glass chamber immersed in water bath system, set at 37°C using hot plate stirrer, represent oral cavity; saliva/test extract/0.12% (v/v) CHX reservoir (using the test tube); (3) bacteria reservoir that flow via rubber tubing and (4) a peristaltic pump, set at 0.3 ml/min (flow rate of resting saliva) to flow bacteria in and out.

3.2.10 Measurement of adherence and anti-adherence capacity of bacteria

Of the nine glass beads, three were taken out randomly whereas the remaining six glass beads were kept for SEM viewing and reserves. The three glass beads were then individually placed in a sterile microcentrifuge tube containing 1 ml sterile 0.1 M PBS (pH 7.4), sonicated for 10 seconds and vortexed for one minute to dislodge the attached bacteria. The suspension was serially diluted ten-fold (10^{-1} to 10^{-5} dilutions) with PBS. A 100 µl of the homogeneous bacterial suspension from each tube was pipetted out and plated on three separate BHI agar plates using the Lawn method. The plates were incubated for 24 hours at 37°C. The counting of all viable microorganisms formed on the plate done using colony counter. Plates from the dilution which gave a CFU number of between 30-300 colonies were selected and used in the calculation of the growth population. The bacterial suspension used in the plating was 100 µl and hence it was expressed as CFU/ml based on the following formula:

$$\text{Total CFU/ml} = \frac{\text{Number of colonies}}{\text{Volume plated (ml)} \times \text{Dilution factor}}$$

The deionized water treated experimental pellicle served as negative control for *S. sanguinis* in single-species biofilm and the bacterial population (total CFU/ml) assumed as 100% bacterial adherence. A similar procedure was repeated with *S. mutans* (standardised to 0.144 absorbances at 550 nm) for single-species biofilm. For dual-species biofilms, the individual strain was mixed in equal amount (1:1 v/v) and a similar procedure was repeated. In the dual-species biofilms, the two bacteria were differentiated according to their colony sizes. *S. mutans* colony is larger than *S. sanguinis* allowing the colonies to be counted separately (please refer Appendix A).

The experiment was subsequently repeated where the experimental pellicle was pre-treated with respective plant extract (*Psidium* sp. / *Mangifera* sp. / *Mentha* sp. / PEM) at

sub-MIC (0.5 mg/ml) before inoculation with bacterial suspension used for the development of single- and dual-species biofilms. For positive control, the experimental pellicle was pre-treated with 0.12% (v/v) chlorhexidine gluconate (CHX). The individual experiment was carried out in triplicate. The NAM model was run parallel with negative, positive controls and test as shown in Figure 3.1.

Percentage of adherence (X) was calculated using the equation below:

$$X = \frac{\text{adhered cells (test or positive control)}}{\text{adhered cells (negative control)}} \times 100$$

Assuming the bacterial adherence to negative control was 100%, the percentage of anti-adherence was calculated as:

$$\% \text{ Anti-adherence} = 100 - X.$$

3.2.11 Scanning Electron Microscopy (SEM) analysis of cell population on glass beads

The remaining beads from the experiments were processed for SEM viewing according to the method described by Rahim and Thurairajah (2011) with slight modification. Briefly, three glass beads for each of experiment were fixed with 1 ml of 4% (v/v) glutaraldehyde solution (prepared in sodium cacodylate buffer, pH 7.4) and kept at 4^{o}C until the subsequent analysis. Prior the analysis, 4% (v/v) glutaraldehyde solution was pipetted out and washed twice with sodium cacodylate buffer for 15 min, followed by postfixing with 2% (v/v) osmium tetroxide in 1% (w/v) sodium cacodylate buffer solution for 1 hour and kept at 4^{o}C. Then, the glass beads were rinsed with deionised water twice for 15 min before dehydration process. A series of 30, 50, 70, 80, and 95% (v/v) ethanol at an interval of 15 min was used for dehydration process. This was followed by dehydration carried out twice for 15 min each using 100% ethanol. Ethanol was

gradually displaced with acetone in the following ratios (v/v); Ethanol: Acetone 3:1, 1:1, 1:3 each for 20 min and finally followed by 100% acetone three times for 20 min. The dehydrated samples were then subjected to Critical Point Drying (CPD) (Balzers CPD 030, Liechtenstein) for 1 hour 40 min in liquid CO_2 under 95 bar pressures. The glass beads were then kept in a tight container in a desiccator. Prior to SEM viewing, the beads were gold-coated under low pressure with ion sputter coater (Joel JFC1100, Japan). The beads were viewed for cell population at 10,000X magnification using Scanning Electron Microscope (Quante FEG 250, Holland) (refer to Appendix B for photograph of SEM).

3.2.12 Effect of plant extracts on bacterial population in biofilm

Prior to use, each of the plant extracts (*Psidium* sp., *Mangifera* sp., *Mentha* sp.) was reconstituted separately in sterile deionised water to a final concentration of 0.5 mg/ml. The respective reconstituted extracts were sonicated for 1 min, vortexed for 30 min and centrifuged at 1500 *g*, 4^{o}C for 15 min. The respective supernatants were then sterilised using 0.22 µm (pore size) nylon syringe filters (Sartorius, USA). PEM contained an equal volume of the reconstituted individual extracts at sub-MIC of 0.5 mg/ml. Then the mixture was sterilised by filtration using 0.22 µm (pore size) nylon syringe filters and kept in sterile Falcon tubes for subsequent use.

S. sanguinis and *S. mutans* singly and mixed were used to develop 24-hour biofilm in a dynamic environment using Nordini's Artificial Mouth (NAM) model with some modification (Rahim *et al.*, 2014; Shafiei *et al.*, 2016; Wan Nordini Hasnor *et al.*, 2013). This biofilm study was designed to resemble the use of plant extract after meal whereas the one reported by previous studies mentioned above were designed to resemble the use of the plant extracts before a meal for the bacterial adherence study.

In this experiment, the NAM model consisted of nine glass beads in a glass chamber that mimicked the surface of a tooth in the oral cavity. The glass chamber was immersed in a water bath system that was set at 37^{o}C to represent human oral cavity. For the development of a biofilm, sterilised saliva was set to flow into NAM model for 2 min at a constant flow rate of resting saliva, (0.3 ml/min) to form the experimental pellicle on glass beads. Then, the excesses saliva was rinsed off for 2 min with sterile deionised water. The respective standardised bacterial suspension (*S. sanguinis*) grown in BHI broth was then allowed to flow for 24 hours to develop a 24-hour biofilm on experimental pellicle-coated glass beads. This represented the bacterial growth in absence of sucrose. The respective plant extract (*Psidium* sp.) at a final concentration of 0.5 mg/ml flowed into NAM model for 2 min to treat the 24-hour biofilm, followed by rinsing off the excess plant extract by letting sterile deionised water flow for 2 min. Similar steps were carried out for other plant extracts of *Mangifera* sp., *Mentha* sp. and PEM. Sterile deionised water and 0.12% (v/v) CHX gluconate were served as negative and positive controls in place of plant extracts. All the experiments stated above were tested towards respective single- and dual-species of *S. sanguinis* and *S. mutans* biofilms.

This study was also designed to investigate the anti-biofilm effect of plant extracts towards single- and dual-species biofilms developed in the presence of sucrose. Similar procedures were adopted, except that standardised bacterial suspension grown in BHI containing 1% (w/v) sucrose broth was used to represent a 24-hour biofilm grown in the presence of sucrose. Each of the experiment was carried out in triplicate.

3.2.13 Measurement of bacterial population in biofilm after treatment with plant extracts

The glass beads with the biofilms were randomly removed from the NAM model for enumeration of bacterial population retained after treatment with the plant extracts. The individual glass bead was diluted with 1 ml Phosphate Buffered Saline (0.1 M PBS; pH 7.4) in corresponding 1.5 ml microcentrifuge tube, followed by sonication for 10 s and vortexing for 1 min to homogeneously mix the bacterial suspension. A ten-fold serially diluted in 0.1 M PBS (10^{-1} to 10^{-5} dilutions) was prepared and 100 µl of each dilution was spread onto three BHI agar plates. The plates were incubated aerobically at 37^{o}C for 24-48 hours. The plates that gave colonies counts between 30-300 CFU were chosen for bacterial enumeration and the bacterial population was expressed as colony forming unit per ml (CFU/ml). Percentage of the bacterial population retained in the biofilm (X) was calculated using the equation below:

$$X = \frac{\text{cell population in the plant extract- OR in the CHX-treated biofilm}}{\text{cell population in the deionised water-treated biofilm (negative control)}} \times 100$$

The negative control was assumed as 100% of the bacterial population in the biofilm.

3.2.14 Effect of plant extracts on cell-surface hydrophobicity of bacteria

This was measured according to the microbial adherence test to hydrocarbon (*n*-hexadecane) (MATH) with modifications (Rahim *et al.*, 2008; Rosenberg *et al.*, 1980). A bacterial suspension of *S. sanguinis* (0.5% v/v) in sterile fresh Brain Heart Infusion (BHI) broth was incubated at 37^{o}C, shaking at 150 rpm for 14 hours until the late exponential phase and then harvested by centrifugation at 5800 *g*, at 4^{o}C for 10 min. The cells pellet was collected, washed twice with phosphate urea magnesium (PUM) buffer of pH 7.1, re-suspended in the PUM buffer and standardised to an optical density (OD) of 1.2 at 550

nm using spectrophotometer (Shimadzu UV-1700, Japan). An equal amount (2 ml) of the bacterial suspension and plant extract (*Psidium* sp.; at a final concentration of 0.5 mg/ml) were mixed in the test tubes and was allowed to stand for 10 min at room temperature. One ml of the mixture was used for OD measurement at 550 nm. This would serve as the initial OD of the bacterial suspension treated with plant extract before agitation (A_0).

Following that, 400 µl of *n*-hexadecane (Sigma-Aldrich, Germany) was added to the plant-bacterial suspension mixture. The mixture was agitated uniformly in a vortex mixer for 1 min and allowed to equilibrate at room temperature for 15 min. After *n*-hexadecane phase separation from the aqueous phase, the OD of the aqueous phase was measured spectrophotometrically at 550 nm. This OD would serve as the final OD of bacterial suspension treated with plant extract in the aqueous phase after agitation (A_1). For blank controls, PUM buffer with plant extracts and sterile deionised water were used, respectively. The experiment was repeated with *S. mutans*. The individual experiment was carried out in triplicate and repeated three times independently for the other plant extracts (*Mangifera* sp., *Mentha* sp. and PEM) on the two bacteria. Results were expressed as the proportion of cells that were excluded from the aqueous phase, determined by the equation: $[(A_0-A_1)/A_0] \times 100$, where A_0 and A_1 are, respectively, the initial and final ODs of the aqueous phases. Strain with a hydrophobic index greater than 70% was arbitrarily classified as hydrophobic, moderate hydrophobic (50-70%) and low hydrophobic (< 50%) (Nostro *et al.*, 2004; Tahmourespour *et al.*, 2008).

3.2.15 Effect of plant extracts on acid production of bacteria

Glycolytic pH drop assay was used to determine acid production with some modifications (Hasan *et al.*, 2015). A thawed bacterial stock of -80ºC (*S. sanguinis*) (0.5% v/v) was incubated in 30 ml BHI broth at 37ºC for 15 hours and was harvested by

centrifugation at 5800 *g*, 4°C for 10 min. The cells pellet was washed twice with a salt solution containing 50 mM KCl and 1 mM $MgCl_2$, pH 7.0, and was resuspended with the same salt solution. The suspension was standardised to an OD of 0.144 at 550 nm, where the salt solution was used as a blank. An equal mixture (25 ml each) of the standardised bacterial suspension and test plant extract (*Psidium* sp.) at sub-MIC of 0.5 mg/ml was titrated with 0.2M KOH to pH 7.2-7.4. Sufficient glucose was added to obtain a concentration of 1% (w/v) and the decrease in pH was assessed at 5 min intervals over a period of 90 min at 37°C. Sterile deionised water and CHX (0.12% v/v), respectively served as negative and positive controls. The initial rate of pH drop for the acid production capacity of the cell was best measured within 10 min which depicted the linear portion (Hasan *et al.*, 2015). A similar procedure was carried out using other plant extracts (*Mangifera* sp., *Mentha* sp. and PEM) towards *S. sanguinis*. The experiment was repeated using *S. mutans* in single- and a mixture of *S. sanguinis* and *S. mutans* (dual-species bacteria). Each experiment was done in triplicate.

3.2.16 Determination of total phenolic content of plant extracts

The total phenolic content (TPC) of the crude aqueous extracts were determined using the methods outlined by Shetty *et al.* (1995) with some modification. Fifteen mg of the crude aqueous extract (*Psidium* sp., *Mangifera* sp., *Mentha* sp. and PEM, respectively) were reconstituted in 30 ml sterile deionised water and referred to a stock solution of the extract. Then, 1 ml of the respective stock (0.5 mg/ml) was diluted with 4 ml of sterile deionised water giving a final concentration of 0.1 mg/ml. Then, the diluted extract was sonicated for 10 s, vortexed for 5 min and centrifuged at 1500 *g*, 4°C for 15 min. The supernatant was collected and used for the determination of phenolic content as follows. One ml of each of the sample supernatant (0.1 mg/ml) was further diluted with 5 ml sterile deionised water to give a final concentration of 0.017 mg/ml. To each sample, 0.5 ml of

Folin-Ciocalteu phenol reagent (50% v/v diluted with deionised water; Merck) was added and vortexed. After 5 min incubation at room temperature, 1 ml of 5% (w/v) sodium carbonate (Na_2CO_3) was added to the reaction mixture and vortexed. The test tubes were covered with aluminium foil and placed in the dark for 1 hour. The absorbance of the blue-coloured complex solution was then read at 725 nm using ELISA reader by pipetting 200 µl of the complex solution to the respective wells of the 96-well plate. Deionised water (plus reagent) was used as a blank. Tannic acid at 0, 20, 40, 60, 80, 100 µg in 1 ml deionised water (v/v) and further diluted with 5 ml deionised water and subsequently prepared for TPC assay was used as a standard for a calibration curve (refer to Appendix C). The results were expressed as mg tannic acid equivalents (TAE) per mg of dry weight of test extract. All samples were analysed in triplicate.

3.2.17 Determination of bioactive compounds using UHPLCMS/MS

A bioactive compound in the plant extracts was analysed using ultra-high-performance liquid chromatography system coupled with a tandem mass spectrometer (UHPLCMS/MS). Prior to the analysis, the powdered leaves of selected plant extracts were accurately weighed (5 mg each) and dissolved in 1 ml of distilled water, respectively. All sample solutions were filtered through a 0.45 µm (pore size) nylon syringe membrane filter before the UHPLCMS/MS analysis.

A Perkin Elmer Flexar FX15 (Massachusetts, USA) ultra-high-performance liquid chromatography system coupled with an AB Sciex 3200QTrap (Toronto, Canada) tandem mass spectrometer (UHPLCMS/MS) was used for the analysis. A reverse-phase analytical column, Zorbax C18, (4.6 mm × 150 mm and 5 µm particle size) was used for the separation. The mobile phase used was water containing 0.1% (v/v) formic acid and 5mM ammonium formate (solvent A) and acetonitrile containing 0.1% (v/v) formic acid

and 5mM ammonium formate (solvent B) at a flow rate of 1.0 ml/min. The gradient profile of the mobile phase was as follows: 10% (v/v) A to 90% (v/v) B from 0.01 min to 8.0 min, hold for 2 min and back to 10% (v/v) A in 0.1 min and re-equilibrated for 5 min. The sample injection volume used was 20 µl and total run time was 20 min.

AB Sciex mass spectrometer control software Analyst version 1.5.2 (Toronto, Canada) was used to control the mass spectrometer as well as for data processing and interpretation. Chemical compounds were identified by matching the compound parent mass and fragment ions (MS/MS spectrum) with AB Sciex natural product library (Toronto, Canada). Further identification and confirmation were performed via literature search and cross-referencing.

3.2.18 Molecular biology assays

3.2.18.1 Real time-PCR primer design

A complete genome sequence of *S. mutans* ATCC 25175 has been deposited in GenBank under accession number AOCB01000000 (Song *et al.*, 2013). Sequences of primers for real-time PCR of the gene of interest (target gene) and internal control gene were adapted from a method published by Shemesh *et al*. (2007) based on the following criteria: primer length 20-22 nucleotides, Tm $\geq 60^{o}$C and the expected length of PCR product or amplicon 85-150 base pairs (bp) (Table 3.1). The sequences of primers for real-time PCR can be designed by using Primer 3 tool and then Primer-BLAST. The global alignment algorithm was used to screen primers against user-selected database in order to avoid primer pairs (all combinations including forward-reverse primer pair, forward-forward as well as reverse-reverse pairs) that can cause non-specific amplifications as described previously (Ye *et al.*, 2012). The 16S rRNA gene was chosen as the internal control gene for normalisations. The choice of the 16S rRNA gene as

internal control is dependent on the individual model and is based on relevant literature (Shemesh *et al.*, 2007a; Shemesh *et al.*, 2007b). Table 3.1 shows the primer sequences used for DNA sequencing and gene expression studies.

Table 3.1: Nucleotide sequences of primers used in this study. *Based on the NCBI *S. mutans* genome database using GS5 clinical isolate (Shemesh *et al*., 2007a).

*Gene	Description	Primer sequence (5S'–3S')	
		Forward	Reverse
gtfB	GTFB, glucan production	AGCAATGCAGCCAATCTACAAAT	ACGAACTTTGCCGTTATTGTCA
gtfC	GTFC, glucan production	GGTTTAACGTCAAAATTAGCTGTATTAGC	CTCAACCAACCGCCACTGTT
gbpB	GBP, glucan-binding protein	ATGGCGGTTATGGACACGTT	TTTGGCCACCTTGAACACCT
spaP	Cell surface antigen, SpaP	GACTTTGGTAATGGTTATGCATCAA	TTTGTATCAGCCGGATCAAGTG
16S	Normalizing internal control gene	CCTACGGGAGGCAGCAGTAG	CAACAGAGCTTTACGATCCGAAA

3.2.18.2 Determination of *S. mutans* ATCC 25175 using 16S rRNA gene sequencing

Genetic stability of the *S. mutans* ATCC 25175 culture used in this study was confirmed using 16S rRNA gene sequencing. The strain was obtained from American Type Culture Collection (ATCC, USA) and kept frozen at -80^{o}C in BHI containing 15% (v/v) glycerol at Faculty of Dentistry, University of Malaya, Malaysia. For DNA extraction, the *S. mutans* stock at -80^{o}C was thawed at room temperature and 1% of bacterial suspension was inoculated in 30 ml BHI broth, followed by incubation at 37^{o}C for 18 hours. Then, the *S. mutans* suspension was centrifuged at 5800 *g* for 10 min at 4^{o}C and the pellet was washed three times with 0.1 M PBS buffer (pH 7.4). The purity of the

strain in the suspension was checked by streaking on BHI agar plate, following incubation at 37^{o}C for 24 hours.

The pellet was then subjected to DNA extraction using Ultraclean Microbial DNA extraction kit (MO Bio, USA) according to the protocol provided by the manufacturer. Briefly, the cell pellet was lysed using combined chemical and mechanical methods to extract the nucleic acid from the microbial cells. The DNA was selectively bound to the silica membrane in the Spin Filter device. The washing step using ethanol-based wash solution to remove residues of salt and other the contaminants followed by centrifugation at 10,000 *g* for 1 min allowing the DNA to bind to the silica membrane. The DNA was collected by elution buffer. The DNA quality and quantity were detected using Nanodrop 2000 (Thermofisher, USA) and was qualitatively detected using 1% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer, stained with GelRed and visualised under UV transilluminator (Thermofisher, USA).

The primer of the 16S rRNA gene from *S. mutans* was selected for DNA sequencing. The sequences of the two primers (forward and reverse primers) of the 16S rRNA gene were displayed in Table 3.1. The specificity of these primer sequences was confirmed using Basic Local Alignment Search Tool (BLAST) nucleotide analysis of *S. mutans* (Shemesh *et al.*, 2007a). Amplification of *S. mutans* genomic DNA with primers 16S rRNA-F and 16S-rRNA-R produces a 104 bp amplicon. PCR amplification was performed with 1 µl (140.3 ng/µl) of template DNA of *S. mutans* in a total reaction volume of 25 µl consisting of 13 µl of EconoTaq PLUS GREEN 2x Master Mix (Lucigen), 2 µl of Forward Primer (10 µM), 2 µl Reverse Primer (10 µM) and 7 µl Nuclease free water. The PCR program consisted of 40 cycles of initial denaturation (95°C for 2 min), denaturation (95°C for 5 s), combined annealing/extension (59°C for 10 s) and then maintained at 4^{o}C to amplify the genes (*gtfB* and 16S rRNA). A 1 kb and 100 bp DNA ladder were used for positive DNA marker. An Applied Biosystem

Thermocycler gradient (Applied Biosystem, USA) was used for PCR analysis. The positive result of the genes was confirmed by 2.5% (w/v) agarose gel electrophoresis stained with gelRED nucleic acid, electrophoresed in 100 volts for 40 min in 0.5% Tris-acetate-EDTA (TAE) buffer (pH 8.0) and photographed under ultraviolet (UV) transilluminator.

The PCR product containing 16S rRNA nucleotides sequences were sent to Macrogen Company in South Korea for DNA sequencing. The sequencing result of 16S rRNA nucleotides sequence was combined and checked for the identification of *S. mutans* ATCC 25175 using NCBI BLAST at http://www.ncbi.nlm.nih.gov website.

3.2.18.3 Determination of the effect of a plant extract mixture (PEM) on regulation of virulent-genes expression of *S. mutans*

3.2.18.3.1 Preparation of bacterial suspensions

In this study, the bacterial suspension of *S. mutans* in single- and dual-species with *S. sanguinis* were developed in planktonic and biofilm forms. *S. sanguinis* and *S. mutans* stock cultures were thawed at room temperature and 150 µl of each stock culture suspension was inoculated in 30 ml sterile fresh BHI broth followed by incubation at 37^{o}C, shaking at 150 rpm for 15 hours (OD 550 nm of 1.4; the late exponential phase of *S. sanguinis* and *S. mutans*). The bacterial suspensions were centrifuged at 5800 *g*, 4^{o}C for 10 min and the pellets were washed three times with ice-cold sterile deionised water, suspended in fresh BHI broth containing either 2% (w/v) sucrose for planktonic or 1% (w/v) sucrose for biofilm experiments and then re-incubated at 37^{o}C for 15 min to reactivate their growth phase. The turbidity of each bacterial species was standardised by adjusting the absorbance to 0.144 (equivalent to 1.00 x 10^{8} CFU/ml and 1.53 x 10^{7} CFU/ml for *S. sanguinis* and *S. mutans*, respectively) at 550 nm using a

spectrophotometer (Shimadzu UV-1700, Japan) (Shafiei *et al.*, 2016). For the preparation of dual-species bacteria, the standardised suspensions of the two bacteria were mixed in equal volumes prior to the experiment.

3.2.18.3.2 Preparation of a plant extract mixture (PEM)

A plant extract mixture (PEM) was used in this study for treatment. PEM containing *Psidium* sp., *Mangifera* sp. and *Mentha* sp. (30mg/30ml; 1 mg/ml) was prepared using sterile deionised water. The plant extract mixture (PEM) suspension was sonicated for 30 s to dislodge plant aggregation, homogeneously vortexed for 10 min and centrifuged at 1500 *g*, 4^{o}C for 15 min. The supernatant was sterilised with 0.22 µm (pore size) nylon membrane syringe filter and the sterilised PEM at concentration 1 mg/ml was kept in 50 ml polystyrene Falcon tubes until further use.

3.2.18.3.3 Effect of PEM on planktonic cells

The effect of PEM on *S. mutans* in single- and dual-species with *S. sanguinis* prior to attachment or if serving as anti-plaque was determined using planktonic cells. In this planktonic experiment, an equal volume (1:1 v/v) mixture of 4 ml *S. mutans* suspension in BHI containing 2% (w/v) sucrose [BHI+2% (w/v) sucrose] broth was treated with 4 ml plant extract (*Psidium* sp.; 1 mg/ml) in a 6-well plate to give a final concentration of BHI+1% (w/v) sucrose and 0.5 mg/ml extract, respectively. The plant-bacterial suspension mixtures were allowed to grow at 37°C for 24 hours. After incubation, the cells were harvested by centrifugation at 5800 *g*, 4^{o}C for 10 min and the pellets were washed once with 5 ml PBS (pH 7.4; 0.1 M) and collected by centrifugation. The pellets were resuspended in 500 µl of fresh BHI+1% (w/v) sucrose broth and transferred into 2 ml microcentrifuge tube, then treated with 1000 µl RNA Protect Bacteria Reagent. The

mixture was vortexed for 5 s, incubated for 5 min at room temperature and centrifuged at 5000 *g* for 10 min at room temperature. The supernatant was decanted, and the microcentrifuge tube was dried by dabbing the tubes on sterile tissue paper. The pellets were stored at -80^{o}C for more than 4 weeks for further RNA extraction.

3.2.18.3.4 Effect of PEM on bacterial population in biofilms

The effect of PEM to disperse the biofilm or act as an anti-caries agent was carried out using *S. mutans* in single- and dual-species with *S. sanguinis* biofilms. For the biofilm experimental set-up, the 6-well plates were first preconditioned with 8 ml sterile saliva for 2 min at 37^{o}C, then the excess saliva were decanted and rinsed once with an equal volume of sterile deionised water for 2 min. Then, 8 ml of the standardised bacterial suspension (e.g., *S. mutans*) in fresh BHI+1% (w/v) sucrose was pipetted in the 6-well plates and incubated at 37^{o}C without any disturbance for 24 hours to form a 24-hour biofilm. After 24 hours of incubation, the bacterial suspension was decanted, the 24-hour biofilm was washed once with 5 ml of ice-cold PBS (pH 7.4; 0.1 M) to remove unattached cells. An equal volume (1:1 v/v) mixture of 4 ml of fresh BHI+2% sucrose broth and 4 ml of sterile PEM was transferred to each 6-wells plate. The final concentration of the reaction mixture was BHI+1% (w/v) sucrose and 0.5 mg/ml extract in a total volume of reaction mixture of 8 ml per well. All the 6-well plates were incubated at 37^{o}C, without disturbance for 24 hours. After 24 hours treatment, the supernatant was decanted, and the biofilm formed was gently rinsed once with 5 ml of ice-cold PBS (pH 7.4; 0.1 M) to remove unattached cells and excess extract. The cell pellets were collected by centrifugation at 5800 *g*, 4^{o}C for 10 min, resuspended in 500 µl of fresh BHI+1% (w/v) sucrose broth and transferred to 2 ml microcentrifuge tubes. Following that, 1000 µl of RNA Protect Bacteria Reagent was added into the respective cell suspension treated-PEM, vortexed for 5 s and incubated for 5 min at room temperature. After centrifugation

at 5000 *g* for 10 min at room temperature, the pellet was dried by dabbing on sterile tissue paper and stored at -80^{o}C for more than 4 weeks for further RNA extraction.

The assays of planktonic and biofilm (both in 6-well plates) were carried out in triplicates to get enough pellet for RNA extraction. Similar procedures were repeated twice independently. The sterile deionised water served as the negative control in place of plant extract (PEM). As for the positive control, 0.12% (v/v) CHX gluconate was not prepared because it is bactericidal to all the bacteria cells. A similar experiment using a mixture-species of *S. mutans* and *S. sanguinis* in planktonic and biofilm were also carried out. The pellets collected from the triplicate wells were combined into one tube and only two independent biological samples were used for RNA extraction.

3.2.18.3.5 Total RNA extraction

The -80^{o}C of stock bacterial pellet-treated with RNA Protect Bacteria Reagent was thawed at room temperature and suspended in 1500 µl of ice-cold 0.1 M PBS (pH 7.4). The suspension was sonicated for 30 s to dislodge the pellet and centrifuged at 5500 *g* (8000 rpm) for 10 min at 4^{o}C. The washing step above was repeated twice (Decker *et al.*, 2014). The RNA extraction process was continued using RNeasy Mini kit (Qiagen, Germany) according to the protocol provided by the manufacturer. The pellet was suspended in 100 µl of TE buffer containing lysozyme and 20 µl Proteinase K. The suspension was mixed by pipetting up and down several times followed by vortexing for 10 s. Then, the suspension was incubated at room temperature for 10 min. During incubation, the suspension was vortexed for 10 s at least for every 2 min. Then, 700 µl of RNA lysing buffer was added for lying cells (RLT buffer; Qiagen, Germany), vortex vigorously for 5-10 s, followed by addition of 50 mg acid-washed glass beads (1 mm in diameter) and vortexed for 5 min at maximum speed to disrupt the cell. The cell

suspension was cooled on ice for 1 min. The homogenising step was repeated twice. Then, the cell suspension was centrifuged at 12000 *g* (10 000 rpm) for 5 min at room temperature. Approximately 760 µl of supernatant was transferred to a new tube, followed by addition of 590 µl (80%) ethanol and then mixed well by pipetting.

For purification of total RNA from the bacterial lysate, 700 µl bacterial lysates were transferred into the RNeasy spin column, centrifuged for 15 seconds at 10 000 rpm and then the flow-through was discarded. This procedure was repeated two times for the remaining bacterial lysate. To remove genomic DNA, the RNase-free DNase set was used as follows. About 350 µl of Buffer RW1 was added to the RNeasy spin column, centrifuged for 15 seconds at 10 000 rpm to wash the spin column and the flow-through was discarded. The DNase 1 stock solution was prepared by adding the 10 µl of DNase 1 with 70 µl Buffer RDD and mixed by inverting the tube and centrifuged briefly. Then, 80 µl DNase 1 incubation mix was added directly to the spin column membrane and incubated at room temperature (25^{o}C) for 15 min. Following that, 350 µl buffer RW1 was added to RNase spin column, left for 5 min and centrifuged at 10 000 rpm for 15 seconds. The flow-through and collection tube was discarded. The RNeasy spin column was transferred to new 2 ml of collection tube. As for washing the step, 500 µl of buffer RPE was added to RNeasy spin column and centrifuged for 15 s at 10 000 rpm to wash the pellet, and the flow-through was discarded. The washing step was repeated twice, followed by centrifugation for 2 min at 10 000 rpm. Then, the RNeasy spin column was transferred to new 1.5-ml collection tube. Then, 30 µl of RNase-free water was added directly to the spin column and centrifuged at 10 000 rpm for 1 min to elute the RNA. The RNA was stored at -80^{o}C for further use.

3.2.18.3.6 Determination of RNA quantity and quality

The RNA quantity and purity were determined by the A260/A280 spectrophotometric absorption ratio using Nanodrop 2000 (Thermofisher, USA). One µl of RNA was dropped on the platform and RNase-free water was used for blank control. The acceptable purity RNA was 1.8-2.0.

The integrity of total RNA obtained was checked using 1% (w/v) denaturing agarose gel electrophoresis, stained with gelRED nucleic acid, electrophoresed in 100 volts for 40 min in 0.5% (v/v) Tris-acetate-EDTA (TAE) buffer (pH 8.0) and photographed under ultraviolet (UV) transilluminator. cDNA was synthesised from the isolated high-quality RNA by using QuantiNova Reverse Transcriptase kit (Qiagen, Germany).

3.2.18.3.7 cDNA synthesis

Due to lower concentration of total RNA obtained (< 500 ng/µl), all the isolated RNA samples were diluted with RNAse-free water and standardised at concentration of 200 ng/µl before converted to cDNA using QuantiNova Reverse Transcription kit (Qiagen, Germany) based on the protocol provided by the manufacturer. Prior to use, all the chemicals were thawed on ice. Genomic DNA removal reaction components contained 2 µl gDNA removal mix, 7.5 µl (of 200 ng/µl) template RNA, 4.5 µl RNase-free water and 1 µl internal control (optional) were mixed and kept on ice. The total reaction volume was 15 µl/PCR tube. The Reverse-transcription Master mix (RT) components contained 1 µl Reverse Transcription (RT) enzyme and 4 µl Reverse transcription mix which included Mg^{2+} and dNTPs were freshly prepared. The reaction mixture was mixed and stored on ice. Then, the PCR cycler was set for the gDNA elimination reaction and RT temperature protocol as follow: gDNA elimination reaction at 45ºC for 2 min, paused at 25ºC, and then the sample was placed on ice and RT components (5 µl) was added to the tube

containing 15 µl genomic DNA removal reaction components followed by annealing step at 25^{o}C for 3 min, reverse-transcription step at 45^{o}C for 10 min, inactivation of reverse transcription enzyme at 85^{o}C for 5 min and cooled at 4^{o}C. The cDNA sample in a total of 20 µl volume was then stored at -20^{o}C until further use for real-time PCR analysis.

3.2.18.3.8 Gene expression analysis with Real-time PCR

In this study, due to different primers used, the PCR efficiency of different targets and internal control genes were determined by the shape of the PCR amplification plot in the logarithmic phase. As shown in Appendix C, Figure A3, the melt curve analysis for genes of interest and internal control (16S rRNA gene) during the amplification were almost similar, indicating that all the primers tested have similar efficiency. Previous studies revealed that all the primers that produce a nearly identically shaped amplification plot was expected to have similar efficiency (Schmittgen & Livak, 2008). The relative comparative quantification, $2^{-\Delta\Delta Ct}$ method also assumes that the amplification efficiency for both the targets (gene of interest) and internal control genes is similar (Livak & Schmittgen, 2001).

Real-time PCR assays were performed in StepOne Plus (Applied System, USA), using the standard thermal cycling protocol as provided by the manufacturer with a modification of annealing temperature. The reaction mixture of 20 µl contained 10 µl of 2x QuantiNova SYBR® Green Master mix (Qiagen, Germany), 2 µl ROX reference dye (for Applied System qPCR), 0.7 µM of forward and reverse primers (1.4 µl each of 10 µM), 100 ng cDNA (1.33 µl of 75 ng cDNA) and RNase-free water (3.87 µl) to reach the final volume. The Real-time PCR was performed in triplicate for each sample. A negative control without the cDNA template, known as non-template control (NTC) where the template cDNA in the reaction mixture was replaced with PCR grade water was also

included. The plate was covered using optical adhesive cover and sealed using the sealing tool. The 96-well plate layout for each assay was prepared for two independent biological samples in triplicate were displayed in the Appendix I.

The thermal amplification program used was as follows: PCR initial heat activation at 95°C for 2 min, denaturation at 95°C for 5 s. and 40 cycles of combined annealing and extension at 59°C for 10 s, followed by the thermal dissociation protocol for SYBR green detection (including melt curve analysis) that set up by the system using GeneAmp® 5700 SDS software (Applied Biosystems). The melting curve (Tm) or also called heat dissociation curve analysis was performed within the temperature range of 60°C to 95°C to detect specific amplification of the target sequence (studied genes of interest and internal control gene).

3.2.18.3.9 Real-time PCR data analysis

The real-time PCR data was analysed using the comparative threshold cycle (Ct) method. Relative quantification was used and the real-time PCR data of the gene of interest (target) relative to internal control gene (16S rRNA gene; also called house-keeping gene or reference gene or endogenous control gene or calibrator) was presented. Gene expression levels was measured as the Ct of the studied gene of interest normalised with the Ct of the internal control gene, 16S rRNA (as calculated qPCR data in Table S1 in the Appendix E). The relative difference in gene expression is the comparative Ct method also referred to as the $2^{-\Delta\Delta Ct}$ method. This equation was used to compare the gene in two different samples (PEM-treated & untreated control) as described in the following equation:

Relative fold change in gene expression = $2^{-\Delta\Delta Ct}$,

$2^{-\Delta\Delta Ct} = 2^{-}$ [(Ct gene of interest – Ct internal control) PEM-treated – (Ct gene of interest – Ct internal control) untreated control]

Using this formula, all the data was analysed by taking the mean of the PCR replicates and the statistical tests and standard deviation (SD) should always be calculated after the $2^{-\Delta\Delta Ct}$, $2^{-\Delta Ct,}$ or 2^{-Ct} transformation was performed (Schmittgen & Livak, 2008).

3.2.19 Statistical analysis

The experiments were repeated three times independently, analysed in triplicate and expressed as a mean ± standard deviation (SD), where the number of determinations are as follows: for growth curve study (n = 9), MIC, MBC and FIC (n = 3), adherence and bacterial population in biofilm assays (n = 27), cell-surface hydrophobicity (n = 9), acid production (n = 57) and TPC (n = 9). For real-time PCR data, the two-independent biological replicate samples were analysed in triplicate per sample (n = 6). Expression data for the target and internal control genes were obtained in the form of threshold cycle (Ct) values. The data were analysed using the $2^{-\Delta\Delta Ct}$ method. Using this analysis, the value of the mean fold change at time zero should be very close to one. Data were analysed using IBM SPSS statistical software version 23. The Shapiro-Wilk test was used to test assumptions of normality. One-way ANOVA and Kruskal-Wallis tests was used for multiple comparison of normal and not normal distribution data. Mann-Whitney test and independent *t*-test were used for non-parametric and parametric statistical analysis, respectively to compare the difference between the two independent samples. Results were considered significant at $p < 0.05$.

CHAPTER 4: RESULTS

4.1 Yield of crude aqueous plant extracts

Table 4.1 shows the yield of dried crude aqueous extracts (g % w/w) obtained from 100g of the respective plants (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.). The yields were different between different plants, with *Mangifera* sp. (12.00 ± 1.63%) demonstrating the highest yield and *Mentha* sp. (3.23 ± 0.20%) the least ($p < 0.05$).

Table 4.1: Yield of the crude aqueous extracts. The determination was carried out in triplicates and the yield was expressed as mean ± SD. * $p < 0.05$: *t*-test.

Plant extract	Yield of crude aqueous extract (mean ± SD) (g % w/w)
Psidium sp.	6.46 ± 0.97*
Mangifera sp.	12.00 ± 1.63*
Mentha sp.	3.23 ± 0.20*

4.2 Determination of mid-log phase growth of bacteria

Figure 4.1 shows the growth curve displayed by *S. sanguinis* and *S. mutans* individually and in a mixture. It was shown that the mid-log phase of *S. sanguinis* and *S. mutans* is different. The mid-log phase of *S. mutans* was 6 hours (OD of 0.790 ± 0.003) while *S. sanguinis* was 8 hours (OD of 0.80 ± 0.05). When in a mixture, the mid-log phase of the two bacteria was 7 hours (OD of 0.858 ± 0.144).

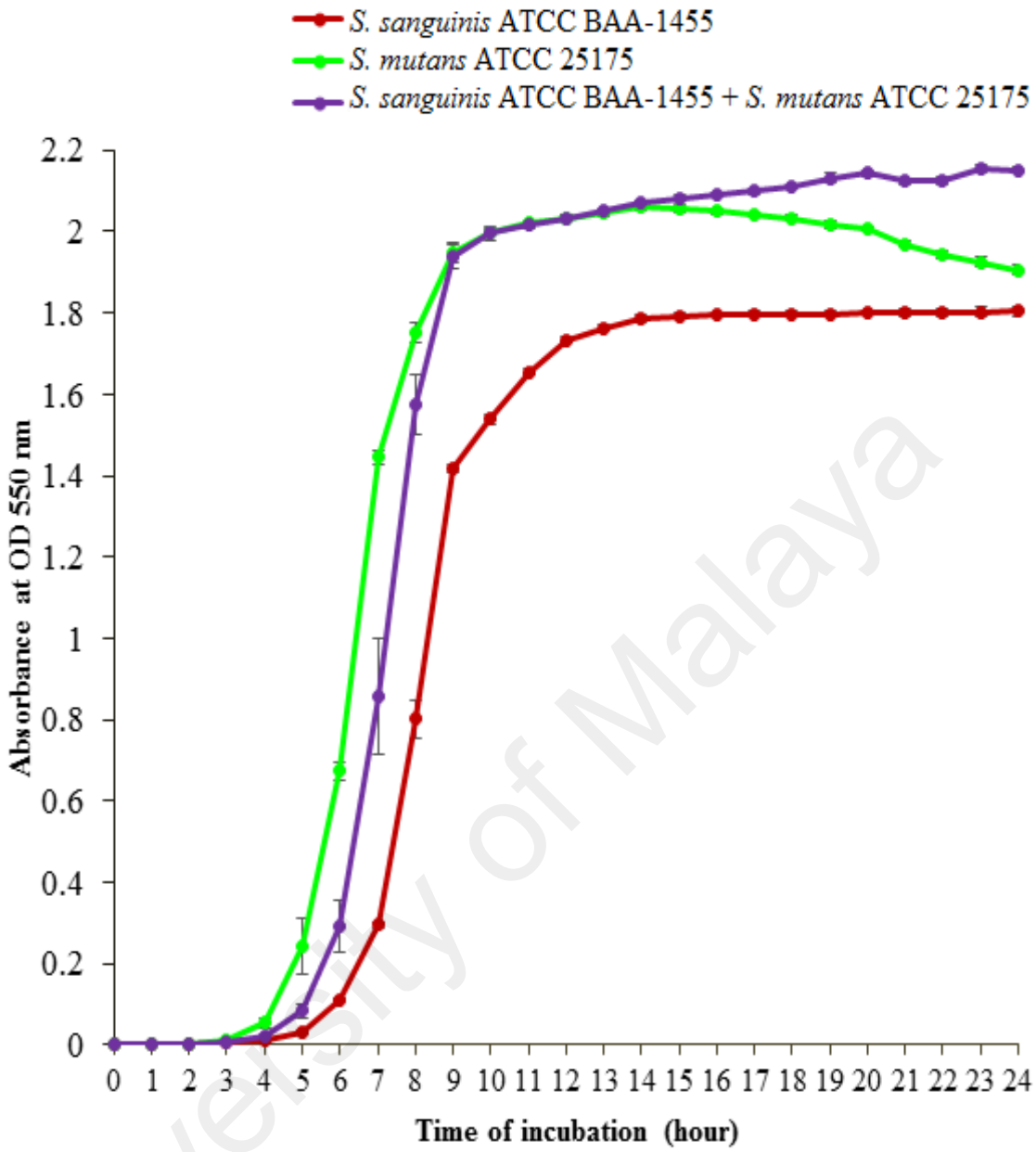


Figure 4.1: Growth curve of bacteria in *S. sanguinis*, *S. mutans,* and its mixture. Data of triplicate from the determination of the two-independent biological duplicate, (n = 6), where comparison of the mid-log phase of *S. sanguinis* to that of *S. mutans* ($p < 0.05$: *t*-test).

4.3 Minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and the sum of the fractional inhibitory concentration (ΣFIC) index of plant extracts

Table 4.2 shows the MIC, MBC and ΣFIC index of the PEM and its individual constituent plant extracts against the early (*S. sanguinis*) and late (*S. mutans*) plaque colonisers. The MIC values of PEM (*S. sanguinis* 3.81 mg/ml; *S. mutans* 1.91 mg/ml) were lower than those of the individual extracts for the two bacteria. Among its individual constituent plant extracts, the *Psidium* sp. showed the lowest MIC against *S. mutans* (3.81 mg/ml) whereas *Mentha* sp. and *Psidium* sp. has almost similar MIC against *S. sanguinis* (7.62 mg/ml). It is shown that the MBC were higher than the MIC for all the extracts. *Psidium* sp. (MBC value of 15.24 mg/ml) showed the higher bactericidal effect compared with *Mangifera* sp., *Mentha* sp. and PEM (MBC value of 30.48 mg/ml) against *S. sanguinis*. The MBC value exhibited by PEM and *Psidium* sp. against *S. mutans* (30.48 mg/ml) was lower compared with *Mangifera* sp. and *Mentha* sp. (60.95 mg/ml). It was found from the calculation of the ΣFIC index, the interactions of the plant extracts are synergistic for the two bacteria where the index is lower for *S. mutans* (ΣFIC = 0.25) compared with *S. sanguinis* (ΣFIC = 0.42).

Table 4.2: The MIC, MBC, and FIC values of PEM and its individual constituent plant extracts against tested oral bacteria. [a]MIC(MBC) = minimum inhibitory concentration (minimum bactericidal concentration); [b]PEM = plant extract mixture and [c]ΣFIC = the sum of the fractional inhibitory concentration (ΣFIC) index.

	[a]MIC (MBC) (mg/ml)				Fractional inhibitory concentration index				
	***Psidium* sp.**	***Mangifera* sp.**	***Mentha* sp.**	**[b]PEM**	**FIC_I**	**FIC_{II}**	**FIC_{III}**	**[c]ΣFIC = 1/3 (FIC_I + FIC_{II} + FIC_{III})**	**Interaction**
S. sanguinis ATCC BAA-1455	7.62 (15.24)	15.24 (30.48)	7.62 (30.48)	3.81 (30.48)	0.50	0.25	0.50	1/3(0.50 + 0.25 + 0.50) = 0.42	synergistic
S. mutans ATCC 25175	3.81 (30.48)	15.24 (60.95)	15.24 (60.95)	1.91 (30.48)	0.50	0.13	0.13	1/3(0.50 + 0.13 + 0.13) = 0.25	synergistic

4.4 Bacterial population adhering to untreated experimental pellicle

The bacterial populations for *S. sanguinis* and *S. mutans* adhering to the experimental pellicle without treatment (negative control) either individually (single-species) or together (dual-species) is shown in Table 4.3. In a single-species biofilm, the population of *S. mutans* [(2.48 ± 0.16) x 10^7 CFU/ml] adhering to the experimental pellicle was ten times higher compared with *S. sanguinis* (2.30 ± 0.37) x 10^6 CFU/ml) and it is statistically significant ($p < 0.05$). However, in dual-species biofilms, the *S. mutans* population [(1.54 ± 0.35) x 10^7 CFU/ml] adhered in almost equal proportions with *S. sanguinis* [(1.49 ± 0.36) x 10^7 CFU/ml].

Table 4.3: Bacterial population adhering to the untreated experimental pellicle (negative control) for *S. sanguinis* and *S. mutans* in single- and dual-species biofilms. The results are shown as average values of triplicate (n = 27) and are expressed in a mean CFU/ml ± standard deviation (SD). [a]Negative control represents bacteria population adhered to the experimental pellicle-coated glass beads and was assumed as 100%. *$p < 0.05$ considered as significant: Mann-Whitney test.

Bacteria		Bacteria population adhered to the experimental pellicle (negative control)[a] (mean ± SD) (CFU/ml)
Single-species	*S. sanguinis* ATCC BAA-1455	(2.30 ± 0.37) x 10^{6} *
	S. mutans ATCC 25175	(2.48 ± 0.16) x 10^{7} *
Dual-species	*S. sanguinis* ATCC BAA-1455	(1.49 ± 0.36) x 10^7
	S. mutans ATCC 25175	(1.54 ± 0.35) x 10^7

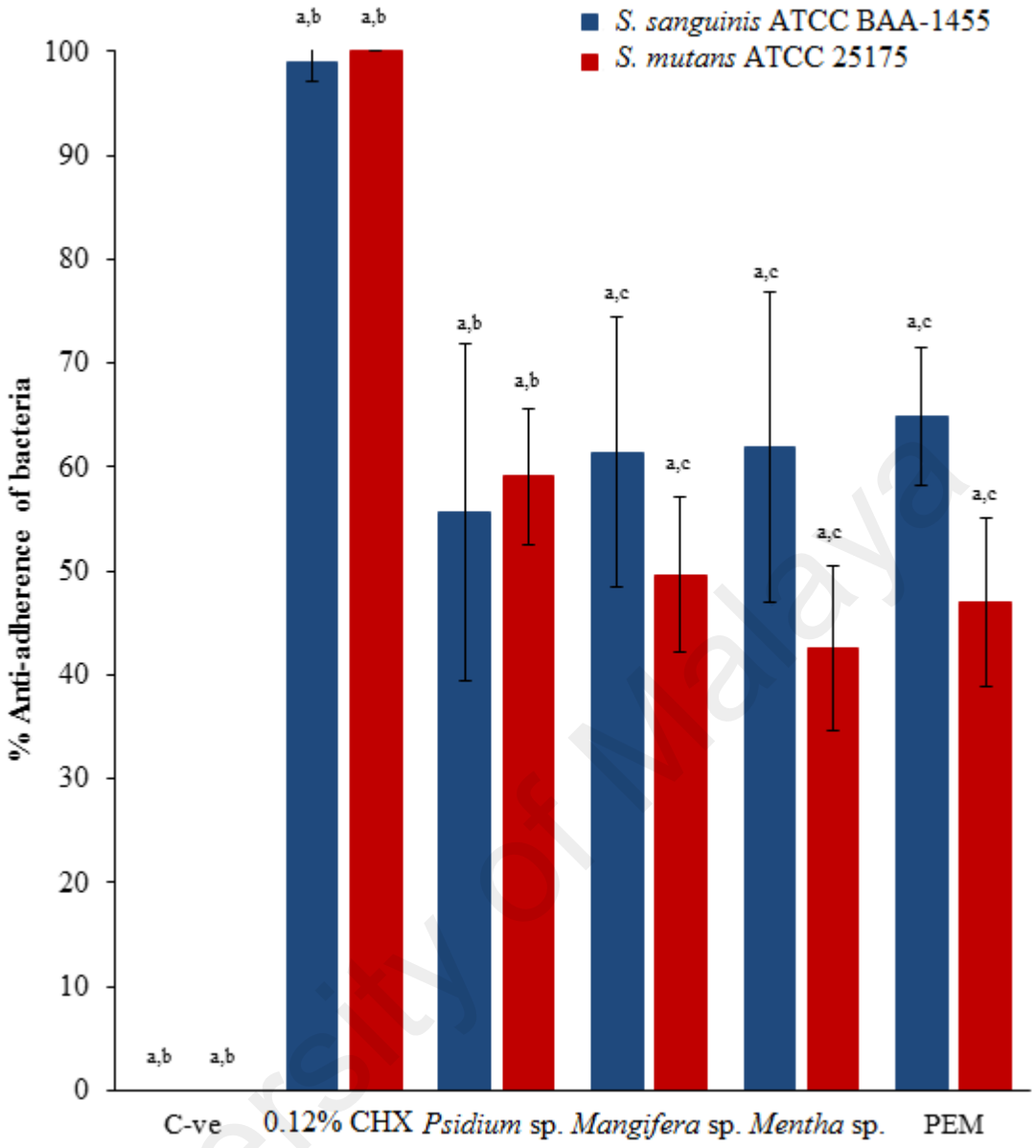


Figure 4.2: The anti-adherence effect of PEM and its respective plant extracts in single-species biofilm (*S. sanguinis* and *S. mutans*). Percentage of anti-adhered bacterial cells present in the biofilm was expressed as the mean ± standard deviation (SD) and the number of determinations (n) = 27.

[a] - $p < 0.05$ comparing between PEM, *Psidium* sp., *Mangifera* sp., *Mentha* sp., 0.12% (v/v) CHX (positive control) and negative control (Kruskal-Wallis test).
[b] - $p < 0.05$ comparing between PEM and *Psidium* sp., PEM and positive control and PEM and negative control (Mann-Whitney test).
[c] - $p < 0.05$ comparing between *S. sanguinis* and *S. mutans* (treated with *Mangifera* sp.), *S. sanguinis* and *S. mutans* (treated with *Mentha* sp.) and *S. sanguinis* and *S. mutans* (treated with PEM) (Mann-Whitney test).

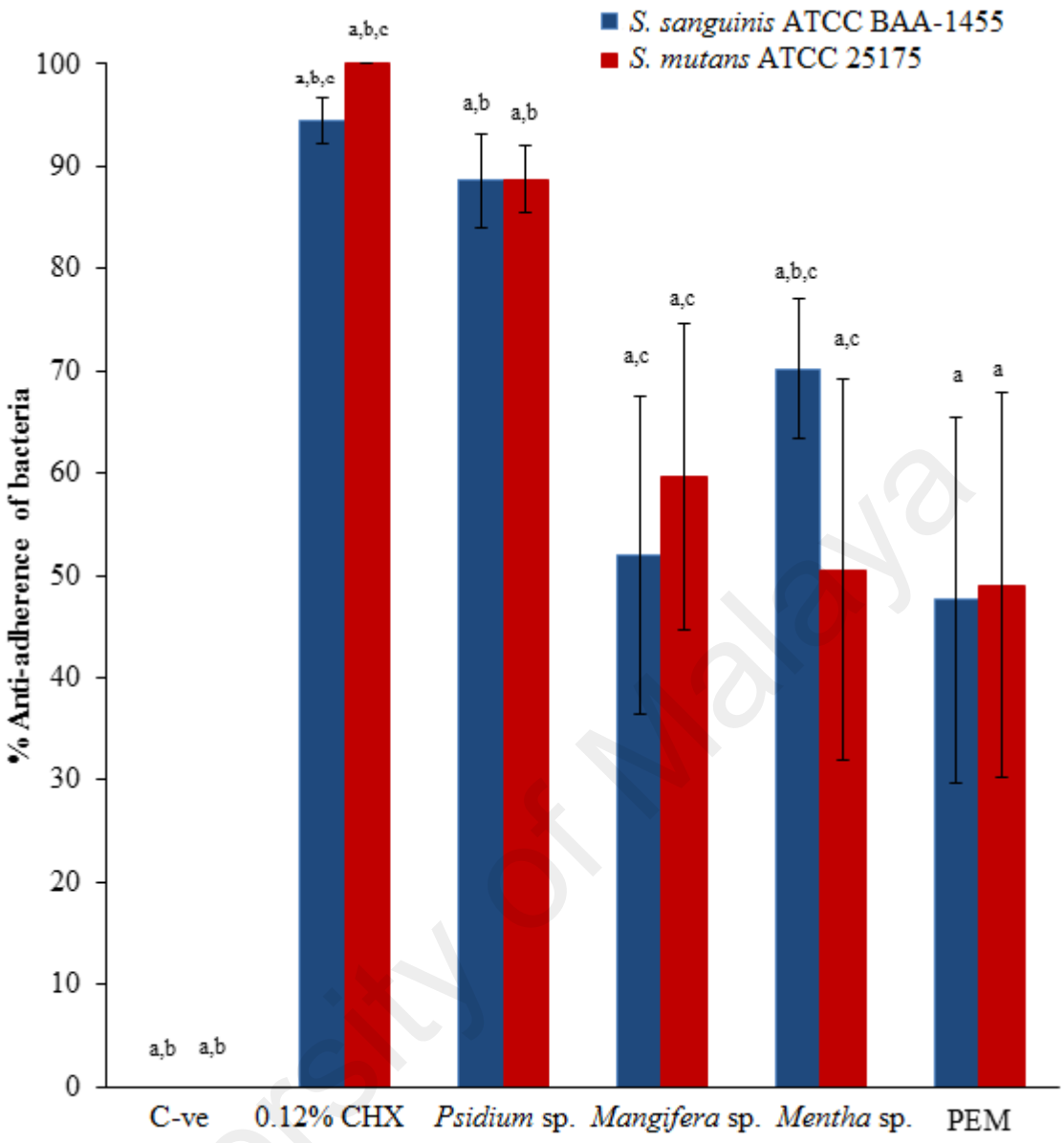


Figure 4.3: The anti-adherence effect of PEM and its respective plant extract on dual-species (*S. sanguinis* + *S. mutans*) biofilms. Percentage of anti-adhered bacterial cells present in the biofilm was expressed as the mean ± standard deviation (SD) and the number of determinations (n) = 27.

[a] - $p < 0.05$ comparing between the respective PEM, *Psidium* sp., *Mangifera* sp., *Mentha* sp., 0.12% (v/v) CHX (positive control) and the negative control (Kruskal-Wallis test).
[b] - $p < 0.05$ comparing between PEM and *Psidium* sp., PEM and *Mentha* sp. (for *S. sanguinis*), PEM and positive control and, PEM and negative control (Mann-Whitney test).
[c] - $p < 0.05$ comparing between *S. mutans* and *S. sanguinis* (treated with *Mangifera* sp.), *S. mutans* and *S. sanguinis* (treated with *Mentha* sp.), *S. mutans* and *S. sanguinis* (treated with positive control) and, *S. mutans* and *S. sanguinis* (treated with negative control) (Mann-Whitney test).

4.5 Anti-adherence effect of plant extracts in single- and dual-species biofilms

Figure 4.2 shows the anti-adherence effects of PEM and its respective constituent plant extracts in single-species biofilm (*S. sanguinis* and *S. mutans*). The anti-adhering effect of all the extracts (except *Psidium* sp.) towards *S. sanguinis* was significantly higher when compared with *S. mutans.*

Figure 4.3 shows the anti-adherence activities of the PEM and its individual constituent plant extracts in the dual-species biofilms. It was observed that *Psidium* sp. also demonstrated similar and higher anti-adherence activities towards *S. sanguinis* (88.54 ± 4.47%) and *S. mutans* (88.65 ± 3.22%) compared with PEM, *Mangifera* sp. and *Mentha* sp. The *Mentha* sp. exhibited significantly higher anti-adherence activity towards *S. sanguinis* (70.12 ± 6.90%) compared with *S. mutans* (50.54 ± 18.64%). PEM demonstrated comparable anti-adherence effect towards *S. sanguinis* and *S. mutans* (47.50 ± 17.93% and 49.08 ± 18.90%, respectively) ($p > 0.05$).

4.6 Bacterial adherence to plant extracts treated-experimental pellicle viewed under SEM

Figures 4.4 and 4.5 show the SEM photographs of single-species biofilm viewed at 10,000X magnification displaying the cell population adhering to the experimental pellicle (saliva-coated glass beads) treated with plant extracts as well those of the negative and positive controls. The population of *S. mutans* and *S. sanguinis* were almost zero in single-species biofilm adhering to 0.12% (v/v) CHX-treated experimental pellicle (positive control) and very much reduced with plant extract-treated when compared with the negative control.

Figure 4.6 shows the SEM photographs of the dual-species biofilms. It was observed that the population of the two bacteria adhering to *Psidium* sp.-treated experimental pellicle was highly reduced (Figure 4.6D). It was different for *Mentha* sp-treated experimental pellicle, the adhered population of *S. sanguinis* was less compared with *S. mutans* (Figure 4.6F). The adherence of the two bacteria to *Mangifera* sp.-treated (Figure 4.6E) and PEM-treated experimental pellicle (Figure 4.6C), respectively were almost comparable.

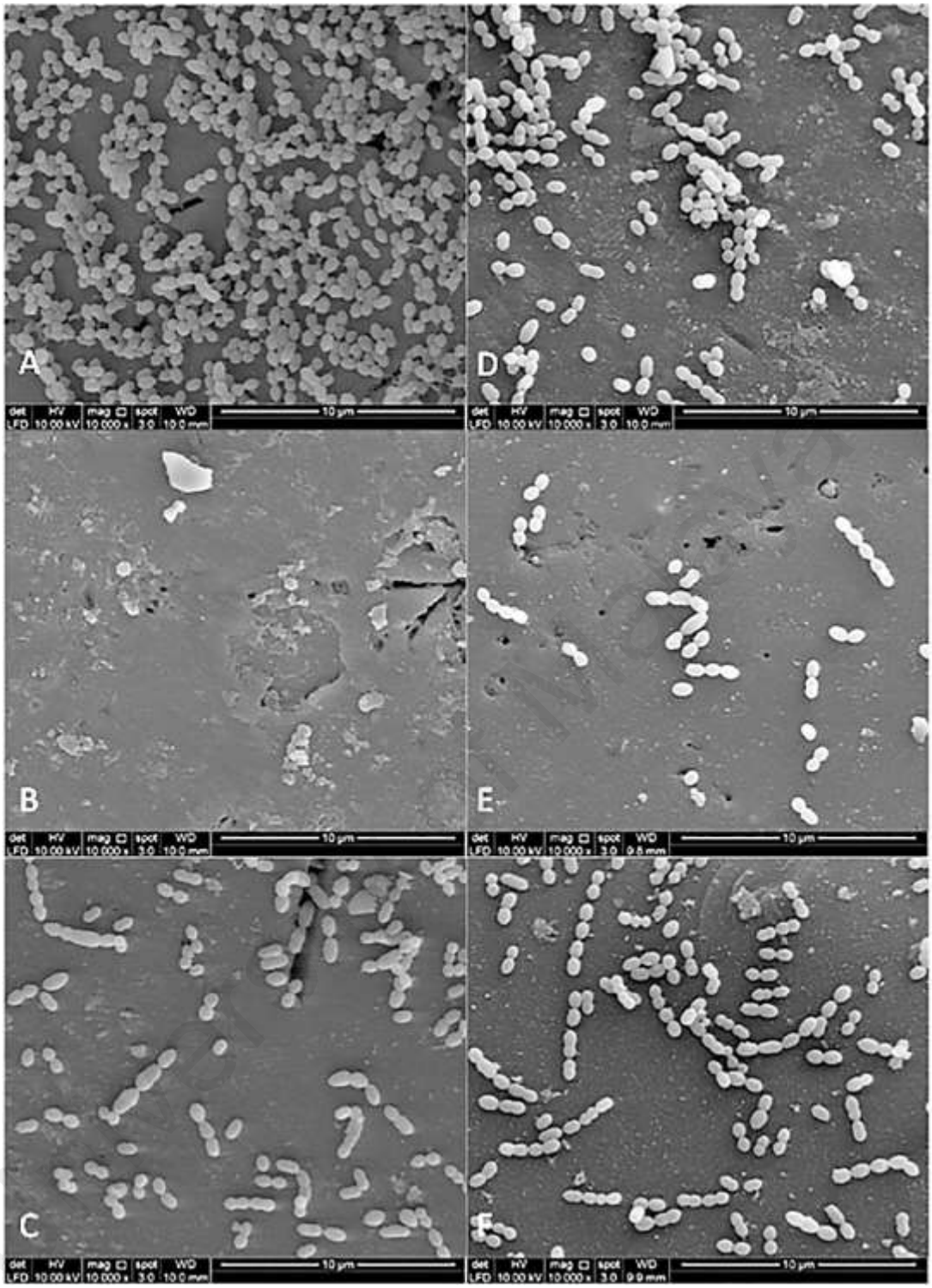


Figure 4.4: The cell population of *S. sanguinis* viewed by Scanning Electron Microscopy (SEM) where the pellicle was treated with PEM {C} and its respective plant extracts (*Psidium* sp {D}; *Mangifera* sp. {E} and *Mentha* sp.{F}). The 0.12% (v/v) chlorhexidine gluconate {B} and deionised water {A} served as positive and negative controls, respectively. Magnification: 10,000X.

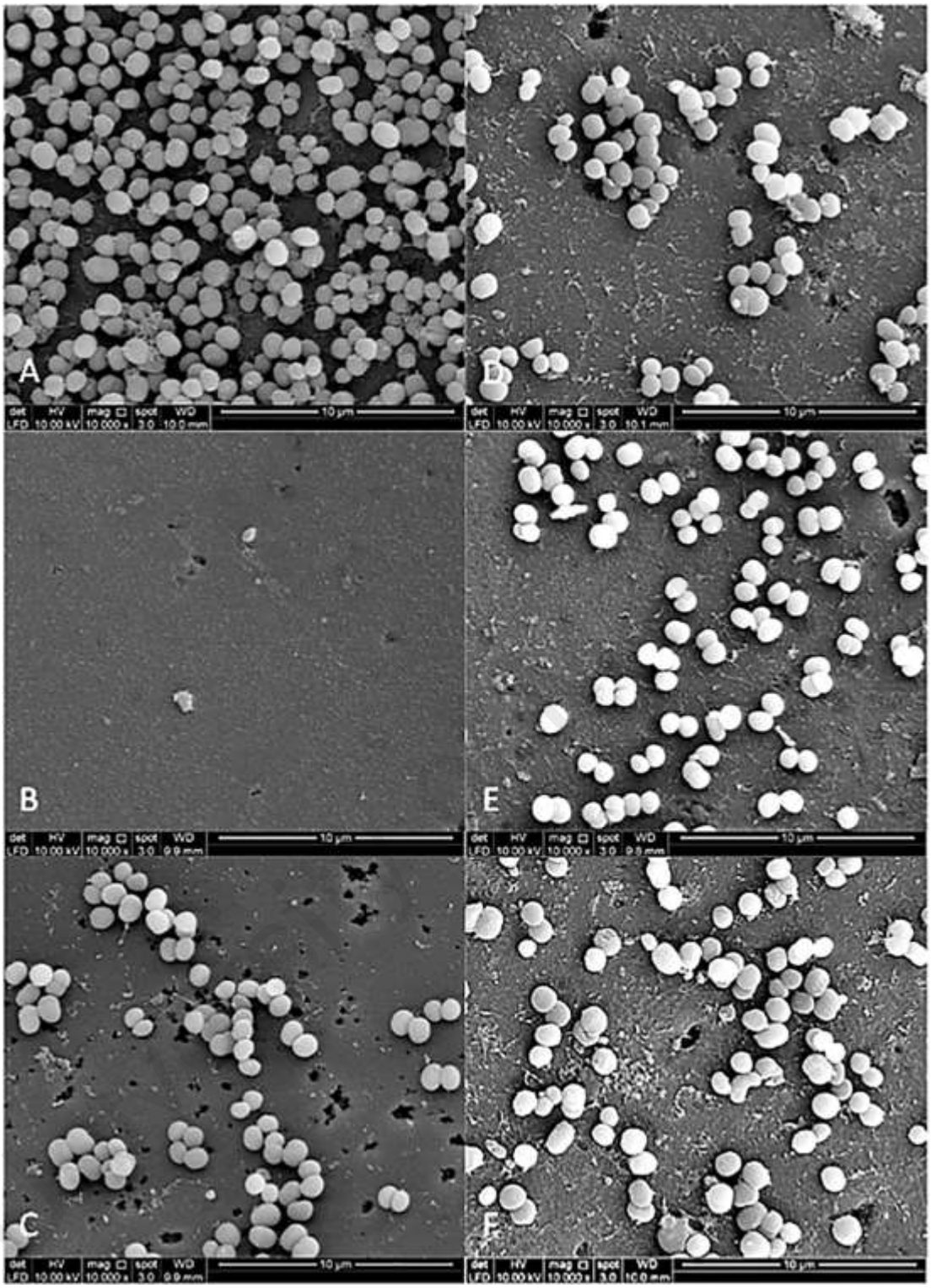


Figure 4.5: The cell population of *S. mutans* viewed by Scanning Electron Microscopy (SEM) where the pellicle was treated with PEM {C} and its respective plant extracts (*Psidium* sp {D}; *Mangifera* sp. {E} and *Mentha* sp. {F}). The 0.12% (w/v) chlorhexidine gluconate {B} and deionised water {A} were served as positive and negative controls, respectively. Magnification: 10,000X.

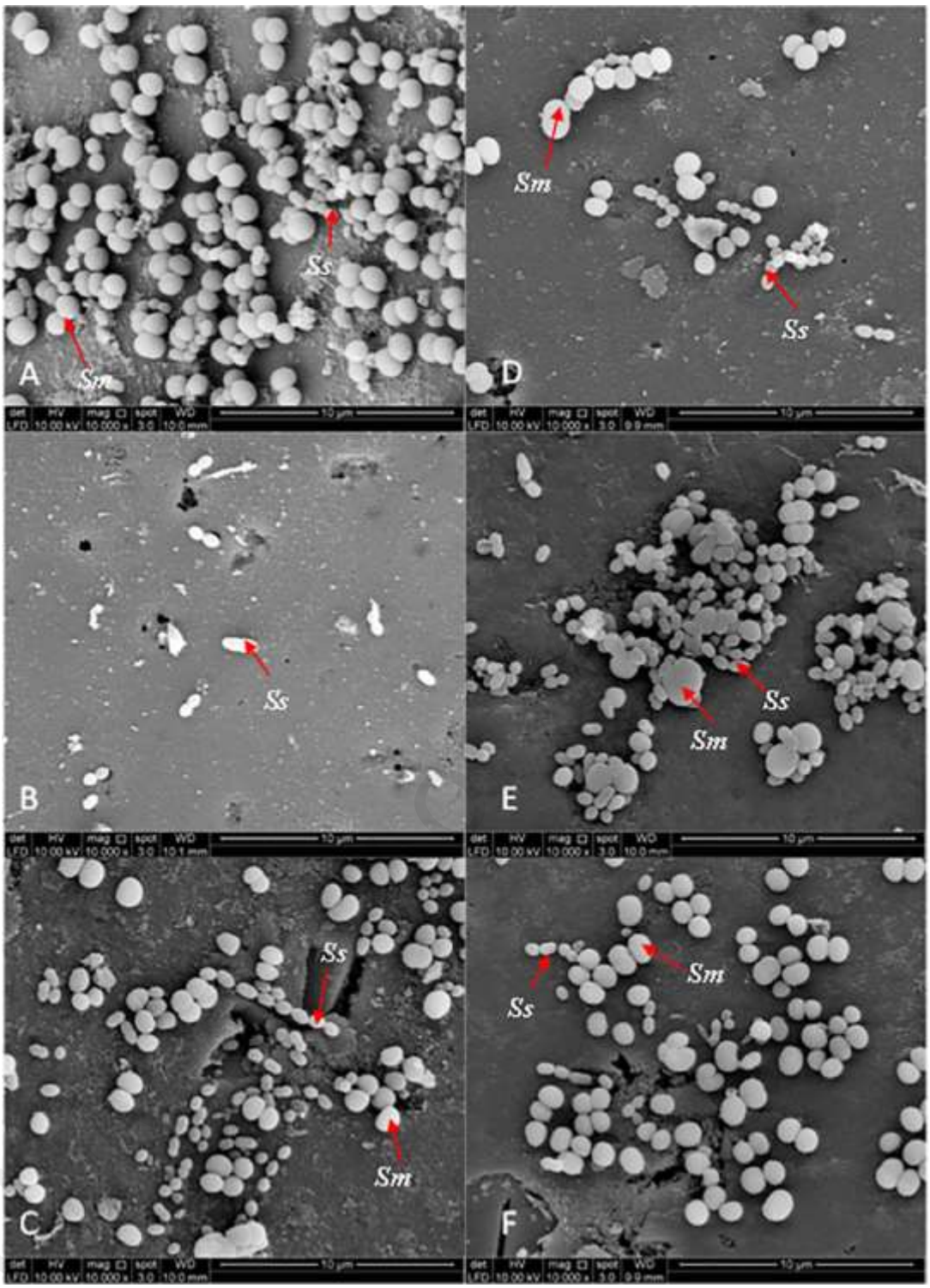


Figure 4.6: The cell population of dual-species biofilms (*S. sanguinis* (*Ss*) + *S. mutans* (*Sm*)) viewed by Scanning Electron Microscopy (SEM) where the pellicle was treated with PEM {C} and its respective plant extracts (*Psidium* sp. {D}; *Mangifera* sp. {E} and *Mentha* sp. {F}) on cell adherence. The 0.12% (v/v) chlorhexidine gluconate {B} and deionised water {A} were served as positive and negative controls, respectively. Magnification: 10,000X.

4.7 Bacterial population in biofilms grown in the absence and presence of sucrose for untreated and plant extract-treated experimental pellicle

Table 4.4 shows the bacterial population formed on experimental pellicle (negative control) in the absence and presence of sucrose. The presence of sucrose enhanced *S. mutans* growth in single-species biofilm [(5.21 ± 0.01) x 10^7 CFU/ml], which was higher (2.1 times) than in absence of sucrose [(2.43 ± 0.29) x 10^7 CFU/ml]. Meanwhile, the *S. sanguinis* population [(4.60 ± 1.14) x 10^5 CFU/ml] was retarded ($p < 0.05$) in media containing 1% (w/v) sucrose. The population of *S. sanguinis* was 4.3 times higher in the presence of sucrose [(5.00 ± 0.11) x 10^6 CFU/ml], while the *S. mutans* population was 0.95 times lower compared to that of its population in the absence of sucrose ($p < 0.05$) (Table 4.4).

Table 4.4: The bacterial populations on untreated experimental pellicle (negative control biofilms) grown in the absence and presence of sucrose. Comparing bacterial population in biofilm between [b] presence and [a] absence of sucrose.
*($p < 0.05$: *t*-test).

Biofilm		**(Mean ± SD) (CFU/ml)**	**(Mean ± SD) (CFU/ml)**
		[a]BHI broth	**[b]BHI + 1% (w/v) sucrose broth**
Single-species	*S. sanguinis* ATCC BAA 1455	(7.04 ± 1.14) x 10^5	(4.60 ± 1.14) x 10^{5} *
	S. mutans ATCC 25175	(2.43 ± 0.29) x 10^7	(5.21 ± 0.01) x 10^{7} *
Dual-species	*S. sanguinis* ATCC BAA 1455	(1.17 ± 0.11) x 10^6	(5.00 ± 0.11) x 10^{6} *
	S. mutans ATCC 25175	(7.21 ± 0.11) x 10^6	(6.88 ± 0.11) x 10^{6} *

Figure 4.7 shows bacterial population in single-species biofilm in the absence and presence of sucrose upon treatment with plant extracts. Comparison of the effect of plant extracts towards respective *S. sanguinis* and *S. mutans* in the absence of sucrose was carried out separately with the two bacteria grown in the presence of sucrose. It was shown that all the plant extracts have the potential to eliminate *S. mutans* population in

single-species biofilms grown in the absence of sucrose and presence of sucrose, with different efficacies. PEM (1.02 ± 0.14%) and *Mangifera* sp. (0.59 ± 0.12%) reduced *S. mutans* population in the biofilm by more than 99% comparable to that of CHX ($p > 0.05$) and retained significant higher population of *S. sanguinis* with PEM (32.94 ± 6.25%) and *Mangifera* sp. (38.6 ± 7.93%), respectively. Meanwhile, *Psidium* sp. (18.9 ± 5.17%) and *Mentha* sp. (13.83 ± 3.70%) exhibited an intermediate effect in reducing *S. mutans* and *S. sanguinis* population in biofilm grown in the absence of sucrose.

For the single-species biofilm grown in the presence of sucrose, it was shown that *Psidium* sp. (9.85 ± 4.65%) and *Mentha* sp. (9.85 ± 4.65%) were the most effective anti-caries agents due to significantly higher elimination of *S. mutans* population and at the same time retained the highest *S. sanguinis* population [*Psidium* sp. (48.33 ± 19.57%) and *Mentha* sp. (21.18 ± 7.56%), respectively] in single-species biofilm in order to create a healthy environment in the oral cavity. *Mangifera* sp. was highly effective in reducing both *S. sanguinis* (5.71 ± 1.58%) and *S. mutans* (3.85 ± 1.56%) population and created a balanced population between them ($p > 0.05$) (Figure 4.7).

Figure 4.8 shows the effect of plant extracts [individually and mixture (PEM)] towards the bacterial population in dual-species biofilms grown in the absence of sucrose. It was shown that *Psidium* sp. (6.23 ± 2.53%) and *Mangifera* sp. (10.96 ± 3.60%) effectively eliminated *S. mutans* population and retained the highest and almost equal to *S. sanguinis* population in the biofilms [*Psidium* sp. (51.38 ±13.88%) and *Mangifera* sp. (52.12 ± 20.37%), respectively] ($p > 0.05$). *Mentha* sp. had effectively reduced both of *S. sanguinis* (14.53 ± 5.05%) and *S. mutans* (1.49 ± 0.48%) populations, with the *S. mutans* as the highest population. Meanwhile, PEM was also effective in reducing the two bacteria and regulated a balanced population between *S. sanguinis* (12.71 ± 1.83%) and *S. mutans* (15.86 ± 6.99%) population in biofilms ($p > 0.05$). This created a harmonious interaction between the two bacteria in biofilms (Figure 4.8).

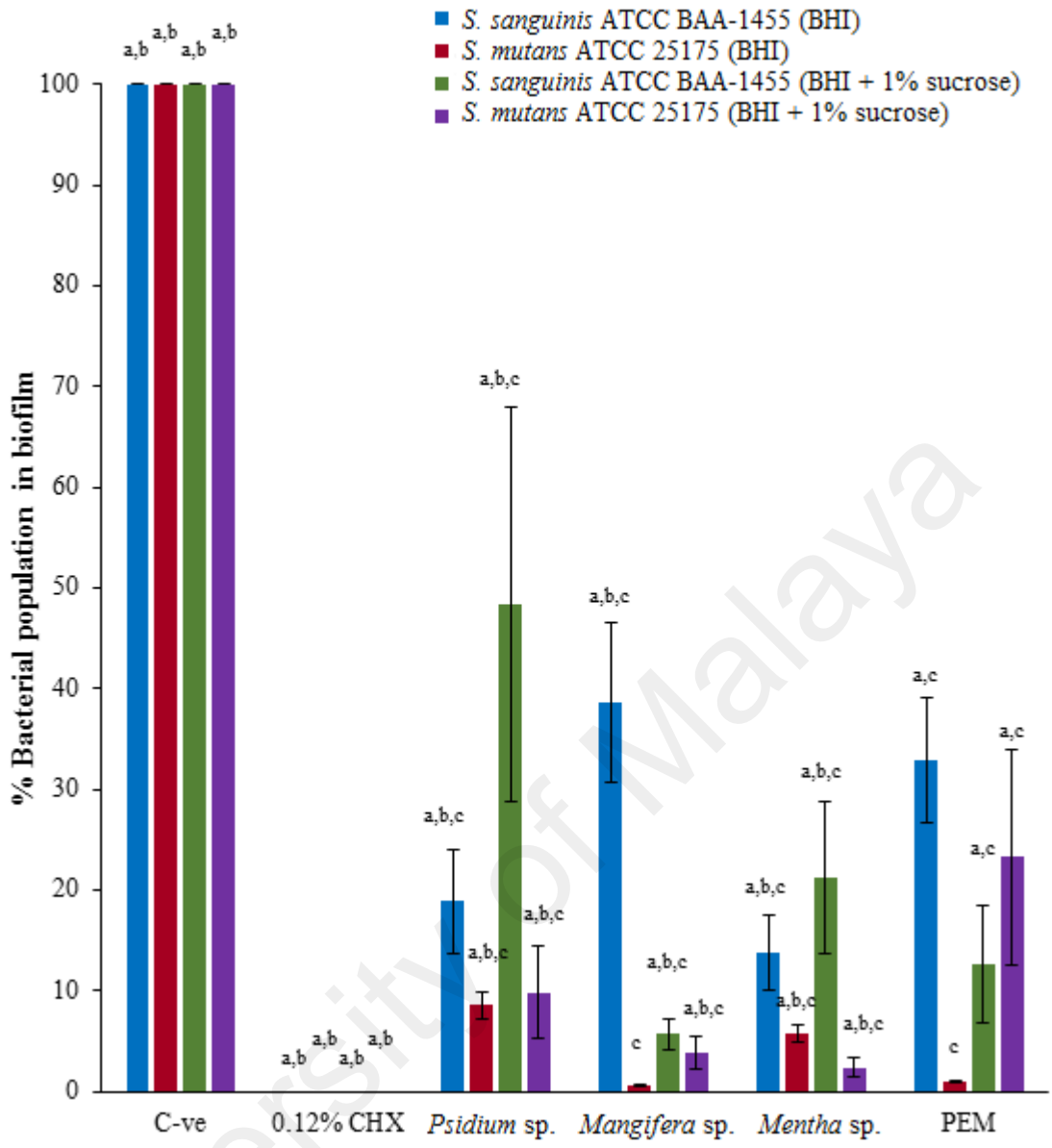


Figure 4.7: The effect of PEM and its constituent plant extracts on the population of *S. sanguinis* and *S. mutans* in single-species biofilm grown in the absence (BHI broth) and the presence of sucrose (BHI + 1% (w/v) sucrose broth). The percentage of the bacterial population in the biofilm was expressed as the mean ± standard deviation (SD) where a number of determinations (n = 27.

Comparing between

[a] - plant extracts (PEM, *Psidium* sp., *Mangifera* sp., *Mentha* sp.), positive [0.12% (v/v) CHX] and negative controls ($p < 0.05$: one-way ANOVA test).

[b] - PEM to that of the respective individual constituent plant extracts, positive and negative controls towards *S. sanguinis* and *S. mutans*, respectively ($p < 0.05$; *t*-test).

[c] - PEM and the respective individual constituents towards *S. sanguinis* to that of *S. mutans* ($p < 0.05$: *t*-test).

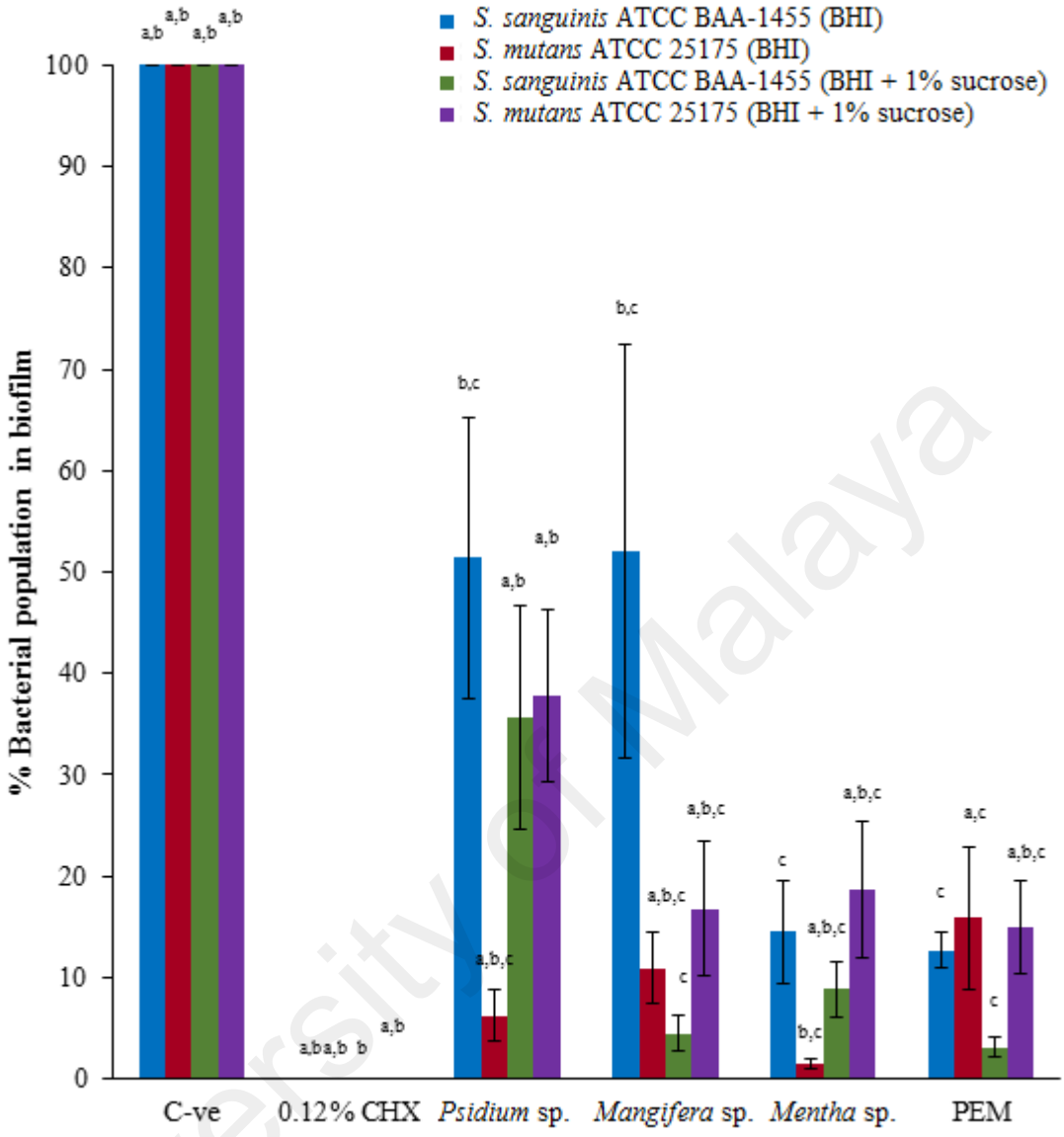


Figure 4.8: The effect of PEM and its constituent plant extracts on the population of *S. sanguinis* and *S. mutans* in dual-species biofilms grown in the absence (BHI broth) and the presence of sucrose (BHI + 1% (w/v) sucrose broth). Percentage of the bacterial population in the biofilm was expressed as the mean ± standard deviation (SD) where a number of determinations (n = 27).

Comparing between

[a] - plant extracts (PEM, *Psidium* sp., *Mangifera* sp., *Mentha* sp.), positive [0.12% (w/v) CHX] and negative controls ($p < 0.05$: one-way ANOVA test).

[b] - PEM to that of the respective individual constituent plant extracts, positive and negative controls towards *S. sanguinis* and *S. mutans*, respectively ($p < 0.05$; *t*-test).

[c] - PEM and the respective individual constituents towards *S. sanguinis* to that of *S. mutans* ($p < 0.05$: *t*-test).

For the bacterial population in dual-species biofilms grown in the presence of sucrose, the result showed that it was difficult for *S. mutans* population to be eliminated by any of the plant extracts compared to that of *S. sanguinis*. *Psidium* sp. retained higher and almost equal propositions (36-38%) of the two bacteria in biofilms ($p > 0.05$), while PEM, *Mangifera* sp. and *Mentha* sp. retained lower *S. sanguinis* population (3-9%) compared to that of *S. mutans* (15-19%), thus allowing the *S. mutans* to create colonisation resistance in the presence of low *S. sanguinis* population. This would enhance biofilm formation and lead to the initiation of dental caries later.

This study also confirmed that 0.12% (v/v) CHX gluconate is a bactericidal agent for the two types of bacteria either in single- or dual-species biofilms.

4.8 Effect of plant extracts on cell-surface hydrophobicity of bacteria

Figure 4.9 shows that *S. sanguinis* (76.05 ± 2.69%) exhibited a higher percentage of cell-surface hydrophobicity to *n*-hexadecane compared to *S. mutans* (62.88 ± 8.58%) ($p < 0.05$). The effect of the different plant extracts towards *S. sanguinis* and *S. mutans*, respectively showed that *Psidium* sp. (24.43 ± 3.93%; 17.20 ± 3.35%) and PEM (29.58 ± 3.80%; 24.66 ± 2.43%) have a similar trend and have the lowest cell-surface hydrophobicity compared to the other constituent plant extracts. The *Mentha* sp. reduced the cell-surface hydrophobicity of *S. mutans* (31.63 ± 5.11%) but did not show any effect on *S. sanguinis* (77.62 ± 8.67%).

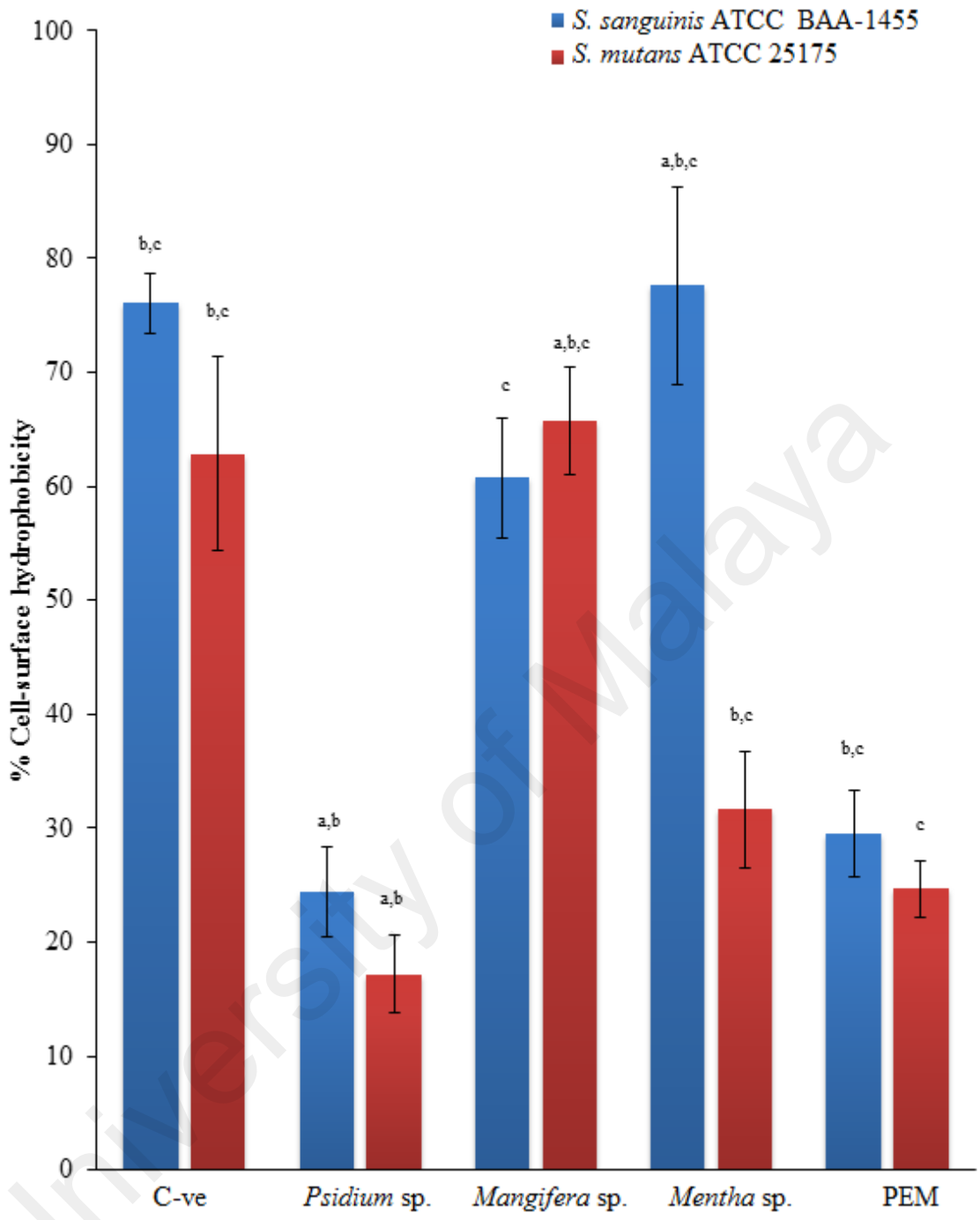


Figure 4.9: Percentage of cell-surface hydrophobicity of *S. sanguinis* and *S. mutans* to *n*-hexadecane, which was expressed as the mean ± standard deviation (SD). Number of determinations (n) = 9.
Comparison of the effect of
[a] - PEM and its constituents with that of the negative control ($p < 0.05$: *t*-test);
[b] - PEM to that of the respective individual constituent plant extracts (including negative control) towards *S. sanguinis* and *S. mutans*, respectively ($p < 0.05$: *t*-test) and
[c] - PEM and the respective individual constituents towards *S. sanguinis* to that of *S. mutans* ($p < 0.05$: *t*-test).

4.9 Effect of plant extracts on acid production of bacteria

Figure 4.10 shows the effect of the plant extracts on the acid production of *S. sanguinis* and *S. mutans* in both individual and mixed species compared with the negative control. It was shown that all of the plant extracts decreased the acid production of *S. sanguinis* with the initial ΔpH reduced by -0.16. Comparison between different plant extracts did not show any significant different in the initial ΔpH ($p > 0.05$) [Figure 4.10(a)]. For the *S. mutans*, it was found that the initial ΔpH in PEM-treated was lower (-0.15) compared to its individual constituent plant extracts and it is significantly different ($p < 0.05$) [Fig. 4.10(b)].

For the mixture of the two bacteria, the *Mangifera* sp. and PEM appeared to exhibit the lowest initial ΔpH (-0.07 and -0.12, respectively) [Fig. 4.10(c)].

4.10 Total phenolic content (TPC) of plant extracts

Table 4.5 shows that the total phenolic content was the highest in the PEM (795 ± 0.05 µg TAE/mg extract) by far, followed by *Psidium* sp. (530 ± 0.04 µg TAE/mg extract), *Mangifera* sp. (317.5 ± 0.02 µg TAE/mg extract) and the least in the *Mentha* sp. (230 ± 0.03 µg TAE/mg extract).

Table 4.5: Total phenolic content of the plant extracts. * Comparing between PEM and its individual plant extract ($p < 0.05$: *t*-test). Number of determination (n) = 9.

Plant extract	Total phenolic content (µg TAE/mg extract)
Psidium sp.	530.00 ± 0.04 *
Mangifera sp.	317.50 ± 0.02 *
Mentha sp.	230.00 ± 0.03 *
PEM	795.00 ± 0.05 *

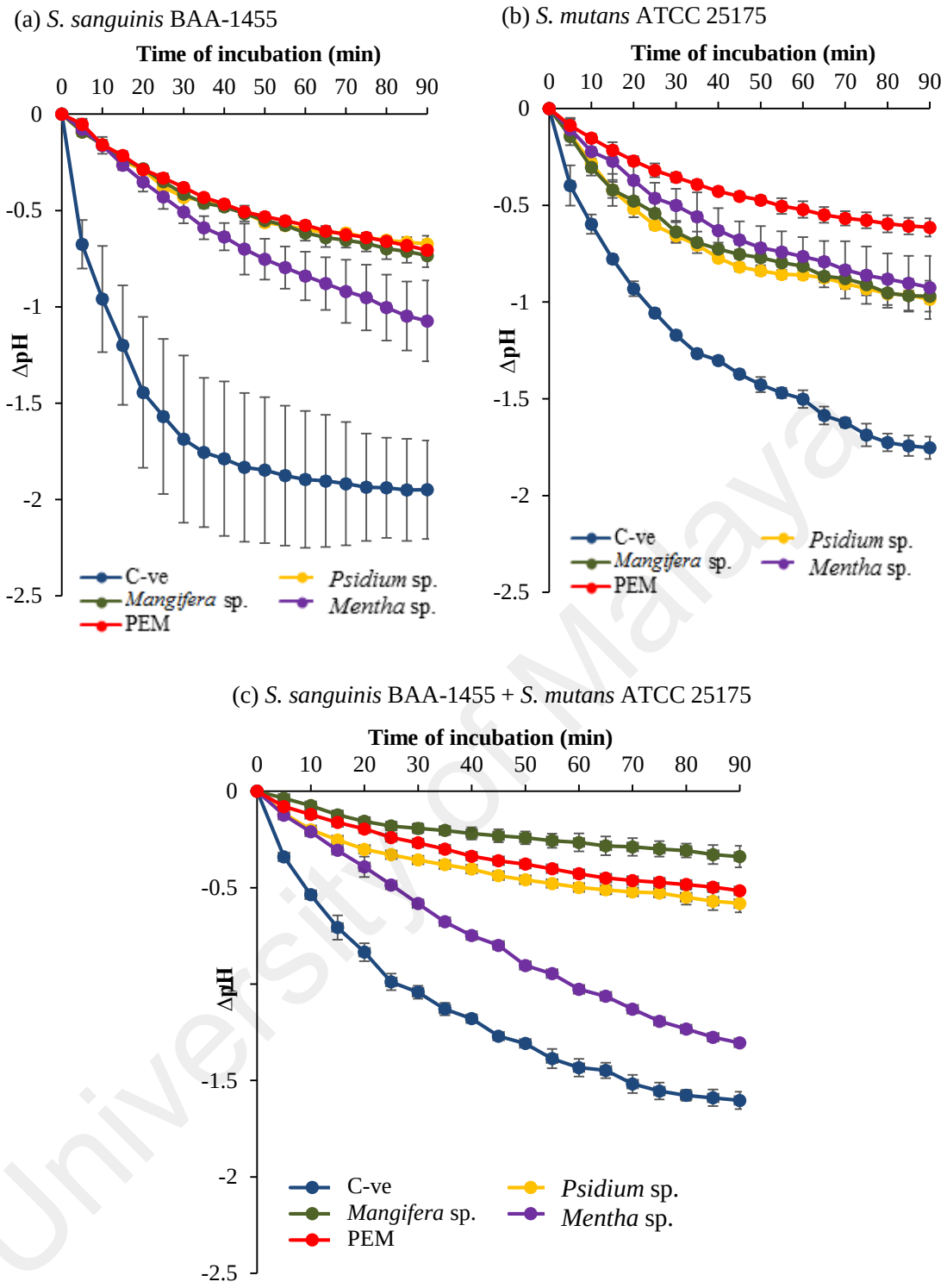


Figure 4.10: Glycolytic ΔpH drops of (a) *S. sanguinis* and (b) *S. mutans* in an individual and (c) dual-species bacteria. The values were expressed as mean ± SD. Number of determinations (n) = 9. Statistical analysis of initial ΔpH showed that all significant comparing between plant extracts and negative controls ($p < 0.05$: Mann-Whitney test); PEM and its constituent plant extracts (except for *Mangifera* sp. towards *S. sanguinis*; *Psidium* sp. and *Mentha* sp. towards dual-species) ($p < 0.05$: Mann-Whitney test) and the two bacteria in single- and dual-species treated with plant extracts (except for the treatment with *Psidium* sp, *Mentha* sp. and PEM, respectively) ($p < 0.05$: Mann-Whitney test).

4.11 UPHLCMS/MS screening of phenolic compounds

The composition of known and unknown phenolic compounds of the crude aqueous extracts of *Psidium* sp., *Mangifera* sp. *Mentha* sp. and their mixture, PEM as analysed using UHPLCMS/MS are shown in Table 4.6. The concentration of each compound was calculated based on the percentage (%) peak area. It was observed that the PEM had the most abundant phenolic compounds. However, most of them were unknown compounds, such as Unknown 6 (9.84%) which exhibited the highest amount. The identified major compounds showed the percentage peak area in the following descending order: apigenin (3.29%) > benzophenone C-glycoside (3.25%) > rosmarinic acid conjugate (3.21%) > methoxyflavone derivates (2.92%) > quercetin-3-*O*-glucoside (2.54%) > succinic acid (2.49%). The rest of the phenolic compounds were present in small amounts (Table 4.6).

For the individual plant extracts, the major compounds in *Psidium* sp. were methylquercetin sulfate (8.12%) and 2,6-dihydroxy-3-methyl-4-O-(6''-*O*-galloyl-b-D-glucopyranosyl)-benzophenone (6.18%). Meanwhile, oxo-octadecanoic acid (4.92%), quercetin sulfate (4.91%), brevifolin carboxylic acid (4.22%), ellagic acid (2.52%), quinic acid (1.51%) and gallic acid (0.30%) were present in smaller amount in *Psidium* sp. The *Mangifera* sp. contained major phenolic compounds identified as benzophenone C-glycoside (23.32%) with percentage peak area higher than quinic acid (14.14%), followed by benzophenone C-glycoside isomer (9.08%) and quercetin-3-*O*-glucoside (7.84%). There were also phenolic compounds [such as quercetin (3.46%), mangiferin (2.46%) and isorhamnetin-3-*O*-hexoside (0.19%)] identified in smaller amount (Table 4.6). The *Mentha* sp. possessed lesser number of phenolic compounds in which methyl 2-cyclohex-2-en-l-yl(hydroxy)-methyl-3-hydroxy-4-2-hydroxyethyl-3-methyl-5-oxoprolinate (9.71%) was the major compound. The chromatograms for the identified phenolic compounds were displayed in Appendices D-G.

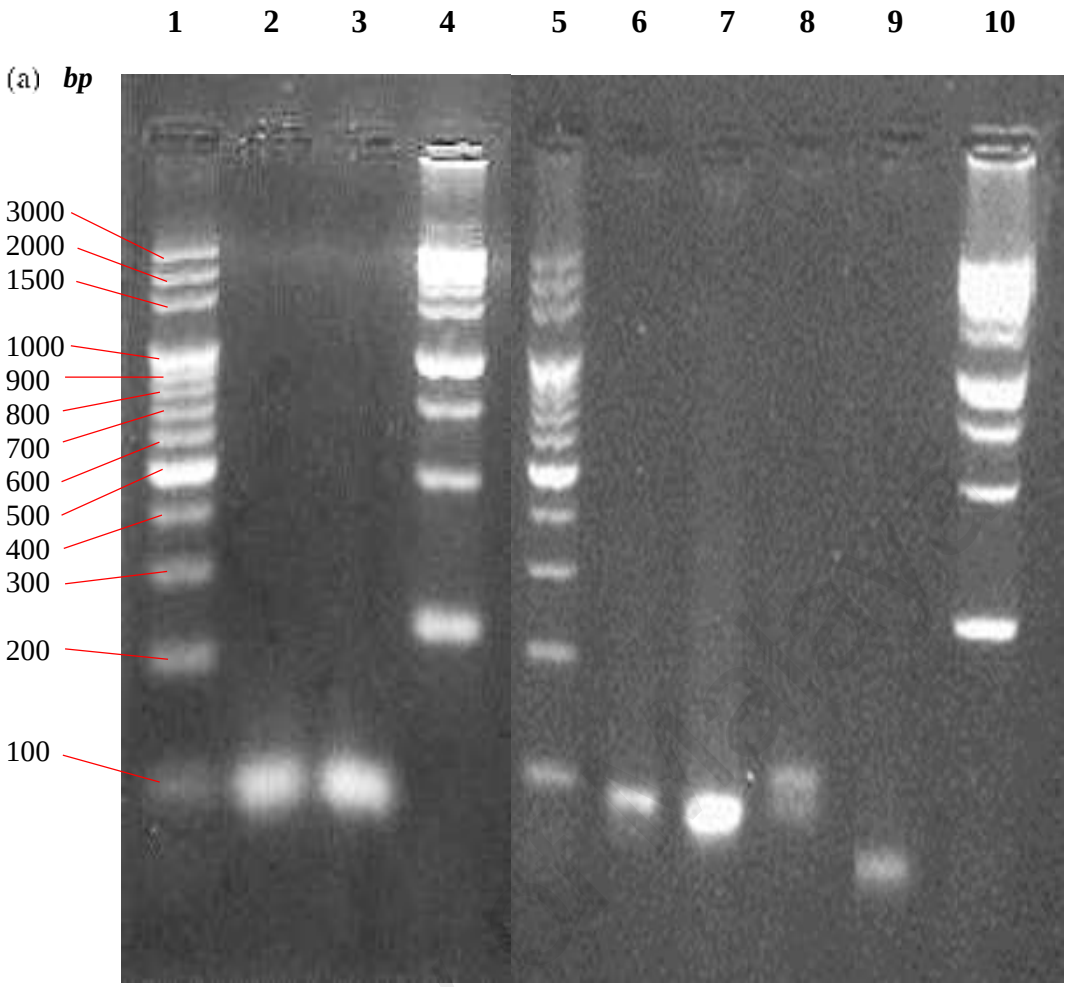
Table 4.6: The composition of phenolic compounds in *Psidium* sp., *Mangifera* sp., *Mentha* sp. and its mixture (PEM). [a] - % Peak area = (peak area/total peak area) x 100; [*] - Major compound(s) in the aqueous extract.

T_R (min)	% Peak Area[a]	$[M-H]^-$ (m/z)	Compound	T_R (min)	% Peak Area[a]	$[M-H]^-$ (m/z)	Compound
***Psidium* sp.**				**PEM**			
2.090	1.51	192.30	Quinic Acid	0.121	2.49	117.05	*Succinic acid
2.613	0.30	168.89	Gallic Acid	0.444	2.68	257.00	Unknown 1
3.791	4.22	290.95	Brevifolin carboxylic acid	0.686	2.92	301.20	*Methoxyflavone derivates
4.577	4.91	380.94	Quercetin sulfate	0.888	1.66	251.06	Unknown 2
4.970	8.12	394.88	*Methylquercetin sulfate	1.456	0.36	191.22	Quinic acid
5.363	6.18	557.00	*2,6-dihydroxy-3-methyl-4-*O*-(6"-*O*-galloyl-b-D-glucopyranosyl)-benzophenone	1.659	0.28	407.75	Benzophenone C-glycoside isomer
6.803	1.74	300.94	Unknown 1	2.145	0.61	169.51	Gallic acid
7.458	2.52	300.96	Ellagic acid	3.201	3.23	408.37	*Benzophenone C-glycoside
8.244	4.92	297.13	Oxo-octadecanoic acid	3.85	0.42	289.20	Catechin
8.637	5.18	312.11	Unknown 2	4.541	2.54	462.88	*Quercetin-3-O-glucoside
9.030	14.91	491.17	Unknown 3	4.784	3.25	720.55	*Rosmarinic acid conjugate
***Mangifera* sp.**							
1.828	14.14	190.95	*Quinic acid	5.149	0.52	572.60	Gallic acid conjugate
3.006	9.08	406.99	*Benzophenone C-glycoside isomer	5.839	0.58	285.35	Luetolin
3.268	2.46	421.00	Mangiferin	6.326	0.60	423.65	Unknown 3
3.661	23.32	407.04	*Benzophenone C-glycoside	6.773	1.72	849.23	Unknown 4
4.447	7.84	463.03	*Quercetin-3-*O*-glucoside	7.463	3.11	293.15	Unknown 5
5.365	0.19	477.00	Isorhamnetin-3-*O*-hexoside	7.706	9.84	801.57	Unknown 6
5.758	3.46	300.98	Quercetin	8.071	3.29	269.12	*Apigenin
6.413	2.69	536.95	Unknown 1	8.314	3.25	577.79	Unknown 7
***Mentha* sp.**							
1.965	0.38	133.09	Chavicol	8.761	3.50	633.73	Unknown 8
2.228	0.71	191.06	Chinic acid	9.085	2.66	325.72	Unknown 9
2.623	1.22	277.00	Caffeic acid conjugate	9.166	1.31	555.80	Unknown 10
3.414	0.48	153.06	Protocatechuic acid	9.451	0.51	339.79	Unknown 11
4.208	5.45	305.25	Unknown 1				
4.605	1.28	371.45	Unknown 2				
4.870	1.23	497.63	Ganoderic acid B				
6.852	9.71	327.40	*Methyl-2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate				

4.12 16S rRNA gene sequencing of *S. mutans*

An amplification of 16S rRNA gene sequence of *S. mutans* was performed to confirm bacterial identification. Primers for conserved regions of 16S rRNA and *gtfB* genes were used for amplification of DNA of *S. mutans* by PCR after which the PCR product was separated on 2.5% (w/v) agarose gel electrophoresis to check the specificity of these primer sequences (Figure 4.11a). The result demonstrated that *S. mutans* had one band for each *gtfB* and 16S rRNA genes with amplicon size of 98-120 bp, confirming the presence of *S. mutans* genes in the tested strain. Identification using 16S rRNA gene showed that the strain was 99% similar to *S. mutans* ATCC 25175 from the BLAST NCBI as a result of the stated nucleotide sequence and the alignments of the sequences of 16S rRNA sequencing data in the Appendix H. However, it also identified other strains due to shorter length of the 16S rRNA gene sequence used in this study that was composed of 104 nucleotides which was too short for an accurate bacteria identification (Figure 4.12).

Primers for conserved regions of *gtfC, gbpB* and *spaP* genes were also used for amplifications using DNA of *S. mutans* by PCR. Then, the PCR products were separated on 2.5% (w/v) agarose gel electrophoresis and a fragment with amplicon size of 95-102 bp for each gene was separated using a similar PCR condition. This indicated that all the PCR products for the primers tested had showed similar PCR specificity for these primer sequences (Figure 4.11a)



(b)

TCCTACGGGAGGCAGCAGTAGGGAATCTATCGGCA
ATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAG
TGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGA

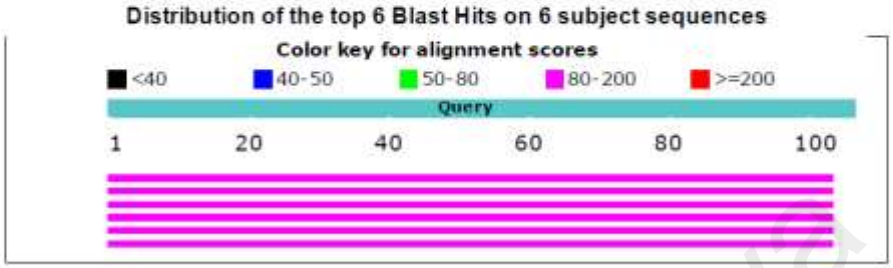
Figure 4.11: Photograph of (a) PCR product of *gtfB* (Lane 2 & 6), 16S rRNA (Lane 3), *gtfC*, *gbpB*, *spaP*) genes of *S. mutans* (Lane 7-9 and (b) the sequencing result of cleaned up PCR product of 16S rRNA. Lanes 1 & 5 and 4 & 10 are 100bp and 1kb DNA ladder, respectively.

RID FAZC1TPW016 (Expires on 04-19 10:44 am)

Query ID lcl|Query_116399
Description None
Molecule type nucleic acid
Query Length 104

Database Name rRNA_typestrains/prokaryotic_16S_ribos
Description 16S ribosomal RNA (Bacteria and Archaea)
Program BLASTN 2.6.0+

Graphic Summary



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus mutans strain NBRC 13955 16S ribosomal RNA gene, partial sequence	183	183	99%	3e-50	99%	NR_113641.1
Streptococcus mutans strain ATCC 25175 16S ribosomal RNA gene, partial sequence	183	183	99%	3e-50	99%	NR_116208.1
Streptococcus mutans strain ATCC 25175 16S ribosomal RNA gene, partial sequence	183	183	99%	3e-50	99%	NR_115733.1
Streptococcus mutans strain ATCC 25175 16S ribosomal RNA gene, complete sequence	183	183	99%	3e-50	99%	NR_042772.1
Streptococcus mutans strain NCTC 10449 16S ribosomal RNA gene, complete sequence	183	183	99%	3e-50	99%	NR_114726.1
Streptococcus mutans strain NCTC 10449 16S ribosomal RNA gene, complete sequence	183	183	99%	3e-50	99%	NR_118933.1

Figure 4.12: Sequencing result for 16SrRNA primer set by Macrogen Company. The sequence was blasted in NCBI against standard strain of *S. mutans* ATCC 25175 complete genome. The identifying result showed 99% similarity.

4.13 Effect of PEM on virulent-genes expression of *S. mutans* in single- and dual-species grew in planktonic and biofilm conditions

Table 4.7 shows the expression of the genes of interest (*gtfB, gtfC, gbpB, spaP* genes) relative to the internal control (16S rRNA gene) in the PEM-treated sample compared to

untreated control for the experiments done in planktonic and biofilm conditions. The detailed calculation changes in mean Ct and standard deviation (SD) of the genes of interest and internal control is shared in the Appendix J. The glucosyltransferases (*gtfB, gtfC* genes) and glucan-binding protein (*gbpB* gene) were the genes regulated in sucrose-dependent adherence, while cell surface protein antigen (*spaP* gene) was sucrose-independent adherence. When *S. mutans* in single-species grew in media containing 1% (w/v) sucrose and PEM-treated, the real-time RT-PCR data showed that the expression of *gtfB* gene of *S. mutans* in planktonic was reduced by 2.04-fold for the PEM-treated compared to the untreated control. Meanwhile, the other genes of interest *spaP, gbpB* and *gtfC* were upregulated by 8.37, 19.37 and 20.68-folds, respectively due to treatment with PEM. As for *S. mutans* in dual-species with *S. sanguinis* grown in planktonic condition, the expression of all genes of interest was dramatically increased by 32-fold to 250-fold, respectively (Table 4.7). The fold changes in gene expression between *S. mutans* in dual-species was increased by 251.95-fold for *gtfB*, 7.71-fold for *gtfC*,13.42-fold for *gbpB* and 3.89-fold for *spaP* genes compared to the single-*S. mutans* grown in planktonic conditions.

The regulation of gene expression of *S. mutans* grown in the presence of 1% (w/v) sucrose and PEM-treated media in biofilm was different compared to the planktonic condition. The result showed that the gene expression of *gtfB* of *S. mutans* in dual-species biofilms was reduced by 2.08-fold for the PEM-treated compared to the untreated control, while the other genes expression (*gtfC, gbpB* and *spaP*) were upregulated by 5 to 7-fold due to the treatment. The fold change in genes expression of *S. mutans* in dual-species biofilms were decreased by 0 to 0.5-fold compared to the *S. mutans* in single-species biofilm (Table 4.7). The overall data also showed that *gtfC* gene was upregulated higher than *gtfB* gene in the planktonic and biofilm conditions. Meanwhile, the expression of *spaP* gene was higher in dual-species with *S. sanguinis* compared to a single species of

S. mutans either in planktonic or biofilm conditions. The expression of *spaP* gene was sucrose-independent and it allowed the bacteria to bind salivary component. Thus, *S. sanguinis* as early coloniser grew best in the absence of sucrose, also expressed the *spaP* gene and allowed to adhere to the saliva-coated tooth surface.

Table 4.7: Regulation expression genes of *S. mutans* in single- and dual-species grew in BHI+1% (w/v) sucrose under planktonic and biofilm conditions due to PEM treatment. The data are expressed as the means and SDs of two biologically independent experiments performed in triplicates. *Statistically significant differences ($p < 0.05$: *t*-test) comparing gene expression in planktonic ([b]*S. mutans*-dualP relative to [a]*S. mutans*-P) and biofilms ([d]*S. mutans*-dualBio relative to [c]*S. mutans*-Bio). **If fold change due to PEM-treated ($2^{-\Delta\Delta Ct} < 1$); fold change due to PEM-treated was calculated as $\frac{-1}{2^{-\Delta\Delta Ct}}$

Gene of *S. mutans*	**Fold change of the PEM-treated in planktonic (P) = $2^{-\Delta\Delta Ct}$ (mean ± SD)**		**Fold change of the PEM-treated in biofilm (Bio) = $2^{-\Delta\Delta Ct}$ (mean ± SD)**	
	[a]*S. mutans*-P	**[b]*S. mutans*-dualP**	**[c]*S. mutans*-Bio**	**[d]*S. mutans*-dualBio**
gtfB	**0.49 ± 0.61	123.45 ± 3.32*	29.14 ± 1.51	**0.48 ± 0.21*
gtfC	20.68 ± 0.10	159.37 ± 0.98*	38.02 ± 1.49	5.21 ± 0.68*
gbpB	19.37 ± 0.44	259.98 ± 0.50*	29.89 ± 1.48	5.78 ± 0.51*
spaP	8.37 ± 0.13	32.55 ± 0.74*	14.43 ± 1.60	7.28 ± 0.36*

CHAPTER 5: DISCUSSION

This study was conducted to investigate the potential of selected plant extracts mixture (PEM) and its respective constituents (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.) as anti-plaque and anti-caries agents. Distilled water was a most polar solvent and higher solubility was used in the extraction of crude aqueous plant extracts from their fresh leaves using boiling method. This method was chosen due to their low cost, safety and eco-friendly procedures. This will also prevent any possible side effects from exposure to any solvent if chemical solvents are used in the extraction process. This procedure is suitable for extracting water soluble and heat stable constituents. Using the boiling method, only 3-12% (w/w) of the dried powdered plant extract was obtained from 1 kg of fresh leaves. The yield percentage varied according to the plant species used in this study. The boiling method (2-6 minutes) resulted in a significant reduction (18.3%) of total phenolic compounds of pumpkin but increased the content of its carotenoids (fat-soluble compounds) (Azizah *et al.*, 2009). Reduction in the quantity of dissolved phenolic compounds during the boiling process could be attributed to the instability of most of the bioactive compounds to heat (Zhang & Hamauzu, 2004). Phenolic compounds consist of water-soluble (flavonoids) and water-insoluble compounds (tannin). All the potential effects that have been exhibited by the selected plant extracts in this study were from the thermally stable phenolic compounds.

In this study, *S. sanguinis* and *S. mutans* had been used to examine the plant extracts being studied as potential candidates as anti-plaque and anti-caries agents. Both bacteria had been chosen because (1) it has been reported that a high proportion of *S. sanguinis* colonies play a role in creating a healthy oral cavity and preventing dental caries and periodontitis (Becker *et al.*, 2002; Caufield *et al.*, 2000; Stingu *et al.*, 2008). *S. sanguinis* is an early coloniser of dental plaque whereas *S. mutans* is a late coloniser which has cariogenic property (Kolenbrander *et al.*, 2010; Valdez *et al.*, 2017); and (2) the two

bacteria can be calculated separately because they have different morphological sizes. The colonies of *S. mutans* that were cultured on BHI agar plate for 24-48 hour appear white, smooth and large whereas those of *S. sanguinis* appear small and translucent (Please see Appendix A, Figure A1, for the photograph). In the determination of the anti-adherence and reduction of bacterial population in the biofilm assays, the bacteria population at mid-log phase of the growth curve (6 hours for *S. mutans* and 8 hours for *S. sanguinis*) and exists in chains was used. The mid-log phase of growth was used to ensure the inhibition of the two bacteria was due to the action of plant extracts and not due to depletion of nutrients. Hasan *et al.* (2012), Lima *et al.* (2014) and Wang *et al.* (2017) have also reported employing the mid-log phase of bacterial growth to investigate the anti-adherence and anti-biofilms effects of the plant extracts they used in their studies.

Pre-screening of antibacterial susceptibility expressed by MIC value of the *Psidium* sp., *Mangifera* sp., *Mentha* sp. and its mixture, PEM against *S. sanguinis* and *S. mutans* were carried out using the microdilution method. Lower MIC value indicates higher antibacterial susceptibility (Riedel *et al.,* 2014). The MIC value of *Psidium* sp. against *S. sanguinis* was higher (7.62 mg/ml) compared with what has been reported by Fathilah (2011) which was 4.69 mg/ml. The discrepancy could be due to the bacterial strain used; where in this study the bacterial strain was purchased from ATCC whereas Fathilah had used clinical isolate. Prabu *et al.* (2006) used pure compound extracted from *Psidium guajava* (guaijaverin) and reported the MIC value of 4 mg/ml against *S. mutans* MTCC 1943. Guaijaverin is a biologically active flavonoid compound of *Psidium guajava* (Prabu *et al.,* 2006). The higher MIC value obtained in this study could be due to the extracts used being crude.

PEM which is a mixture of equal amounts of *Psidium* sp., *Mangifera* sp. and *Mentha* sp. has a lower MIC value than the individual plant extracts. In addition, it exhibited a

synergistic interaction as indicated by the ΣFIC index. This suggests that PEM is a better growth inhibitor compared to its individual constituents.

PEM has the same MBC value against *S. sanguinis* and *S. mutans,* indicating that it has similar bactericidal effect on both bacteria. However, *Psidium* sp. has a lower MBC value compared to PEM against *S. sanguinis*. The *S. sanguinis* tend to aggregate in the presence of *Psidium* sp. (Fathilah, 2011). This may account for the lower MBC value obtained for *Psidium* sp. against *S. sanguinis.* The similar MBC value of PEM against the two bacteria indicates that PEM has a component(s) that may be bactericidal. The synergistic effect of the individual constituent plant extracts in PEM contributes for the better bactericidal and growth inhibitory effects. It has been reported that there are synergistic interactions for constituents in the overall extract of a single herb, as well as between different herbs in a formulation (Nahrstedt & Butterweck, 2010; Williamson, 2001).

Bacterial adhesion plays an important role in the formation of dental plaques. It is initiated by the formation of pellicles on the surface of tooth which then becomes the substratum for bacterial adherence and colonisation (Jakubovics & Kolenbrander, 2010). Dental plaque, if not controlled, will cause dental caries and periodontitis. It has been a common practice to clean teeth after meals. This scenario may be different if oral health care products are used before meals. In this study, I simulated the use of health care products before meals by treating experimental pellicles with plant extracts before inoculating them with the bacteria. This determines the ability of the bacteria to be attached to the treated experimental pellicle. Using plant extracts at sub-MIC values will allow adhesion by viable cells only (Hasan *et al.*, 2015). The final concentration of PEM and its individual constituent plant extracts (0.5 mg/ml) used in this study is far below the MIC value which had also been used for PEM in previous studies in a single species

biofilm (Rahim *et al.*, 2014). The sub-MIC value (0.5 mg/ml) of the plant extracts would not kill the test bacteria used in the study. Fathilah *et al.* (2009) have reported that *Psidium* sp. at sub-MIC value (4 mg/ml) was non-toxic to epithelial cells as well as bacterial cells such as *S. sanguinis*, *S. mitis* and *Actinomyces* sp.

In this study, the trend of bacterial populations attached to the experimental pellicle surface was different in single-species compared to dual-species biofilms. In single-species biofilm, the population of *S. sanguinis* was ten-fold lower than that of *S. mutans*. This corresponds to what was reported by Rahim *et al.* (2014). This may suggest that in a single species biofilm, bacteria (*S. sanguinis* and *S. mutans*, respectively) interact separately with the components of the experimental pellicle. The behaviour of the bacterial populations is different when they co-exist in dual-species in which both bacteria are inoculated at the same time. The population of bacteria filled with both bacteria is almost the same. This can be attributed to reduced competition between the bacteria when they are inoculated at the same time as reported by Kreth *et al.* (2005).

The normal adherence process for early and late colonisers (*S. sanguinis* and *S. mutans*, respectively) was disrupted when experimental pellicle was treated with plant extracts prior to bacterial inoculation (Rahim *et al.*, 2014). Their study involves a single species biofilm while our study includes single- and dual-species biofilms in a dynamic environment. In a single species biofilm, PEM showed higher anti-adhering activity to *S. sanguinis* than *S. mutans*. The individual plant extracts except *Psidium* sp. also showed similar anti-adherence profile. The latter is not in agreement with what was reported by Rahim *et al.* (2014) which was conducted in the same dynamic environment. The discrepancy could be due to the different strain of *S. sanguinis* used in this study. In dual-species biofilms, the pre-treatment of experimental pellicle with the plant extracts demonstrated a different profile of anti-adhering activity compared to single- species. The anti-adhering activity exhibited by *Psidium* sp. against both bacteria is the highest

compared to other plant extracts. However, the large removal of *S. sanguinis* population will cause the oral cavity to be colonised by other bacteria. For example, a dietary intake of carbohydrates will promote *S. mutans* growth and subsequent biofilm formation leading to the initiation of dental caries. This makes *Psidium* sp. unsuitable as an anti-plaque agent. The PEM is capable of reducing about 50% of the population of both bacteria when compared to negative control. The *S. sanguinis* attachment to experimental pellicle treated by PEM provides receptors to *S. mutans* to attach to pellicle or *S. sanguinis* cells when they are cultured together in dual-species. A balanced bacterial population is essential for maintaining oral health by preventing the emergence of pathogenic bacteria following an imbalance in oral resident microbiotas (Marsh *et al.,* 2009). For example, the excess *S. mutans* population can lead to the onset of dental caries (Bowen, 2002). The increase in the adhesion of *S. sanguinis* preceded *S. mutans* in the dual-species can be attributed to modification of complementary binding sites between adhesins on the bacterial surface and receptors on the treated pellicles (Duque *et al*., 2011). The *S. sanguinis* population needs to be maintained because it has the ability to produce antibacterial substances against putative periodontal pathogens where the loss of its colonisation appears to be associated with aggressive periodontitis (Ma *et al.,* 2014; Stingu *et al.*, 2008). The *S. sanguinis* colonisation provides suitable substratum for colonisation of *S. mutans* (Caufield *et al.,* 2000; Kreth *et al.,* 2005). The positive control, 0.12% (v/v) CHX has no adhering activity as the concentration used favours bactericidal activity (Rahim & Thurairajah, 2011).

The bacterial population viewed under SEM corresponded to the colonies attached to the experimental pellicle surface (treated with plant extracts and untreated). This confirmed the potential impact of these plant extracts. *Psidium* sp. appeared to aggregate *S. sanguinis* colonies (Figure 4.6 (i) D) and this corresponded to what was reported by Fathilah (2011). Future studies may include the inoculation of early coloniser prior to the

late coloniser for dual-species biofilms. This is to investigate whether early coloniser has any effect on the adherence of the later coloniser.

It has been reported that the cell-surface hydrophobicity played an important role in the initial adherence of bacteria to the tooth surface. Thus, study on the effect of the selected plant extracts on cell-surface hydrophobicity of the two bacteria was carried out. *S. sanguinis* in this study was shown to be more hydrophobic than *S. mutans,* which are in accordance with what has been reported from a previous study (Yamaguchi *et al.*, 2006). The cell-surface hydrophobicity of the Streptococci is said to be associated with the lipoteichoic acid, capsule, outer membrane proteins, lipids, surface fibrils and fimbriae present on the cell surface (Krasowska & Sigler, 2014). The *S. sanguinis,* which is an early plaque coloniser and is more hydrophobic than *S. mutans* would henceforth have a stronger binding capacity towards the salivary glycoprotein on the tooth surfaces, forming what is known as the acquired pellicle. However, in BHI media (including glucose) and BHI + 1% (w/v) sucrose, *S. mutans* which has low capacity to bind to the experimental pellicle but has higher ability to metabolise (or effectively metabolise) glucose/sucrose in the media to produce glucans. The production of glucans increases the efficiency of adhesion and enhances the proportion of *S. mutans* (Banas, 2004). This is why the *S. mutans* colonisation was 100-fold higher compared to the *S. sanguinis* (Table 4.4).

The reduction of cell-surface hydrophobicity of *S. sanguinis* and *S. mutans* by PEM and *Psidium* sp., respectively may imply that they can control the adherence of the two bacteria to the tooth surface, thus limiting the development of dental biofilm. There are numerous reports available that the leaves extract of *P. guajava* and its active compound, guaijaverin has the potential as an anti-plaque agent due to its ability to inhibit the growth *S. sanguinis, S. mitis, Actinomyces* sp., *S. mutans* and *Staphylococcus aureus* (*S. aureus*) on tooth surface during plaque formation (Fathilah *et al.,* 2009; Limsong *et al.,* 2004).

Guaijaverin binds to the cell surface proteins, reducing the overall cell hydrophobicity of *S. mutans* (Prabu *et al.*, 2006). The effect of *Psidium* sp. in reducing the cell-surface hydrophobicity of *S. sanguinis* has also been reported by Razak *et al.*, (2006). The aqueous extracts *Piper betle* and *Psidium* sp. were also found to have anti-plaque activity by their effect on ultrastructure of plaque bacteria by interfering with normal growth cycle and development, reducing the adhering capacity of the acquired pellicle which forms on the surface of tooth during early plaque formation, diminishing the cell-surface hydrophobicity of bacteria which are necessary to assist the adherence process (Fathilah *et al.*, 2009; Razak *et al.*, 2006; Razak & Rahim, 2003).

The anti-adhering activity exhibited by the plant extracts treated-experimental pellicle reflected surviving cell adhered as the concentration used in this study is within the sub-MIC range. The phenolic content of the plant extracts may interfere with the adhesion of bacterial cells in the experimental pellicle (Thimothe *et al.*, 2007). Decreased attachment activity can affect biofilm formation (Barnabe *et al.*, 2014; Rahim *et al.*, 2014).

The effect of selected plant extracts on bacterial population in biofilm was also studied. This is to determine the potential effect of selected plant extracts to detach the biofilms formed and serve as anti-caries agent. In this study, the biofilm was developed in a dynamic environment for 24 hours in medium BHI broth (without sucrose) and BHI broth containing 1% (w/v) sucrose (with sucrose), respectively. For the single-species biofilm formed in the medium without sucrose, *S. mutans* population adhered to the untreated experimental pellicle was 35-fold higher compared to the *S. sanguinis* population. The BHI broth (Oxoid brand) used in this study contained glucose. *S. mutans* rapidly metabolises glucose to produce energy. It was shown that in single-species biofilm, the population of *S. mutans* grown in the presence of sucrose increases rapidly whereas that of *S. sanguinis* decreases. The decrease in *S. sanguinis* population might be due to the lack of glucosyltransferases (Gtfs) and fructosyltransferase (Ftf) enzymes enabling them

to metabolise sucrose to produce extracellular polysaccharide (EPS). It is well documented that sucrose is considered the most cariogenic dietary carbohydrate because it is fermentable by *S. mutans* and serves as a substrate for the synthesis of extracellular polysaccharides (EPS) and intracellular polysaccharides (IPS) in dental plaques (Bowen, 2002). Thus, sucrose enhances the growth of cariogenic *S. mutans* leading to the pathogenesis of dental caries. It has been reported that the *S. mutans* produces three Gtfs and one Ftf enzymes for sucrose metabolism to produce EPS (glucans) and IPS (fructan), respectively. The GtfB is responsible for the production of water-insoluble glucan, GtfC produces a mixture of water-soluble and water-insoluble glucans and GtfD synthesises water-soluble glucan (Bowen & Koo, 2011; Koo *et al.*, 2010). The Gtfs produced by *S. mutans* and the EPS production have gained a lot of attention for their role in the formation of biofilm and initiation of dental caries (Bowen & Koo, 2011; Takahashi & Nyvad, 2011).

In the dual-species experiment, it was shown that the population of *S. sanguinis* and *S. mutans* grown in the sucrose-enriched medium (1%) in dynamic environment were almost equal. While in the absence of sucrose, the growth of *S. sanguinis* was almost 7-fold lower than that of *S. mutans.* In the biofilm formation, *S. sanguinis* being an early coloniser would be the first to adhere to the pellicle followed by *S. mutans*. The *S. sanguinis* has surface adhesins to which the *S. mutans* attach. There are several environmental factors involved in the viability and growth of *S. mutans* in biofilms such as nutrient supply, metabolite outflux, pH gradient and oxygen tension (Bowden & Li, 1997; Marsh, 2005). In addition, Bradshaw and Marsh (1998) reported that lack of nutrition and accumulation of lactic acid by-products reduced the growth of the acid-sensitive species such as *S. sanguinis.* With sucrose in the media, *S. mutans* would be metabolising it to produce EPS and energy which might have a role in maintaining an equal population of the two bacteria. When the two bacteria were deprived of sucrose,

they compete for nutrient for energy. Furthermore, *S. sanguinis* does not possess Gtf and Ftf enzymes. This may explain for the reduced population of *S. sanguinis*. This phenomenon shows that biofilm formation is influenced by the composition of growth medium (Loo *et al.*, 2000).

When the biofilm was treated with the selected plant extracts, the two bacteria showed different growth characteristics. All the plant extracts effectively reduced the bacterial population in single- and dual-species biofilms. It was shown that in single-species biofilm grown in the absence of sucrose, the *S. mutans* population was significantly reduced compared to *S. sanguinis* ($p < 0.05$) when treated with PEM and *Mangifera* sp., respectively. In the oral cavity, bacteria exist in multi-species biofilms. Thus, the discussion more focus on the effect of plant extracts on dual-species biofilms. In the dual-species biofilms grown in the absence of sucrose, all the individual plant extracts reduced and retained more *S. sanguinis* population than that of *S. mutans.* The PEM appeared to retain *S. sanguinis* and *S. mutans* in almost equal proportion (13-16%). While in the presence of sucrose, *S. mutans* population was highly reduced but it was found hard to be removed in the biofilms compared to that of *S. sanguinis*.

In addition, it was shown that *Psidium* sp. exhibited properties as a better candidate in reducing bacterial population in biofilms where *Psidium* sp. had retained the *S. sanguinis* and *S. mutans* population in almost equal proportions (36-38%; $p > 0.05$) and created a balanced population between them in the presence of sucrose. Meanwhile, the other plant extracts (PEM, *Mangifera* sp. and *Mentha* sp.) had lowered down the *S. sanguinis* population, allowing the *S. mutans* population to enhance biofilm development and initiate caries formation in the presence of sucrose. There is no report on the potential of *Psidium* sp. to reduce bacterial population in biofilms (*S. sanguinis* or/and *S. mutans*) so far. However, most of the studies reported that guava leaves altered and disturbed the cell-surface hydrophobicity of early coloniser (*S. sanguinis*) of dental plaque, making

them less adherent and also delayed in the development of dental biofilm by targeting growth, adherence and co-aggregation (Fathilah *et al.*, 2009; John *et al.*, 2013b; Ravi & Divyashree, 2014; Razak *et al.*, 2006). This property could be due to the presence of flavonoids and tannins detected in *P. guajava* (John *et al.*, 2013a). Previous studies reported that difference in total phenolic and flavonoid contents obtained from different solvents extraction of *Psidium* sp. leaf extracts play a role as antioxidant, antitumor, anticancer and cytotoxic effects (Ashraf *et al.*, 2016). Furthermore, the specific combination of phytochemicals in *Psidium* sp. may be far more effective in protecting against cancer than isolated compounds, suggesting the synergy interactions occur among active compounds in the plants (de Kok *et al.*, 2008).

Dental caries was develops from demineralisation of enamel of the tooth surfaces due to bacterial metabolism of dietary carbohydrate to produce lactic acid by-product (Strużycka, 2014). Thus, the ability of *S. sanguinis* and *S. mutans* cells to metabolise glucose for energy and produce lactic acid by-product was studied in planktonic condition. Acidogenicity is one of the main cariogenic factors of *S. mutans* (Banas, 2004). In glycolysis, *S. mutans* and *S. sanguinis* metabolise glucose to pyruvate and finally to lactate by lactate dehydrogenase when glucose is in excess, as well as formate, acetate and ethanol via pyruvate-formate-lyase system when glucose is limiting (Marsh *et al.*, 2009). These activities create an acidic environment in dental plaque which becomes accessible to the tooth enamel, initiating dental caries.

In this study, all the plant extracts reduced the acid-producing capacity of *S. sanguinis* and *S. mutans* (single- and dual-species) with the PEM demonstrating the highest reduction of acid production as indicated by the small pH change. It has also been reported that plant extracts reduced acid production of *S. mutans* (Ban *et al.*, 2012; Hasan *et al.*, 2015). The reduction in acid production of bacteria could be due to the limitation of glucose uptake into the bacterial cell resulting in reduced energy production. The uptake

of the glucose may be affected by the inhibition of the membrane-associated phosphoenolpyruvate (PEP)-phosphotransferase system (PTS) (Marsh *et al.*, 2009). In their study, they were using solvent-plant extracts and the activity was fixed at room temperature. The acid production of *S. sanguinis* and *S. mutans* throughout this study was fixed at 37^{o}C with slow flow rate (rotation at 40 rpm) in aerobic condition to mimic the environment of the oral cavity and using crude aqueous plant extracts.

In single-species cells, it was shown that PEM, *Psidium* sp. and *Mangifera* sp. had an inhibitory effect on glucose metabolism by *S. sanguinis* as indicated by a reduction in acid production compared to the negative control. The metabolism of *S. mutans* was highly affected by PEM compared to the other two plant extracts. The inhibition of glucose metabolism would limit the energy production which may explain the reduction in the population of the bacteria in the plant extracts-treated biofilms. In the dual-species cells, the effect of PEM was intermediate between *Psidium* sp. and *Mangifera* sp. This may be attributed to the harmonious population between the two bacteria (*S. sanguinis* and *S. mutans*) as observed in the PEM-treated biofilms. The intake of glucose by the bacterial cells in biofilms and acid production may be slower compared to the bacterial cells in planktonic condition (Kreth & Herzberg, 2015).

The phenolic compounds in the plant extract reduced the adherence and glycolytic pH-drop of *S. mutans* cells and subsequently affected the biofilm formation (Rahim *et al.*, 2014; Thimothe *et al.*, 2007). The same attribute can be used to explain their effect on reduction of bacterial population in biofilm. In this study, the level of TPC in PEM was higher compared to that of *Psidium* sp., *Mangifera* sp. and *Mentha* sp. This may explain the difference or comparable effect between them towards the bacteria.

The result from the UHPLCMS/MS analysis indicated that the phenolic compounds identified in PEM have been studied widely by researchers worldwide for the prevention

of oral diseases related to *S. mutans*. The identified phenolic compounds in PEM include gallic acid, apigenin, quercetin, quercetin-3-*O*-glucoside, succinic acid and quinic acid. Quinic acid, gallic acid, quercetin sulfate and ellagic acid are the phenolic compounds present in *Psidium* sp. While quinic acid, quercetin-3-*O*-glycoside and quercetin are detected in *Mangifera* sp. All of these phenolic compounds may play a role in inhibiting virulence properties of *S. mutans*. Previous studies have shown that gallic acid extracted from *Melaphis chinensis* significantly inhibited the growth and aggregation of *S. mutans* (Wu-Yuan *et al.,* 1988). The gallic acid in *Galla rhois* also inhibited the growth of cariogenic bacteria in the planktonic environment and the *in vitro* formation of *S. mutans* biofilms (Kang *et al.,* 2008). Apigenin (4',5,7-trihydroxyflavone) obtained from propolis (a natural beehive product), reduce the bacterial population of *S. mutans* (in the sucrose-dependent environment) which lead to the decreased the biofilm formation (Koo *et al.,* 2003). Quercetin, extracted using solvent extraction of *Nidus vespae* effectively inhibited the growth of *S. mutans* as well as reduced acid production and acid tolerance of *S. mutans* (Guan *et al.,* 2012). Quercetin, an active compound of *P. guajava* has shown excellent antibacterial actions against periodontopathogens such as *A. actinomycetemcomitans, P. gingivalis, P. intermedia* and *F. nucleatum* (Geoghegan *et al.,* 2010). It has shown inhibitory actions against *S. mutans, S. sanguinis* and *Actinomyces* sp. (Shu *et al.,* 2011). The antibacterial action of quercetin is probably due to the disruption of membrane and inactivation of extracellular proteins by forming irreversible complexes (Shu *et al.,* 2011). Quercetin-3-*O*-glucoside isolated from *Vaccinium macrocarpon* (cranberry fruit) showed the moderate effect towards the acid-producing capacity of *S. mutans*. It was also suggested that the biological activity might be due to the synergistic effect of a complex mixture of the active compounds rather than an individual compound of cranberry fruit (Gregoire *et al.,* 2007). Succinic acid and quinic acid extract of *Cichorium intybus* var. *silvestre* (red chicory) has been shown to inhibit adherence to epithelial cell and biofilm

development or promotion of their biofilm disruption of *S. mutans* and *A. naeslundii*, and *P. intermedia* (Papetti *et al.*, 2013). Besides PEM, quinic acid also present in *Psidium* sp. and *Mangifera* sp. may be interfering with the reduced ability of *S. mutans* to adhere and form biofilm.

Gallic acid and ellagic acid which were found in *Psidium* sp. (Table 4.6) may contribute to the inhibition of *S. mutans* growth and acid production. Ismail *et al.* (2012) has reported on the effect of *Punica granatum* (*P. granatum*) on inhibiting the growth, reducing the adherence and biofilm formation as well as acid production of *S. mutans.* They revealed that gallic acid and ellagic acid in *P. granatum* have ability to precipitate membrane proteins and inhibit metabolic enzymes causing cell lysis. Quercetin could significantly inhibit biofilm formation of *S. mutans* and removed the mature biofilm by suppress over 50% of the expression of *gtfB, gtfC, comD* and *comE* genes of *S. mutans* (Yue *et al.*, 2016). Presence of quercetin and quercetin 3-glucoside in *P. americana* exerted antibacterial activity against *P. gingivalis* and *S. mutans* (Patra *et al.*, 2014). Quercetin glycosides present in cranberry fruit inhibited surface-adsorbed glucosyltranferases and F-ATPases activities and acid production of *S. mutans* cells (Duarte *et al.*, 2006).

The molecular assay started with the identity confirmation of *S. mutans* ATCC 25175 using 16S rRNA gene sequencing. This offers a more consistently reliable and accurate method for identification, unaffected by phenotypic variation or experimental bias, and has the potential to reduce laboratory errors (Petti *et al.*, 2005). The identity of the *S. mutans* ATCC 25175 strain used in the study was confirmed as *S. mutans* by the 16S rRNA sequencing analysis. *S. mutans* ATCC 25175 was commercially bought from ATCC and also known as *S. mutans* Clarke (ATCC 25175) serotype c and was isolated from carious dentine tooth as declared from a commercial sheet provided. Its complete genome was described by Song *et al.* (2013). *S. sanguinis* ATCC BAA-1455 (strain

SK36) used in this study to develop dual-species with *S. mutans* was also commercially bought from ATCC. The complete genome of *Streptococcus sanguinis* SK36 chromosome was provided from NCBI Reference Sequence: NC_009009.1 as published by Xu *et al.* (2007).

The sequences of primer pairs (forward and reverse) of genes of interest (*gtfB, gtfC, gbpB, spaP*) and internal control gene (16S rRNA) used in this study was chosen from another study and was blasted using NCBI software (Shemesh *et al.*, 2007a). These short sequence of primer pairs were synthesised for gene expression study using real-time PCR. The specificity (or PCR efficiency) of all the selected primers for each *S. mutans* genes (*gtfB, gtfC, gbpB, spaP* genes) were amplified using PCR and one single band was detected by running the PCR product through 2.5% (w/v) agarose gel electrophoresis (Figure 4.11a). Gangisetty and Reddy (2009) proposed that the specificity of these primer sequences can be checked by (a) running a melting curve analysis at end of PCR where the amplification plot should only yield a single sharp peak (refer Appendix K); (b) running PCR products on a 2% (w/v) agarose gel – the amplification should only yield one band and (c) purifying and sequencing the PCR product.

Real-time PCR is a powerful tool to quantify gene expression. The quantification of gene expression was measured by absolute and relative quantification. In this study, the relative quantification was performed, and the real-time PCR data was presented as the expression level of target genes relative to the internal control. The real-time PCR data were analysed by comparative Ct ($2^{-\Delta\Delta Ct}$) method. The comparative Ct method make several assumptions including the efficiency of the PCR that is close to 1 and the PCR efficiency of the target gene is similar to the internal control (Schmittgen & Livak, 2008). Advantages of the comparative Ct method include its ease to use and that the data can be presented as 'fold-change' in expression. A disadvantage of this method was that the

efficiency of the targets and internal control genes must be determined for each experiment.

It is well known that *S. mutans* expresses several virulent genes such as *gtfB, gtfC, gbpB* and *spaP* to adhere and form a biofilm. The glucosyltransferases (*gtfB, gtfC* genes) and glucan-binding-protein (*gbpB* gene) were regulated in sucrose-dependent adherence and play an important role in the initiation of dental caries (Zhao *et al.*, 2014). Sucrose-independent adherence such as cell surface antigen, spaP contributes to the microbial colonisation by providing binding sites for bacteria (Shemesh & Steinberg, 2006).

It is well known that the *gtfB* gene of *S. mutans* encoded a glucosyltransferase, GtfB, synthesised mostly water-insoluble glucans containing elevated amounts of α-1,3- linked glucose from sucrose to promote adherence to the tooth surface, which contributes to the formation of dental plaque (biofilm). Meanwhile, a glucosyltransferase, GtfC synthesised a mixture of water-insoluble and water-soluble glucans. As shown in real-time PCR data (Table 4.7), the *gtfC* gene was expressed stronger than the *gtfB* gene. This is possible because the *gtfC* has a strong effect on sucrose-dependent adhesion and essential for biofilm formation on tooth surfaces with the help by water-soluble glucan (Tamesada *et al.*, 2004). Bowen and Koo (2011) revealed that the GtfC is adsorbed to enamel-coated salivary pellicle whereas GtfB binds avidly to bacteria promoting tight cell clustering and enhancing the cohesion of plaque. In this study, it was found that the treatment with PEM downregulated the expression of the *gtfB* gene of *S. mutans* in single-species grew planktonically by 2.04-fold change compared to the untreated control. Its adherence capacity was increased in dual-species with *S. sanguinis.* As the GtfB involved in cell to cell clustering, the GtfB might bind to early coloniser, *S. sanguinis* cells on saliva-coated surfaces, producing glucan locally and provide binding sites for late coloniser, *S. mutans.* (Nobbs *et al.*, 2009). The similar adherence was also proposed for the *Candida albicans* co-cultured with *S. mutans* (Hwang *et al.*, 2015). The upregulated expression of all *S.*

mutans genes in dual-species grew planktonically implied in media containing sucrose, *S. mutans* actively metabolised sucrose and synthesised all the sucrose-dependent adherence genes, providing higher affinity to bind to the *S. sanguinis* for colonisation on the saliva-coated surfaces. In media with no competition for colonisation, the expression of *gtfB* gene *S. mutans* was reduced due to the treatment with PEM but the other genes were upregulated to enable *S. mutans* to adhere and form a biofilm.

The ability of PEM to detach the biofilm formed by *S. mutans* in single- and dual-species biofilms was also investigated. Treatment with PEM significantly reduced the expression of *gtfB, gtfC, gbpB* and *spaP* genes of *S. mutans* in dual-species biofilms compared to its single-species biofilm, with the *gtfB* gene being the most downregulated. Meanwhile, in *S. mutans* biofilms, treatment with PEM still allow *S. mutans* to grow, and synthesised biofilm-associated genes of sucrose-dependent adherence (*gtfB* and *gtfC*) were expressed in higher amount in the presence of sucrose. This is comparable with the bacterial population in biofilm study. It was found that *S. mutans* in the dual-species biofilms were reduced by 15% compared to its individual biofilm (which only reduced by 21%) in the presence of sucrose and PEM-treated conditions. This could be due to the competition for binding sites with *S. sanguinis* and PEM-treated environment reduced *S. mutans* population in dual-species biofilms. However, in the media with no competition for growth, *S. mutans* biofilm grew abundantly in the presence of sucrose. This finding was supported by Wen *et al.* (2010) where biofilm formation by *S. mutans* when grown with *S. sanguinis* was significantly decreased compared to *S. mutans* in the single-species biofilm.

A *gbpP* gene encoded a glucan-binding protein and was expressed when the glucan was synthesised from sucrose by the activities of glycosyltransferases (Gtfs) *S. mutans.* It is known that water-insoluble glucans significantly facilitate cell adherence and accumulation of stable biofilm, and are mediated by glucan-binding protein. *S. mutans*

produce at least four types of *S. mutans* glucan-binding protein (Gbp), identified as GbpA, GbpB, GbpC and GbpD. The GbpA and GbpC in *S. mutans* have strong relationships with cariogenicity, while GbpB may have another biological function such as cell shape and cell maintenance as well as some role in in cariogenicity of *S. mutans*. Depletion of GbpB impaired initial phases of sucrose-dependent biofilm formation, leading to altered cell shape, decreased autolysis, increased cell hydrophobicity and sensitivity to antibiotics, osmotic and oxidative stresses (Duque *et al.*, 2011; Matsumoto-Nakano, *et al.*, 2007).

A *spaP* gene encodes cell surface antigen, known as sucrose-independent adherence. It also contributes to the virulence relating to the adherence capacity of cell surface antigen of *S. mutans* to the salivary component and the cell-surface hydrophobicity of the bacteria. A study showed that the presence of *spaP* gene of *S. mutans* highly contributes to the prevalence of childhood caries (Duran-Contreras *et al.*, 2011). Real-time PCR data showed that *spaP* gene was expressed in low affinity in the presence of sucrose, indicating that its expression was related to sucrose-independent adherence. However, a study by Biswas *et al.* (2007) revealed that the glucan-binding capacity (Gbp) are not only associated with Gtfs activity but also the cell surface-associated protein (spaP) and the ability to bind glucan with very high affinity. Therefore, the expression of *spaP* gene is still high in the presence of sucrose, due to its ability to bind to glucans in the media. In addition, *spaP* gene affects the ability of organisms to adhere to saliva-coated surfaces and possibly affects primary colonization of the oral cavity in the absence of a glucan surface but has no effect on glucan-mediated adherence *in vitro* or *in vivo* (Bowen *et al.*, 1991; Wen *et al.* 2010). As compared to single-species *S. mutans*, the *spaP* gene was highly upregulated in dual-species bacteria grown planktonically, indicating that *S. sanguinis* also expressed *spaP* gene in order to adhere to the surfaces. This statement was supported by a previous study that some regions of the *spaP* gene are highly conserved

in *S. mutans* and *S. sanguinis* and could be involved in the bacterial adherence (Ma *et al.*, 1991).

Treatment with PEM affects the expression of multiple *S. mutans* genes involved in adherence and biofilm formation. The degree of expression genes depends on the availability of sucrose, cultures condition either in planktonic or biofilm forms and competition with *S. sanguinis* in the media.

This study implies that the individual plant extracts in PEM have different efficacy in retaining the bacterial population in biofilm compared to PEM itself. PEM creates a harmonious bacteria population (*S. sanguinis* and *S. mutans*) in the biofilm and could be attributed to the better anti-plaque agent. The behaviour of the plant extracts (individual and mixture) towards the bacteria is different in the single- and dual-species biofilms. In all the studies, the *S. sanguinis* was not jeopardised as much as *S. mutans*. This study also suggested that PEM could be a better candidate as anti-caries agent due to capability to detach a biofilm formed by the two bacteria in dual-species biofilms grown during sucrose-limited (fasting) condition.

CHAPTER 6: CONCLUSION

In conclusion, this study has successfully determined the potential effects of a plant extract mixture (PEM) and its individual extracts (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.) as anti-plaque and anti-caries agents. All the extracts were able to inhibit the growth of *S. sanguinis* and S. *mutans*, and PEM was observed to be the best inhibitor. The bacteriostatic and bactericidal effects of PEM may be due to synergistic interactions of active compounds in individual plant extracts. All plant extracts are able to reduce the adhesion and population of both bacteria with different efficacy. PEM showed a significant anti-adhesion effect on *S. sanguinis* and *S. mutans* in a single-species biofilm. For dual-species biofilms, PEM has the highest potential to be an anti-plaque agent compared to the other individual plant extracts because PEM were able to reduce half of the population and yielded an almost balanced population between the two bacteria, which was verified by SEM. Whereas *Psidium* sp. exhibited the highest anti-adhesion activity for both bacteria in dual-species biofilms, but it was not the best anti-plaque candidate because the high reduction of certain bacterial colonisation such as *S. sanguinis* will induce other bacterial pathogens to colonise the oral cavity and form biofilms. This will induce caries formation if the growth of *S. mutans* increases with the intake of a carbohydrate diet by an individual.

PEM also reduced the hydrophobic ability of the bacterial cell surface. This will reduce the ability of both bacteria to attach and form biofilms. This will, in turn, slow down the process of caries formation. The S. *mutans* population in a single species biofilm in the medium without sucrose also decreased with PEM treatment. With the presence of sucrose, PEM sought to reduce both bacterial populations in a single species biofilm significantly. However, the reduction of *S. mutans* population was less than the *S. sanguinis* population. This is because *S. mutans* are able to metabolize sucrose and promote growth, while the presence of sucrose in the media is not suitable for growth of

S. sanguinis. Furthermore, *S. mutans* colonies also interact weakly on salivary glycoproteins and with their own cells in a single-species biofilm. For dual-species biofilms, *S. sanguinis* as the earliest coloniser, got attached to pellicle (salivary glycoprotein) and provided receptors for *S. mutans* to attach later. This will provide a strong adhesion between *S. sanguinis* and *S. mutans* in dual-species biofilms. The *S. mutans* population in the media containing sucrose only decreased slightly compared to *S. sanguinis* with PEM treatment. This is because *S. mutans* are still able to metabolize sucrose to produce glucans to promote the adhesion process and subsequently to form biofilm in the media. *Mangifera* sp. and *Mentha* sp. also showed the same anti-biofilm activities as PEM. The ability of *S. mutans* to form biofilms with the presence of PEM in media containing sucrose had been verified by the quantitative analysis of qRT-PCR. The results showed that of the three types of sucrose-dependent adhesion genes investigated, only *S. mutans gtfB* gene expression [in (single-species; planktonic) and (dual-species; biofilms)] were inhibited, while genes expression of *gtfC* and *gbpB* were increased with PEM treatment in medium containing sucrose. Water-soluble and -insoluble glucans synthesized by *gtfC* gene and *gbpB* gene helps in bonding and forming biofilms on the surface of the 6-wells plate used. Cell surface antigens protein, *spaP* which is sucrose-independent adhesion gene can also be synthesised in media containing sucrose treated by PEM. This is due to several factors: (a) *spaP* gene can be attached to salivary glycoprotein (pellicle), (b) *S. sanguinis* as the earliest plaque coloniser also produces *spaP* gene and helps attachment to pellicle, (c) *spaP* has a role on hydrophobic surfaces of *S. sanguinis* and *S. mutans* cells and (d) glucan production in the media also has a role in *spaP* expression gene. Additionally, PEM can also significantly reduce the production of acid by *S. mutans* and thereby reduce its cognitive potential to unleash the surface of the tooth enamel. The UPHLCMS/MS analysis results supported that chemical compounds in PEM were actively involved in synergistic interactions on bacterial cells.

Based on all the results above, PEM may be incorporated in oral healthcare product with further validation of the product *in vivo*.

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LIST OF PUBLICATIONS AND PAPER PRESENTED

1. Shafiei, Z., Rahim, Z. H. A., Philip, K., & Thurairajah, N. (2016). Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (*Psidium* sp., *Mangifera* sp., and *Mentha* sp.) on single- and dual-species biofilms. *PeerJ, 4*, e2519.

2. Zaleha Shafiei, Zubaidah Hj. Abdul Rahim, Koshy Philip, Nalina Thurairajah & Resni Mona Abdullah. Antibacterial and anti-adherence effects of plant extract mixture (*Psidium* sp., *Mangifera* sp., and *Mentha* sp.) and its individual extracts on single- and dual-species biofilms. *International Congress of The Malaysian Society for Microbiology* (ICMSM), Penang, Malaysia, 7-10 December 2015, Oral Presentation.

3. Z. Shafiei, Z. A. Rahim, K. Philip, N. Thurairajah, R. Mona. Plant extracts' effect on population of bacteria in single/dual-species biofilms. *94th General Session & Exhibition of the International Association for Dental Research (IADR)*, Seoul, Korea, 22-25 June 2016, Poster Presentation.

4. Zaleha Shafiei, Zubaidah Hj. Abdul Rahim, Hashim Yaacob, Koshy Philip, Nalina Thurairajah. *In vitro* effect of plant extract mixture and its individual extracts in reducing bacterial population on single- and dual-species biofilms. *International Conference of Oral Immunology and Oral Microbiology* (ICOIOM), University of Malaya, Malaysia, 14-15 August 2018, Poster Presentation.

<u>Paper under submission:</u>

Shafiei, Z., Rahim, Z. H. A., Philip, K., & Thurairajah, N. *Psidium* sp., *Mangifera* sp., *Mentha* sp. and its mixture (PEM) as potential anti-caries agents. (submitted to Frontier in Microbiology: waiting for reviewers comments).