# EFFECT OF NICOTINE ON THE ADHERENCE AND GENE EXPRESSION OF SELECTED ORAL MICROORGANISMS

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**KUALA LUMPUR**

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# EFFECT OF NICOTINE ON THE ADHERENCE AND GENE EXPRESSION OF SELECTED ORAL MICROORGANISMS

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**DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF DENTAL SCIENCE**

**DEPARTMENT OF ORAL AND CRANIOFACIAL SCIENCE**
**FACULTY OF DENTISTRY**
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## ABSTRACT

The ability of microbes to adhere on tooth surfaces coated with salivary pellicle lead to the formation of biofilm. Some environment risk factor such as smoking, poor oral hygiene and diet can contribute to the formation of plaque on tooth surface. The aim of this study was to investigate the effect of nicotine on the antimicrobial, adherence capacity, expression of adherence-associated genes and extracellular polysaccharide (EPS) and micro-colonies formation of selected oral microorganisms; *Streptococcus sanguinis*, *Streptococcus mutans*, *Candida albicans* and *Candida parapsilosis*. Growth profiles were carried out to determine the survival rate of the selected oral microbes in different concentration of nicotine (1, 2, 4 and 8 mg/ml). The relative number of viable microbial cells was estimated using viable plate count enumeration method and biofilm growth was quantified using crystal violet assay. Cell surface hydrophobicity and the cell aggregation were also studied. The regulations of selected genes associated with adherence were evaluated quantitatively and qualitatively by using real time and reserve transcription-polymerase chain reaction. In addition, the structure of EPS and biofilm maximum depth upon exposure with nicotine were analysed using confocal laser scanning microscope (CLSM). The results indicated that nicotine enhanced the growth of oral candida and bacteria in both planktonic and biofilm cells. Cell surface hydrophobicity and the expression of hyphal wall protein 1 (*HWP1)* and agglutinin-like sequences 3 (*ALS3)* of *C. albicans* and *C. parapsilosis* were found to increase in relative to the nicotine concentrations used. We also found that nicotine could increase the expression of *Streptococcus* sp. adherence-associated genes such as surface protein antigen (*spaP*), glucosyltransferase (*gtfB*), and glucan binding protein B (*gbpB*). Interestingly, the

thickness of biofilm was increased as the nicotine concentration increases. The data concluded that nicotine can promote the growth of *S. sanguinis*, *S. mutans*, *C. albicans* and *C. parapsilosis* and influences its adherence on the plastic wells which mimic tooth surfaces. The findings of the study may have implications in improving and providing better healthcare for heavy smokers to reduce dental biofilm. In addition, the overall outcomes of this research may be applied to smoking cessation measures in smokers and aid in providing guidelines for control and preventive measures of dental biofilm associated oral diseases.

## ABSTRAK

Pembentukan plaque adalah salah satu jangkitan mulut yang paling biasa melibatkan sekumpulan mikroorganisma yang melekat dan membiak pada permukaan gigi dengan kehadiran pelikel. Sesetengah faktor risiko persekitaran juga membantu pembentukan plak pada permukaan gigi seperti merokok, kebersihan mulut yang tidak memuaskan dan pemakanan yang tidak seimbang. Tujuan kajian ini adalah untuk mengkaji kesan nikotin kepada faktor-faktor pertumbuhan, pematuhan sel dan pengawalatur gen yang diekspresikan oleh mikroorganisma oral termasuk *Streptococcus sanguinis, Streptococcus mutans, Candida albicans* dan *Candida parapsilosis*. Profil pertumbuhan telah dijalankan untuk menentukan kadar keterushidupan sel-sel mikrob dalam mulut dengan menggunakan kepekatan nikotin yang berbeza. Bilangan relatif sel mikrob yang masih hidup telah dianggarkan menggunakan kaedah penghitungan sel hidup dan pertumbuhan sel dalam pembentukan biofilm telah diukur menggunakan assay kristal ungu. Sel hidrofobisiti permukaan dan pengagregatan sel-sel mikroorganisma telah dikaji. Pengawalatur gen dipilih berkaitan dengan pematuhan telah dinilai secara kuantitatif dan kualitatif dengan menggunakan 'real time dan reverse transcription-polymerase chain reaction'. Di samping itu, ketebalan biofilm selepas dirawat dengan nikotin telah dianalisisa menggunakan mikroskop konfocal imbasan laser. Keputusan menunjukkan bahawa nikotin meningkatkan pertumbuhan kulat oral dan bakteria dalam kedua-dua pembentukan sel planktonik dan biofilm. Apabila kepekatan nikotin semakin meningkat, sel hidrofobisiti permukaan dan hyphal wall protein 1 (*HWP1*) serta agglutinin-like sequence 3 (*ALS3*) pada *C. albicans* dan *C. parapsilosis* juga didapati meningkat. Kami mendapati bahawa nikotin juga boleh meningkatkan pengawalatur gen untuk spesies

*Streptococcus*. Selain daripada itu, ketebalan biofilm sel mikrob juga didapati telah meningkat dengan peningkatan kepekatan nikotin. Kesimpulannya, nikotin mampu meningkatkan pertumbuhan *S.s sanguinis, S. mutans, C. albicans* dan *C. parapsilosis* telah mempengaruhi pelekatannya pada permukaan gigi. Dapatan kajian ini mungkin mempunyai implikasi dalam meningkatkan dan menyediakan penjagaan kesihatan lebih baik kepada perokok tegar untuk mengurangkan biofilm gigi. Di samping itu, hasil kajian ini secara keseluruhan boleh digunakan untuk langkah-langkah pemberhentian merokok di kalangan perokok dan boleh membantu dalam merangka garis panduan bagi kawalan dan langkah-langkah pencegahan biofilm gigi berkaitan penyakit mulut.

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abs : absorbance unit

CFU : colony forming unit

CLSM : confocal laser scanning microscopy

CSH : cell surface hydrophobicity

EPS : extracellular polysaccharides

et al : et alia or others

GC : gas chromatography

h : hour

l : litres

mg : milligram

min : minutes

ml : millilitre

nm : nanometer

no. : number

PBS : phosphate buffered saline

s : seconds

sp : species

v/v : volume/volume

% : percentage

µg : microgram

µl : millilitre

µm : micrometer

ºC : degree centigrade

## LISTS OF APPENDICES

# CHAPTER 1: INTRODUCTION

## 1.1 Prevalence of microbial infection in oral cavity

In 2015, approximately 22.8% (4,991,458) of Malaysian population aged 15 years and above were smokers; 43.0% (4.85 million) of men and 1.4% (143,566) of women smoked manufactured cigarettes, hand-rolled and smokeless cigarettes. Out of the current smokers, 20.5% were daily smokers; 38.8% of men and 1.1% of women. About one tenth of the Malaysian population aged 15 years and above (20.8% of men and 0.8% of women) take smokeless cigarette (Malaysia Institute for Public Health, 2015).

Factors such as smoking, poor oral hygiene, malnutrition, diabetes, low immunity system, stress, and extensive usage of antibiotics have been shown to affect the first line of the host's defense which may lead to facilitation of microbial colonization (Arendorf & Walker, 1980; Samaranayake *et al.*, 2009).

Microorganisms indigenous to the human mouth vary considerably in their ability to adapt and inhabit different oral surfaces. The proportions in which various *Streptococcus* sp. and *Candida* sp. inhabits surfaces of teeth and epithelial surfaces have been found to be correlated positively with the relative affinity of the organisms for these surfaces. Oral diseases promulgated by *Streptococcus* sp. and *Candida* sp. are the most common diseases reported in the late 19$^{th}$ and 20$^{th}$ century (Samaranayake *et al.,* 2009)

and its prevalence are still overwhelming worldwide as the result of multiple factors such as poor oral hygiene, smoking habits, unhealthy diet and excessive usage of antibiotics. These might favour bacteria and candida to be mutualistic and develop potential pathogenic relationship with the hosts (Arendorf & Walker, 1980; Abaci & Haliki-Uztan, 2011).

### 1.2 *Streptococcus* species in oral infection

Over 400 microbial species are found in the typical adolescent human mouth and this ecosystem is maintained at homeostasis. Each microbe inhabits its own ecological niche such as the hard and soft tissues surfaces within the human mouth. *Streptococcus* sp. is the major resident microbes in the oral cavity. Most oral streptococci are normal flora and non-periodonthopathogenic under normal condition (Ferrithi, 2016). Any changes in the oral cavity such as increase in glucose consumptions can cause the acidogenic bacteria to damage the teeth, resulting in dental caries. The most virulent species in *Streptococcus* genus are *Streptococcus mutans* and *Streptococcus sanguinis* and they are most frequently isolated from dental plaque. *S. mutans* has been found to be the initiator of most dental caries as it is able to metabolize different types of carbohydrates, creating an acidic condition in the dental plaque. Smokers have higher number of cariogenic *Streptococcus* sp. than non-smokers thus, increase the possibility of caries (Hutcherson *et al.*, 2015; Bagaitkar *et al.*, 2008).

### 1.3 *Candida* species in oral infection

*Candida* is a genus of yeast and in human, it has been isolated from three main parts of the body including the oral cavity, gastrointestinal and urogenital tracts. *Candida* sp. are found in 17-75% of both healthy and diseased people (Rindum *et al.,* 1994). In healthy condition, they are usually found as commensal flora (Samaranayake & MacFarlane, 1990). Commensalism is a symbiotic relationship where the symbiont gets benefits, but the hosts are harmless (Arendorf & Walker, 1980). However, any alteration in the ecological balance enables *Candida* to switch its characteristics to become pathogenic and leads to opportunistic diseases such as candidiasis (Samaranayake & MacFarlane, 1990). Oral candidiasis is well defined as thrush. It is a yeast infection of the oral cavity characterised by white lesions occurring on the tongue or any other parts of the mouth lined by oral mucosa. It is generally due to the overgrowth of *Candida*, predominantly by *C. albicans* (William & Lewis, 2011). Recently, *Candida* infections have received increasing attention, presumably due to the increased of its prevalence worldwide (Keten *et al.*, 2015; Pryszcz *et al.,* 2013). For years, *Candida albicans* is the predominant species of yeast and frequently isolated from both commensal state and oral candidiasis cases, based on the data reported in hospitals (Madhavan *et al.,* 2011). *Candida parapsilosis* is the second most frequently oral yeast after *C. albicans* (Pfaller *et al.*, 2004). It is commonly isolated from children and young adults with oral cancers (Gravina *et al.,* 2007; Safdar *et al.,* 2001).

## 1.4 Nicotine

Nicotine is a nitrogen-containing chemical known as alkaloid. It is derived from tobacco (*Nicotiana tabacum*) and is responsible for many of the effects of tobacco which is the main component of cigarette. Nicotine was named after a 16$^{th}$ Century French ambassador and was first synthesized in 1904 by A. Pictet and P. Crepieux. The French ambassador in Portugal, Jean Nicot de Villemain have transported the tobacco leaves and seeds to Paris from Brazil in 1560, claiming that tobacco has beneficial uses (Barlow & Johnson, 1989). However, some journals have reported that nicotine from cigarette smoke contributes to the development of dental plaque and increased the growth of oral microorganisms (Li *et al.*, 2014; Keten *et al.*, 2015; Huang *et al*., 2012).

## 1.5 Association of Nicotine and Oral Health

Up to date, there are many unsolved questions in relating the formation of oral biofilm towards nicotine among which include: how does nicotine induces biofilm formation in the oral cavity? How nicotine stimulates the development of biofilm? Does nicotine alter gene expression of oral microorganisms in smokers? The number of smokers and subsequently biofilm-associated diseases continues to rise globally.

In addition to cancers, heart disease, stroke, premature low-weight birth babies, reproductive effects in women and age-related macular degeneration, cigarette use is also a major risk factor of multiple bacterial and candidal infections in the oral cavity. Since smokers tend to have many oral problems, it is necessary to investigate how nicotine

influences the adhesion of oral microbes and how this compound alters the expression of genes that are responsible for attachment of microbes onto the tooth surface.

### 1.6 Hypothesis of the study

Nicotine enhances the formation of biofilm and affects the gene expression and regulation of *Streptococcus mutans, Streptococcus sanguinis, Candida albicans* and *Candida parapsilosis*.

## 1.7 Aim and Objectives of the Study

Although the relationship between oral microbes and dental plaque has been well established, no reports have focused on the effect of nicotine on the adherence-associated genes expressed by *Streptococcus* sp. and *Candida* sp. and the formation of dental plaque. Therefore, this research was focused on the influences of nicotine on the adherence of *Streptococcus mutans, Streptococcus sanguinis, Candida albicans* and *Candida parapsilosis* to saliva-coated surfaces and how this compound influences the expression of adherence-associated genes.

The above aim will be accomplished by fulfilling the following research objectives:

- To investigate the effect of nicotine on antimicrobial property of *C. albicans* and *C. parapsilosis, S. mutans* and *S. sanguinis*
- To study the influence of nicotine on the non-specific adherence of *S. mutans, S. sanguinis, C. albicans* and *C. parapsilosis*.
- To estimate the effect of nicotine on the structure of extracellular polysaccharides (EPS) and micro-colonies formation by *C. albicans, C. parapsilosis, S. mutans* and *S. sanguinis*.
- To determine the effect of nicotine on selected adherence-associated gene expression of *S. mutans, S. sanguinis, C. albicans* and *C. parapsilosis*.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 The oral cavity and its indigenous microbial inhabitants

In human anatomy, oral cavity is the most favourable environment for the ecological niches of microbial species, which include bacteria, yeasts, protozoa, and mycoplasma. Over the years, scientists have discovered up to 750 microbial species in the human oral cavity and their presence are often as commensals and at times, may be transient (Huang *et al.,* 2011; Kreth *et al.,* 2009). The major constituents of microorganisms in the oral cavity are *Candida, Streptococci, Staphylococci, Neisseria, Lactobacilli, Veillonella, Actinomyces* and *Fusobacteria* (Zollner & Jorge 2003; Kreth *et al.,* 2009).

The presence of hard tissues surfaces in the oral cavity provides an excellent option for microorganisms to adhere and multiply due to their physical nature and biological properties. In addition, other environmental factors such as nutrition, oral hygiene, smoking, dehydration and even stress play major roles in increasing the load of microbial species in the oral cavity (Vellappally *et al.,* 2007). Scientists have special interest to conduct more investigation on development of dental plaque by *Candida* sp. and *Streptococcus* sp. because of uncertain answer remained on the inner mechanisms of pathogenesis. Up to date, investigation on *Streptococcus sanguinis, Streptococcus mutans, Candida albicans,* and *Candida parapsilosis* are overwhelming as they are among the major commensals in the oral cavity (Himratul *et al.,* 2013; Lebeer *et al.,* 2007; Moelling *et al.,* 2007; Wen *et al.,* 2010).

## 2.2 Colonisation sites

Most of the microorganisms are identified to colonise several types of host cells including tooth surface, mucosa layer, tongue, epithelial, endothelial, and phagocytic cells. Different areas of the oral cavity present different ecological niches, and each species has specific properties for colonizing different oral sites. Both *Streptococcus* and *Candida* sp. are commonly found on the tooth surfaces, hard and soft palate regions, buccal and labial mucosa, dorsum or lateral borders of tongue, dental appliances, and denture-bearing areas (Cannon *et al.*, 1995). The production of specific enzymes such as agglutinin-like proteins and binding-like proteins intensified the ability of microbial species to form biofilm onto dental appliances and intraoral surfaces. In addition, some factors such as reduction in salivary flow, low salivary pH, high intake of food containing carbohydrates, oral hygiene, osmotic pressure, and epithelial loss may influence the colonisation of microbial species onto surfaces of the oral cavity (Siar *et al.*, 2003).

## 2.3 Growth requirements of oral microbes

### 2.3.1 Influence of oral fluids

Major and minor salivary glands are responsible to secrete saliva. The major salivary glands are composed of paired parotid, submandibular and sublingual glands; whereas, the minor salivary glands are found in the lower lip, tongue, palate, cheeks, and pharynx. The main function of whole saliva is to maintain the integrity of teeth by clearing off food debris and neutralize the acids produced by oral biofilm or dental plaque microbes. It also helps in lubricating the oral cavity by keeping them moist. Saliva also

promotes production of thin film of approximately 0.1 mm deep over all external surfaces in oral cavity. Few buffering components such as bicarbonate, phosphate and peptides are present in saliva to give a normal saliva mean pH of 6.75 to 7.25 (Marsh & Martin, 2009).

In addition to saliva, the gingival crevicular fluid (GCF) in the oral cavity can increase the colonization of *Streptococcus* and *Candida* sp. onto tooth surfaces. According to Gamonal and his collagenous (2000) study, the flow of GCF is slow and consistent at healthy sites but drastically increased up to 3-fold in gingivitis patients than in healthy volunteers (Gamonal *et al.*, 2000).

### 2.3.2 Influence of body temperature

The optimum growth temperature for *Streptococcus* sp. and *Candida* sp. including normal flora and pathogenic microorganisms is between 30ºC to 37ºC (Marsh & Martin, 2009). However, the temperature of 37ºC is chosen as standard incubation temperature for *Streptococcus* sp. and *Candida* sp. (except *Candida parapsilosis* at 35ºC). Any changes in the normal body temperature will influence the survival rate of normal microbial flora and may increase the chance to develop into opportunistic microorganisms.

## 2.4 Microbial growth

### 2.4.1 Growth curves

In ecological studies, the growth of microorganisms including bacteria and yeasts in batch culture can be modelled with four different phases: lag phase represents the

earliest and most poorly understood stage of the bacterial growth cycle, log phase where cell division proceeds at a constant rate, stationary phase when conditions become unfavourable for growth and microbe stop multiplying, and death phase when cells lose theirs vitality, leading to prolonged stationary phase (Rofle *et al.*, 2012).

### 2.4.2 Measuring microbial growth

There are different methods of measuring microbial growth which involve direct methods and indirect methods of measurement. These are based on different parameters of cells such as dry- and wet-weight measurement, plating method, density, turbidity, metabolic activity measurement, absorbance value, and use of Coulter counter. In our study, we opted for indirect method of absorbance reading using spectrophotometer. A high value of absorbance represents increased number of cells. When the light was passed through the microbial cell suspension, the cells scattered light. Scattering of light increased with higher cell number, causes the transmission of light to reduce. At that point of wavelength, cell concentration of microorganism present in the suspension was measured. Thus, cell growth of cell suspension at a wavelength at different interval can be measured in terms of absorbance and prepare a standard graph (absorbance value against cell concentration) was plotted.

## 2.5 Oral microorganisms

### 2.5.1 Normal microorganisms

Large numbers of bacterial and candidal species that live as commensal and coaggregate in harmony with the host are called indigenous flora. In homeostatic state, this normal flora plays an important role in protecting the host from invasions by pathogens. However, under favourable condition, it can cause diseases, such as dental caries, periodontal disease, oral candidiasis, and gingivitis (Arendorf & Walker, 1980). The human mucosal surface and teeth are the primary locations of inhabitance. Commensal microorganisms will break down the carbohydrate available in saliva via fermentation with the help of enzymes to yield carbon dioxide and alcohol for their survival. Yet, any alteration in the oral cavity environment can change the harmless commensal microbial species to a pathogenic state (Samaranayake, 2009). The causes of such changes are categorized as predisposing factor for bacterial infection (dental caries) and candida infection (oral candidiasis).

### 2.5.2 Opportunistic microorganisms

In recent years, several microbial species such as *Streptococcus* sp. and *Candida* sp. have developed as pathogens. At least 17 *Candida* sp. (Rinaldi, 1993) and 40 *Streptococcus* sp. (Ferrithi, 2016) have been found to develop diseases in human. Poor oral hygiene and consumption of tobacco are the main causative agents of oral infections including dental plaque, and caries due to overgrowth of microorganisms. A commensal microorganism can be pathogenic when there is a slight change in oral cavity environment.

Thus, any alteration in local and systemic changes within the oral environment promotes the proliferation and colonisation of individual's own microbial species which can leads to diseases.

## 2.6 The genus *Streptococcus*

### 2.6.1 Biology and taxonomy

The first description of infection caused by streptococcal genus was introduced by Austrian surgeon, Theodor Billroth in 1874 (Bridge & Sneath, 1983). He discovered the microorganisms in erysipelas and wound infections. In 1879, the importance of the streptococcal infection was revealed in history when Louis Pasteur isolated the microorganisms from the uterus and blood of women with puerperal fever. He also demonstrated that *Streptococcus* sp. was one of the causative agents for the disease that caused highest mortality rates in women and newborns. In 1884, Friedrich Julius Rosenbach examined the bacteria from suppurative lesions and named the species as *Streptococcus pyogens* after the symptoms of pus. However, in 1920s, George and Gladys Dick discovered *Streptococcus* sp. from scarlet fever with a sore throat (Ferrethi, 2016; Whiley & Beighton, 1998).

The human mouth is usually sterile at birth but soon colonized by predominantly streptococcal microbiota. According to the literature, oral streptococci are divided into five different families such as Mutans group (commonly are *Streptococcus mutans* and *Streptococcus sobrinus*), Salivarius group (*Streptococcus salivarius*), Anginosus group

(*S. intermedius* and *S. anginosus*), Sanguinis group (*S. sanguinis* and *S. gordonii*) and Mitis group (*S. oralis* and *S. mitis*). Most of them are normal flora and non-periodonthopathogenic bacteria in normal condition (Caufield *et al.,* 2000; Kreth *et al.,* 2009; Krzyściak *et al.,* 2014). Oral *Streptococci* species are known as early colonizers which play a major role in the initial colonisation of acquired pellicle to form dental plaque. *Streptococcus salivarius* was isolated from 75% infants aged 1 to 15 years, 18 hours after birth. The species *S. salivarius* was dominated on the tongue and oral mucosa. In many cases, *S. sanguinis* and *S. mutans* were discovered in oral cavity. Among the species, *S. mutans* comprise the most prevalent microorganisms in approximately 39% cases implicated with dental caries. It has been reported that the colonisation of *S. mutans* in dental plaque and saliva from 39 children aged 2 to 4 years from the suburbs of Helsinki revealed the close relationship with dental caries (Ferrethi, 2016; Hamada & Slade, 1980).

### 2.6.2 General morphology

*Streptococci* are gram positive cocci which looks spherical or ovoid. Sometimes species occur in pairs and chains. The Greek name of "Strepto" defined as chain and coccus. In 1874, an Austrian surgeon, Theodor Billroth found that *Streptococci* appear in single cocci (berry), in pairs or sometimes in chains (Ferrethi, 2016; Caufield *et al.,* 2000).

### 2.6.3 Structure and functions of cell wall

*Streptococcus* species is gram-positive bacteria and has a thick cell wall which consists of 90% polysaccharides and 10% proteins (Ferrethi, 2016). The cell wall is

composed of peptidoglycan (murein) and teichoic acids that prevent osmotic lysis of cell protoplast and maintain its rigidity and shape of cell. Several strains of *Streptococcus* sp. have capsule surrounding the cell that is composed of polysaccharides (Leme *et al.*, 2006).

One of the virulent factors of *Streptococcus* species is the ability to attach on tooth surfaces and form oral biofilm resulting in dental plaque. Exopolymers that attached in the polysaccharide matrix form its own stability and allow an adhesion site for bacterial cells, promote cohesion and also act as a source of energy (Krzyściak *et al.*, 2014). The primary sources of extracellular polysaccharides (EPS) are glucosyltransferase (gtf) and fructosyltransferase (ftf). Bacterial surface-exposed adhesions also classified as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are responsible to initiate the colonization on the tooth surface. *Streptococcus* sp. adheres specifically to the pellicle of the tooth by means of a protein on the cell surface. The cell grows and synthesizes dextran capsule which binds them to the enamel and forms biofilm on the surfaces (Stanley *et al.*, 2004; Huang *et al.*, 2011).

Studies have provided evidence that defects in genes may contribute to biofilm formation and dental caries (Wen *et al.*, 2010; Yoshida & Kuramitsu, 2002). The colonisation of opportunistic *Streptococcus* sp. to the tooth enamel is controlled by the gene known as multi-functional adhesion (spaP), a surface-associated protein (Krzyściak *et al.*, 2014). *S. mutans* metabolised sucrose and starch into lactic acid and α1,3- and α1,6-linked glucans by the enzyme known as glucosyltransferases (gtfs). Glucans are bind by

glucan binding proteins (gbp) and facilitate bacterial attachment to the tooth surface which then contribute to the development of dental plaque and dental caries (Wen *et al.*, 2010; Krzyściak *et al.*, 2014).

### 2.6.4 *Streptococcus* species

#### 2.6.4.1 *Streptococcus sanguinis*

Approximately 20% of the oral bacteria are streptococci. The most common streptococci found in the oral cavity are *Streptococcus mutans* and *Streptococcus sanguinis*. While *S. sanguinis* previously named as *S. sanguis* appears as gram-positive and non-spore forming cocci in healthy mouth when viewed under microscope (Caufield *et al.*, 2000). *S. sanguinis* is recognized not only for its historical association with life-threatening bacterial endocarditis, but also because of its putative antagonistic role in dental caries and periodontal diseases (Ferrethi, 2016; Leme *et al.*, 2006).

Carlsson and co-workers were among the first to describe both the taxonomic and ecological features of *S. sanguinis* in the oral cavity. In fact, it was the Carlsson group who made the key observation that *S. sanguinis* did not colonize infants until after the emergence of teeth and that colonization by *S. sanguinis* precedes by mutans streptococci (Carlsson *et al.*, 1970).

#### 2.6.4.2 *Streptococcus mutans*

*Streptococcus mutans* is a facultative anaerobic, gram-positive coccus (round bacterium) commonly found in the human oral cavity and is a significant contributor to tooth decay. In 1924, J Kilian Clarke described the morphology of the species. *S. mutans* is most prevalent on the pits and fissures, constituting 39% of the total streptococci in the oral cavity. Approximately 2-9% of *S. mutans* bacteria are found on the buccal surface (Hamada & Slade, 1980; Ferrithi, 2016).

This bacterium, along with the closely related species *Streptococcus sobrinus*, can inhabit in the mouth and contribute to oral diseases, and the expense of differentiating them in laboratory testing is often not clinically necessary. Therefore, for clinical purposes they are often considered together as a group, called the mutans streptococci. This grouping of similar bacteria with similar tropism can also be seen in the viridans streptococci, another group of *Streptococcus* species (Hamada & Slade, 1980).

*S. mutans* is responsible to metabolize different kinds of carbohydrates, creating an acidic condition in the mouth which results in tooth decay. It is very important to study the pathogenicity of *S. mutans* since it is cariogenic.

## 2.7. The genus *Candida*

### 2.7.1 Biology and taxonomy

Literature on the genus *Candida* have been well documented in many original and reviews, covering all aspects of genomic properties, pathogenesis and factors that contribute to the infections (William & Lewis, 2011; Liu & Filler, 2011; Van Wyk *et al.*, 2011). These yeast-like microorganisms have attracted the interest of scientists for years since they were first discovered in 1844 from the sputum of a tuberculosis patient and later were identified in denture-related infections (Bennett *et al.*, 1844). Clearly, scientist has showed deep interested in candidal infections studies due to the outbreak of human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) pandemics in 1980s (Odds, 1988). However, concerning the presence of *Candida* sp. in newborn oral cavity, a study was conducted by Zollner and Jorge (2003) to investigate the distribution of *Candida* sp. among infants. In the study, they found that the colonization of *Candida* sp. occurred during breastfeeding and the microorganisms became permanent resident in oral cavity.

*Candida* sp. is classified under the family of Cryptococcaceae. The family of Cryptococcaceae includes the genera Torupsilosis and Cryptococcus. Members of this genus are ubiquitously disseminated as saprophytes in soil, aquatic environments and colonising several animal reservoirs (Brandão *et al.*, 2010). The *Candida* genus is comprised of about 163 species of yeast-like fungi. Most of the *Candida* sp. are unable to grow at 37ºC, therefore, they are not normally related with human colonisation. However,

*Candida* species such as *C. albicans, C. troplicalis, C. krusei, C. lusitaniae, C. dubliniensis, C. kefyr, C. guilliermondil, C. parapsilosis* and *C. lipolytica* are isolated in oral cavity as commensal microorganisms and these can act as opportunistic pathogens in low immunity system (William & Lewis, 2011). Among all *candida* species, *Candida albicans* is the most frequently isolated species in about 20-60% from the oral cavities that acts as normal flora and also an opportunistic fungal pathogen under favourable condition which causes approximately 50% of candidemia cases and 80% of cases of oropharyngeal diseases (Liu & Filler, 2011). On the other hand, the second commonest yeast isolated in the oral cavity is *C. parapsilosis*, a species that is usually found on human hands (Zollner & Jorge, 2003). In general, some pathogenic and non-pathogenic *Candida* sp. are classified according to the morphological stages of asexual and sexual.

### 2.7.2 General morphology

*Candida* sp. may exist in three morphological forms which are blastospores (yeast-like ovoid cells), filamentous hyphae and chlamydiospores (dormant phases of microorganisms). Chlamydiospores is a thick-walled spherical wall with approximately 10-12 µm in diameter which is attached to the pseudohyphae by elongated, suspensor cells. However, different *Candida* sp. has various shapes and sizes of blastospores. In many cases, *Candida* sp. are found in two basic morphological forms which are the yeast and pseudohyphae. Pseudohyphae is essential in developing biofilm on the surfaces of the host cell. Both morphological forms looked similar thus making it difficult to differentiate between these two basic characteristics (Richardson & Warnock, 2003)

### 2.7.3 Structures and functions of cell wall

The cell envelope of microorganism that is responsible for initial colonisation basically consists of a cell wall or one or two lipid membranes (Beveridge & Graham, 1991. The cell wall of microorganism differs from one organism to another and exhibits a small change due to physiological adaption to the environment contribute to the pathogenesis of microbial species. Cell wall also acts as connection point between the microbe and host surface.

Few articles have suggested that cell wall of *Candida* species is similar to the cell wall of *Saccharomyces cerevisiae* (Ballou, 1982), however it is not found in animal cells. Therefore, it is a prime target for any studies related to *Candida* species. The cell wall of yeast may account for up to 30% of the dry weight of the cell (Orlean, 1997) and it is mainly composed of 90% exopolysaccharides and 10% protein. Antigens and pathogens-associated molecular patterns (PAMPs) contribute the conversion of *Candida* sp. from commensal to pathogenic yeast. These PAMPs form two layers; outer layer and inner layer. The inner layer consists of polysaccharides that promote immunological mechanisms in *Candida* sp. The outer surface of candida cell wall is composed of three basic layers; representing the major polysaccharides of the cell wall: (i) polymers of glucose containing β-1,3 and β-1,6 linkages (β-glucans), (ii) polymers of N-acetyl-D-glucosamine (GlcNAc) consisting of β-1,4 bonds (chitin) and (iii) glycoproteins that formed by covalent links between O and N-linked mannose polymers (mannans) and proteins (Fleet, 1991; Valentin *et al.*, 2000).

Several authors have reported the essential of mannoprotein components were observed in the cell wall of *Candida* sp. particularly *C. albicans* (Chaffin et al., 1998; Ruiz-Herrera *et al.,* 2006). The mannoproteins which appear as a dense network of fibrils were synthesized in the cytoplasm and transported through the secretory pathway. The proteins were attracted to different kinds of receptors either by being loosely attached to the matrix wall or covalently bound to mannan via phosphatidyl bonds during candida pathogenesis. This mechanism explained that cell wall of candida does not only protect the protoplast against osmotic pressure but provide antigenic structure. The structure is composed of many enzymically proteins, have hydrophobic properties, and adhere specifically to host cells and tissues, as well as dental appliances (Casanova & Chaffin, 1991). It is reported that connection between the extracellular materials also promotes the formation of biofilms. Therefore, the cell wall is considered as one of the important determinants in the pathogenicity of *Candida* species (Cassone, 1989).

### 2.7.4 *Candida* species

#### 2.7.4.1 *Candida albicans*

*Candida albicans* was first introduced by Berkhout in 1923 (Kwon-Chung & Bennett, 1992). *C. albicans* has been investigated widely than non-*Candida albicans* sp. because of its predominant medical importance and seriousness of infection. It is generally considered as the most pathogenic member of the genus *Candida* (Roy & Meyer, 1998) and is frequently isolated in 60-80% of nosocomial candidal infections cases from the oral cavities of both healthy and diseased patients (Rautemaa *et al.,* 2006).

*Candida* can grow as yeast cells, pseudohyphae and hyphae, and produces chlamydospores which is round and spore-like structure that are produced at the end of hyphae under specific environment. Characteristics of *C. albicans* on agar are commonly used to differentiate *C. albicans* from non-*Candida albicans* sp. Isolation of *C. albicans* produces fluorescence in Sabouraud methylene blue and gives green smooth colonies on CHROMagar. *C. albicans* usually gives smooth colonies of a whitish-cream colour on Sabouraud agar and tobacco agar (Silveira-Gomes *et al.*, 2011).

There are several known virulent factors that contribute to its pathogenicity such as phenotypic switching and adhesion to the host cells. *C. albicans* can switch its morphology from the single budding cells (blastosphores) to a filamentaous growth form (hyphae) which is called dimorphic transition. The hyphae form is crucial for attachment to host tissues (Calderone & Fonzi, 2001; Cutler, 1991). The morphological transitions occur due to the reaction of the yeast cells to the environmental condition and allow the cells to adapt to different biological niches.

#### 2.7.4.2 *Candida parapsilosis*

*Candida parapsilosis* is the second most frequently isolated yeast from blood culture in Europe, and Canada. This species also ranked third in the United States (Pfaller *et al.*, 1999). On the other hand, *C. parapsilosis* is also the most frequent candidal species isolated from oral candidiasis in children and young adults with oral cancers (Gravina *et al.*, 2007). The discovery of *C. parapsilosis* in the hospital environment is unique where

most of them are found on the hands of health care workers. This explains why the catheters and other medical devices are contaminated with *C. parapsilosis* (Hedderwick *et al.,* 2000). *C. parapsilosis* has been reported to produce an extracellular polysaccharide *in vitro* which contributes to adherence and biofilm formation on artificial surfaces (Levin *et al.,* 1998). In addition, *C. parapsilosis* exhibits different morphology and observed as pseudohyphal and single budding cells.

### 2.8 Adherence as a pathogenic determinant of oral microorganism

Some factors such as the ability to adhere to host cells (Ellepola & Samaranayake, 1998) and dental devices, hydrophobicity properties and expression of virulence genes contribute to the pathogenicity of microbial species.

#### 2.8.1 Adherence mechanisms

The importance of cell adherence in the physiological growth of microbial species has been well documented. Adherence is a key attribute of virulence among microbial species for successful colonization and invasion on host cells, particularly in oral cavity. Adherence can occur either on the hard surfaces or smooth surfaces with the help of oral fluids (Machado *et al.,* 2011; Drumm *et al.,* 1989). Generally, there are two mechanisms in facilitating the adhesion of microorganisms on the tooth surface. Interaction between acquired pellicle and the host cell surface is called specific-binding adherence whereas the mechanisms involving physiological forces are known as non-specific which involves cell surface hydrophobicity.

#### 2.8.1.1 Specific adhesion

Many articles demonstrated that microbial cell wall is covered with variety of ligands on the host cell surface such as protein-protein, protein-carbohydrates and mannoprotein ligands. For example, when a denture is fixed in the oral cavity, surfaces of the denture will be instantaneously coated with saliva. The specific receptors on the bacteria cells were attached on the acquired pellicle. Hence, promotes the attachment of the free-floating cells to the denture surface.

Adhesion to biomaterial may cause infection in the oral cavity and deterioration of the devices. The seriousness of the infection is depended on the microporosity present on the surface of the denture (Powers & Sakaguchi, 2006). The irregular surfaces of dentures enabled the microorganisms to accumulate and aggregate with each other to develop biofilms. This mechanism has been proven that the pathogenicity of microbial sp. is correlated with the adherence.

Besides that, adhering properties such as virulence genes like surface associated protein (*spaP*), glucosyltransferases (*gtf*) and glucan binding protein (*gbp*), hyphal wall protein 1 (*Hwp1*), and agglutinin-like sequence 3 (*Als3*) have been shown to play a crucial role in the ability of the bacteria and yeast to adhere to human buccal epithelial cells and other substrates (Krzyściak *et al.,* 2014; Wachtler *et al.,* 2011; Zhu & Filler, 2011)

#### 2.8.1.2 Non-specific adhesion

Hydrophobic interactions assist microorganisms to adhere on different types of surfaces including dental biomaterials, mammalian cells and insoluble hydrophobic substrates (Hazen & Hazen, 1992; Cannon & Chaffin, 1999). The attachment of microbial cells to surfaces depends on a few factors including van der Waals attraction, surface electrostatic charges, and gravitational forces. One of the important factors is the hydrophobicity of the cells which can enhance the propensity of microorganisms to adhere on abiotic and biotic surface and increase the tendency to form biofilm such as on the inert surface of medical implants. Another important tenet is that microorganisms can switch between hydrophobic and hydrophilic phenotypes in response to changes in environmental conditions including temperature, presence of saliva, composition of nutrients, and growth phases (Borecká-Melkusová & Bujdaková, 2008; Bujdakova *et al.*, 2013). In terms of structure, hydrophobic proteins are usually smaller than 50 kDa while hydrophilic proteins are predominantly larger than 90 kDa. Freeze-fracture was used to examine the differences between hydrophobic and hydrophilic external cell surface layers. Thus, hydrophilic exhibits a dense layer of fibrils, composed mostly of high-molecular-weight mannoproteins that is absent or scant in hydrophobic cells (Hazen & Hazen, 1992). Jabra-Rizk et al. (1999) proposed that each microbial species have different cell surface variation. The microorganisms can adapt to the presence of hydrocarbon by modifying their cell surface composition to either promote or hinder adhesion (Chakarborty *et al.*, 2010). The more hydrophobic cells adhere stronger to hydrophobic surfaces, while hydrophilic cells strongly adhere to hydrophilic surfaces (Giaouris *et al.*, 2009). High CSH enables microorganisms to attach to hydrocarbon

droplets on the surface or cells. Therefore, the hydrophobicity of cells can increase the ability of microorganisms to adhesion.

### 2.8.2 Adherence-associated genes

Most pathogens such as *Streptococcus* sp. and *Candida* sp. have their own virulence traits to overcome the host defenses and invade mucosal tissues in the oral cavity. Although many factors have been listed to be virulence attributes for oral microorganisms, specifically of *Streptococcus sanguinis, Streptococcus mutans, Candida albicans* and *Candida parapsilosis*, the binding-associated genes have widely been studied to understand the mechanisms of the oral microorganisms or the adherence to oral surfaces. Bacteria and fungi are often considered as unicellular organisms, but research has recently shown that many bacteria and yeast possess ability to communicate with one another and appear in groups. They produce extracellular molecular signal to monitor their own population density and to coordinate the expression of specific sets of genes in response to adherences and cell density (Atkinson & Williams, 2009).

#### 2.8.2.1 Surface-associated protein P (*spaP*)

The multi-functional adhesion *spaP* also known as PAc1 or P1 is necessary to produce antigen I/II (Ag I/II) during the initial adherence of *S. mutans* to the tooth (Pieralisi *et al.*, 2013; Krzyściak *et al.*, 2004). The protein family of Ag I/II, represented by *SpaP, SspA,* or *SspB,* is identified not only on the surface of *S. mutans*, but also on other *Streptococci,* such as *Streptococcus pyogenes*, *Streptococcus agalactiae*,

*Streptococcus sanguinis* or *Streptococcus suis*. Genetic sequences encoding Ag I/II comprise six distinct regions. The most important region of this protein is A region which is rich in alanine and P region which is rich in proline. Region V is located between them and it is composed in many different sequences found in individual strains. The A and V regions encode adhesive epitopes appearing on the surface of bacterial cells (so-called adhesive types) responsible for their affinity to salivary glycoproteins (Krzyściak *et al.*, 2004).

The *spaP* protein and other proteins of the Ag I/II family specifically interact with glycoprotein-340 (gp-340) found in saliva. It is interesting that gp-340 when dissolved in the liquid phase of saliva plays a role in the aggregation of bacterial cells and help purify the oral cavity. However, if gp-340 is adsorbed on the surface of teeth or gums, it acts as a receptor for surface bacterial adhesins that initiates the adhesion process. The Ag I/II protein family is also involved in interaction between microorganisms including *Streptococcus gordonii* and *Porphyromonas gingivalis*, and in the aggregation of cells in the absence of gp-340 (Krzyściak *et al.*, 2004). The presence of *spaP* gene in bacterial strains thus, favours the development of dental caries.



#### 2.8.2.2 Glucosyltransferases (*gtf*)

The sucrose-dependent mechanism of plaque formation is dependent on glucosyltransferases (gtfB, -C, and-D) produced by *S. mutans* in combination with glucan-binding proteins (gbps). Glucosyltransferases play critical roles in dental plaque

development and are responsible for glucans formation from sucrose (Krzyściak *et al.*, 2004). The synthesized glucans provide the possibility of both bacterial adhesion to the tooth enamel and cell-to-cell attachment. Gtfs are expressed in three different transcripts; *gtfB, gtfC* and *gtfD*. Each of the three types of gtf plays a different, though similar role in biofilm formation and, therefore, the loss or mutation of one of them impairs the whole process. *GtfB* (known as *gtfI*), a glucosyltransferase, is primarily responsible for the interaction with other *S. mutans* which mainly synthesize insoluble glucan rich in α-1,3-linkages (Donlan, 2002; Krzyściak *et al.*, 2004. It is responsible for the formation of highly differentiated microcolonies forming the structure of biofilm. Its activity significantly increases when there is a glucose in the environment, which is not a typical situation and is observed extremely rarely. Glucosyltransferases also interact with salivary amylase to block the activity and prevent the lysozyme absorption. This can decrease the activity of GtfB, but does not affect the characteristics of glucans formed by the peroxidase (Donlan, 2002).

#### 2.8.2.3 Glucan binding protein (*gbp*)

Another component of the sucrose-dependent mechanism is the non-gtf glucan binding protein (gbps) that mediates the binding of bacteria to glucans. Four types of this protein are known: gbpA, -B, -C, and -D. The gbps protein (and probably *gbpB*) is associated with the bacterial cell wall and, therefore, acts as a specific receptor for glucan. All four types of proteins play a role in microorganism adhesion and biofilm formation. The absence or mutation of the gene encoding *gbpB* results in a change of cell

shape and a slowing down of its growth. This disables the appropriate development of biofilm, which, instead of a diverse, dense formation, becomes a product of non-regular cell clusters surrounded by a matrix of unusual structure. The expression patterns of *gbpB* has been associated with *Streptococcus* sp. virulence (Donlan, 2002; Krzyściak *et al.*, 2004).

#### 2.8.2.4 Hyphal-wall protein 1 (*HWP1*)

Cell wall proteins have been associated with hydrophobic attachment of *Candida* to oral mucosa. The adherence and production of biofilm by *Candida* sp. is influence by surface hydrophobicity of its cell wall (CSH) which is characterized by hydrophobic proteins that are involved in cell-host interaction. Specific adhesion between *Candida* and receptors on host tissues which results in a stronger covalent attachment to epithelial cells is linked to enhance expression of several virulence genes; *HWP1*, hyphal wall protein (Snide & Sundstrom, 2006; Sundstrom *et al.*, 2002) and *ALS*, agglutinin-like sequence (Hoyer & Cota, 2016; Roudbarmohammadi *et al.*, 2016; Phan *et al.*, 2007).

Hyphal cell wall-specific protein 1 (*HWP1*) is one of the well-studied adhesion that is present abundantly in *Candida* sp. It has been reported that the expression of *HWP1* was high during candidal hyphae forms than in yeast forms (Snide & Sundstrom, 2006; Nantel *et al.*, 2002; Sundstrom, 2002). However, there is no evident to support the absence of *HWP1* gene in yeast cells. *HWP1* has unique adherence characteristic where it forms covalent bonds to proteins on human buccal epithelial cells in host tissue. It mainly

involves in adhesions of *Candida* species to tooth surfaces. Therefore, the presence of adhesin is important for the pathogenesis of candidiasis.

Recently, it has been reported that, the expression of *HWP1* was responsible for adhesion production in cell wall, promoting the attachment of *C. albicans* and *C. parapsilosis* to the host mucosal surface and oral epithelial cells (Himratul *et al.*, 2013). In addition, *HWP1* has a critical role in biofilm formation. The high expression of *HWP1* in oral cavity from carriers strongly implicates the role of *HWP1* and hyphae in establishing and maintaining the presence of *C. albicans* on mucosal surfaces (Snide & Sundstrom, 2006). Hence, *HWP1* and hyphal growth are the important factors in invasive interactions of *C. albicans* to human hosts.

#### 2.8.2.5 Agglutinin-like sequence 3 (*ALS3*)

Other than *HWP1*, Liu & Filler (2011) suggested that *ALS3* played a key role in colonization and causative agent in dental plaque by multiple mechanisms including biofilm formation and iron acquisition. *Candida* encoded with ALS gene family mainly *ALS3*, *ALS1* and *ALS5*. This group of genes help *Candida* sp. to adhere to the host epithelial cells, endothelial cells and on tooth surfaces. *ALS3* could also invade into these cells and damage the cells resulting in dental caries.

*ALS3* is a member of the agglutinin-like sequence (ALS) family of protein that is essential for the invasion of *Candida* into host tissues. The main function of *ALS3* is to invade the oral epithelial cells and endothelial cells by inducing their own endocytosis. It binds to host cell receptors such as E-cadherin and N-cadherin on oral epithelial cells to induce host cell to endocytose the organism (Hoyer & Cota, 2016; Roudbarmohammadi *et al.,* 2016; Phan *et al.,* 2007). Besides that, *ALS3* is also responsible for mediating attachment of candidal cells to epithelial cells, endothelial cells and the formation of extracellular matrix proteins (Phan *et al.,* 207). In addition, it is also required in biofilm formation and as a sole iron source from ferritin by hyphal cells (Almeida *et al.,* 2008).

## 2.9 Oral diseases-related to microorganisms

### 2.9.1 Dental caries

Dental caries is also referred as tooth decay or cavities and is one of the most prevalent chronic diseases worldwide (Malaysia Institute for Public Health, 2015). Dental caries forms through a complex interaction over time between acid-producing bacteria such as *S. mutans.* They ferment carbohydrates and produce acid that destroys the tooth's enamel and dentin. In general, biofilms develop after initial attachment of microbes to a surface, followed by formation of highly structured cell clusters in a complex extracellular matrix. Majority of biofilm matrices contain up to 40% of exopolysaccharides which are mostly glucans synthesized by gtfs. *S. mutans* plays a major role in the development of EPS matrix in dental biofilms. The gtfs secreted by *S. mutans* bind avidly to the pellicle formed on the tooth surface and to bacterial surfaces. It provides binding sites for colonization and accumulation of S. *mutans* on the apatite surface and for binding to each

other though interactions with membrane-associated glucan-binding proteins. The gtf promotes tight adherence and coherence of bacterial cells bound to each other and to the tooth surface, which results to the formation of microcolonies by *S. mutans* and hence modulates the initial steps of cariogenic biofilm development. Nutritional transition with easy access to refined carbohydrates, low use of fluoride toothpaste and irregular tooth brushing habits lead to increasing trend in dental caries in developing countries.

### 2.9.2 Dental plaque

In many cases, oral microorganisms also produce extracellular polysaccharides. Extracellular polysaccharide is a network of non-living mass which provides support to cells to attach on the surfaces. This anchorage properties help the microbial species to colonize on hard surfaces and thus, lead to formation of plaque.

The main components of the biofilm formed on the surface of teeth include glucan (10–20% of dry weight), fructan (1–2% of dry weight) and proteins (40% dry matter). Biofilms formed in the oral cavity were three-dimensional structures, consisting of bacterial strains anchored to solid surfaces such as tooth enamel, tooth roots or dental implants. The formation of biofilm happens when the microorganisms irreversibly attach and grow on a surface. They produce extracellular polymers equivalent to 50 % to 90 % of total organic carbon of biofilm that encourage attachment and matrix formation of microbes, resulting in phenotype changes, adjustment in growth curve and gene transcription alteration (Huang *et al.*, 2011).

The three main stages that influences the oral biofilm formation are conditional film or acquired pellicle formation on the tooth enamel, cell-cell interaction between microbial and attachment of cells onto tooth surfaces (Wen *et al.,* 2010; Yoshida & Kuramitsu, 2002). They are embedded in an extracellular polysaccharide (EPS). The structure and composition of the extracellular polysaccharide is determined by the conditions existing in the oral cavity. The presence of EPS also affects the physical and biochemical properties of the biofilm. According to Liu & Filler (2011), oral microbes have ability to colonize the host tissues and cause disease by multiple processes which are adherence to host cells, biofilm formation, invasion into host cells and obtaining iron from host cells.

### 2.9.3 Oral candidiasis

A common way for infectious diseases to spread is through adhesion, growth, and invasion of the host immune system. The ability of *C. albicans* to infect host is supported by a wide range of virulence factors including the morphological transition between yeast cell and hyphal form, phenotypic switching, the expression of adhesins and invasins on the cell surface, biofilm formation and secretion of hydrolytic enzyme. It has been proposed that the yeast cells switch into hyphal forms under certain factors and both growth forms are important for pathogenicity (Nantel *et al.,* 2002; Sudbery, 2011). Hyphal forms *C. albicans* has been shown to be more invasive than the yeast form by penetrating the host cells mucosal and lead to oral candidiasis. Oral candidiasis, also known as thrush is a common opportunistic infection caused by an overgrowth of the genus *Candida*,

predominantly by *C. albicans* (Rautemaa & Ramage, 2011; Gravina *et al.*, 2007). Three main clinical appearances of candidiasis are generally recognized as pseudomembranous, erythematous (atrophic) and hyperplastic. They appeared in creamy white, curd-like pigments or patches on the tongue, buccal mucosa, periodontal tissues, and other intraoral regions.

## 2.10 Risk factors

The most common cause of oral infections is pathogenic conditions of oral microorganisms and impaired local defence mechanisms. This destruction can occur due to several risk factors such as decrease in saliva production, smoking, antibiotics treatment and poor oral hygiene (Welbury *et al.*, 2003; Benítez-páez *et al.*, 2014).

### 2.10.1 Poor oral hygiene

Oral health is important because untreated dental disease and poor gingival management may cause oral infection which is a significant risk factor for oral infection such as dental caries, oral candidiasis, periodontal disease, and oral cancer (Welbury *et al.*, 2003; Milgrom *et al.*, 2000).

### 2.10.2 Smoking

Cigarette smoke (CS) has been found to have a strong impact on oral microflora leading to microbiome deregulation, which may promote the growth of pathogenic microorganisms. It was suggested that CS provides an appropriate environment for

microbial species to grow and colonize the host. In recent years, many evidences showed smoking affects caries (Haber *et al.,* 1993; Benítez-páez *et al.,* 2014; Li *et al.,* 2014) and some journals are focusing on the effect of passive smoking and seriousness to the infants and young children (Shenkin *et al.,* 2004; Avsar *et al.,* 2008; Esa *et al.,* 2014). According to the study, salivary *S. mutans* and *lactobacilli* colonisation were significantly higher in passive smoking children than in control groups. Hence, it was concluded that passive smoking children were at increased risk to dental caries than unexposed children (Avsar *et al.,* 2008).

**2.11 Nicotine**

Nicotine enhanced the formation of biofilm on the host cell. Additionally, *in vitro* studies investigated the risk of smoking on caries and also the effect of nicotine on biofilm production and metabolic activity of *S. mutans* and *S. sanguinis* (Li *et al.,* 2014). Nicotine was found to alter certain cellular functions of oral microorganisms including cell growth, attachment, and matrix protein synthesis (Albandar *et al.,* 2000; Wu *et al.,* 2016; Cunningham *et al.,* 2016; Bouchard *et al.,* 2016). Nicotine has also been reported to be associated with the development of biofilm formation in the oral cavity and influence cell metabolism of other oral microorganisms. However, the mechanisms of how nicotine increases the metabolic activities of microorganisms in oral cavity remain unanswered (Huang *et al.,* 2012).

It has been reported that *Candida* species is more prevalent in tobacco smokers than in non-smokers (Arendorf & Walker, 1980). Recent study on nicotine have supported

that nicotine provide a favourable environment for *Candida* species to colonise the tooth surfaces (Keten *et al.,* 2015). Besides that, cigarette smoke is also able to suppress the activity of immune system which could directly results in *C. albicans* overgrowth and development of biofilm (Soysa & Ellepola, 2005). Studies have demonstrated that cigarette smoke could speed up the production of histolytic enzyme by *C. albicans* that contributed to tissue degradation and invasion to host tissues and increased the expression of genes associated with adherence of *C. albicans* (Semlali *et al.,* 2014; Johnson & Guthmiller, 2007).

### 2.11.1 The main components of cigarette

Cigarette smoke contains more than 4000 chemicals including chemical toxicants where 50 of them are very cariogenic. Approximately 6-30% of cigarettes components are composed of nicotine, an alkaloid in cigarette smoke. A stick of cigarettes contains approximately 9 to 30 mg of nicotine, however about 0.5 to 2 mg of nicotine per cigarette are absorbed by our body through respiration (Gellner *et al.,* 2016; Guerin, 1979). Although the amount is low, it is adequate to cause many serious illnesses, such as cancer, heart problems, and abortion as well as other health problems.

### 2.11.2 Structure of nicotine

Nicotine is the main addictive agent and a potent parasympathomimetic alkaloid found abundantly in cigarette smoke (Figure 2.1). Nicotine is also represented as 3-(1-methyl-2-pyrrolidinyl) pyridine according to the IUPAC nomenclature. It is a bicyclic

compound with a pyridine cycle and a pyrrolidine cycle (Guerin, 1979). The molecule possesses an asymmetric carbon and so exists in two enantiomeric compounds. In nature, nicotine only exists in the S shape, which is levogyre. The properties of nicotine are listed in Table 2.1 (Barlow & Johnson, 1989).

### 2.11.3 The uses of nicotine

Nicotine is the primary ingredient in tobacco products. Despite its disadvantages, nicotine has few advantages where it is used to reduce withdrawal symptoms associated with smoking cessation thus helping resist the urge to smoke cigarettes. It is an aid to smoking cessation, sometimes known as nicotine replacement therapy (NRT). Nicotine gum and lozenges, intranasal nicotine spray and nicotine inhaler are the medical products examples used to aid in smoking cessation in adults (Chauhan *et al.*, 2016).

### 2.11.4 Adverse effect of nicotine

A direct application of nicotine in human causes burning sensation in the mouth and throat, irritation, increased salivation, nausea, vomiting, swelling, red and itchy skin and abnormal heart rhythm. Some effects are not serious but should be taken into consideration such as gastrointestinal and respiratory diseases.


Figure 2.1. Chemical structure of nicotine (Barlow & Johnson, 1989)

| | |
|---|---|
| Formula | $C_{10}H_{14}N_2$ |
| Molecular weight | 162.234 g/mol |
| Melting point | -7.9ºC |
| Boiling point | 247ºC |
| Rotatory index (S) | $A_D$ = -168 at 20ºC |
| Density | D = 1,010 |
| Refractive index | N = 1,530 |
| Colour | Pale yellow with a slight and fishy odour. |

Table 2.1. The properties of nicotine (Barlow & Johnson, 1989)

# CHAPTER 3: MATERIALS AND METHODOLOGY

## 3.1 MATERIALS

### 3.1.1 Microbial species

The listed strains of microbes were purchased from the American Types Cultures Collection (ATCC), Virginia, USA.

- *Streptococcus sanguinis* ATCC 10556
- *Streptococcus mutans* ATCC 25175
- *Candida albicans* ATCC 14053
- *Candida parapsilosis* ATCC 22019

### 3.1.2 Culture media

- Brain Heart Infusion (BHI) Broth (BD Difco™)
- Brain Heart Infusion (BHI) Agar (BD Difco™)
- Yeast Peptone Dextrose (YPD) Agar (BD Difco™)
- Yeast Peptone Dextrose (YPD) Broth (BD Difco™)

### 3.1.3 Commercial kits

- easy-RED total RNA extraction kit (Intron Biotechnology Inc)
- Prime RT-PCR Premix 2X (GENET BIO)

- SuPrimeScript RT Premix (2X)
- EvaGreen Master Mix kit (Applied Biosystem)
- Syto_9 nucleic acid stain (Molecular Probes Inc)
- Exopolysaccharide (EPS) dye Alexa Fluor_647 (Molecular Probes Inc)

### 3.1.4 Chemical reagents

- Nicotine (Merck Millipore)
- Ethanol (Merck Millipore)
- Chloroform (Merck Millipore)
- Absolute Alcohol (Merck Millipore)
- Agarose (Sigma-Aldrich)
- RNase Free Water (Ambion)
- Tris/Borate/EDTA Buffer (Thermo Fisher Scientific)
- Ethidium Bromide (Sigma-Aldrich)
- Crystal Violet (Merck Millipore)
- Isopropanol (Merck Millipore)
- Distilled Water
- Deionized Water
- Glycerol (Sigma-Aldrich)
- Phosphate Buffer Saline (Sigma-Aldrich)
- Sodium Chloride (Sigma-Aldrich)
- Non-fluorescent immersion oil (Leica)

### 3.1.5 Equipments

- 7500 Fast Real-Time PCR System (ABI, USA)
- Autoclave (HICLA VE HVE-50 Hirayama, Japan)
- Analytical Balance (Mettler AJ100J, USA)
- Centrifuge (Jouan A14, France)
- Centrifuge (Refrigerated) (Jouan GR20 22, France)
- Chiller (4°C) (Mutiara, Malaysia)
- Freezer (-20°C) (Zanussi, Germany)
- Freezer (-80°C) (Hetofrig Cl410, Denmark)
- Gel Image Analyzer (Media Cybernetics, USA)
- Gradient Thermocycler (Eppendorf, Germany)
- Hotplate / Stirrer (Thermolyne)
- Incubator (Memmert, Germany)
- Laminar Flow Unit (ERLA CFM Series, Australia)
- NanoDrop 2000 UV–vis Spectrophotometer (Thermo Scientific)
- Spectrophotometer (Shimadzu UV160A, Japan)
- Vortex Mixer (Snijders Scientific, Holland)
- Water Bath (Grants SS-40-A2, Cambridge, England)
- Water Distiller (J Bibby Merit)
- Confocal laser scanning microscope (Leica, USA)
- Microplate reader (Multiskan EX, Thermo Electron Corp., USA)

## 3.2 METHODS

### 3.2.1 Research Outline

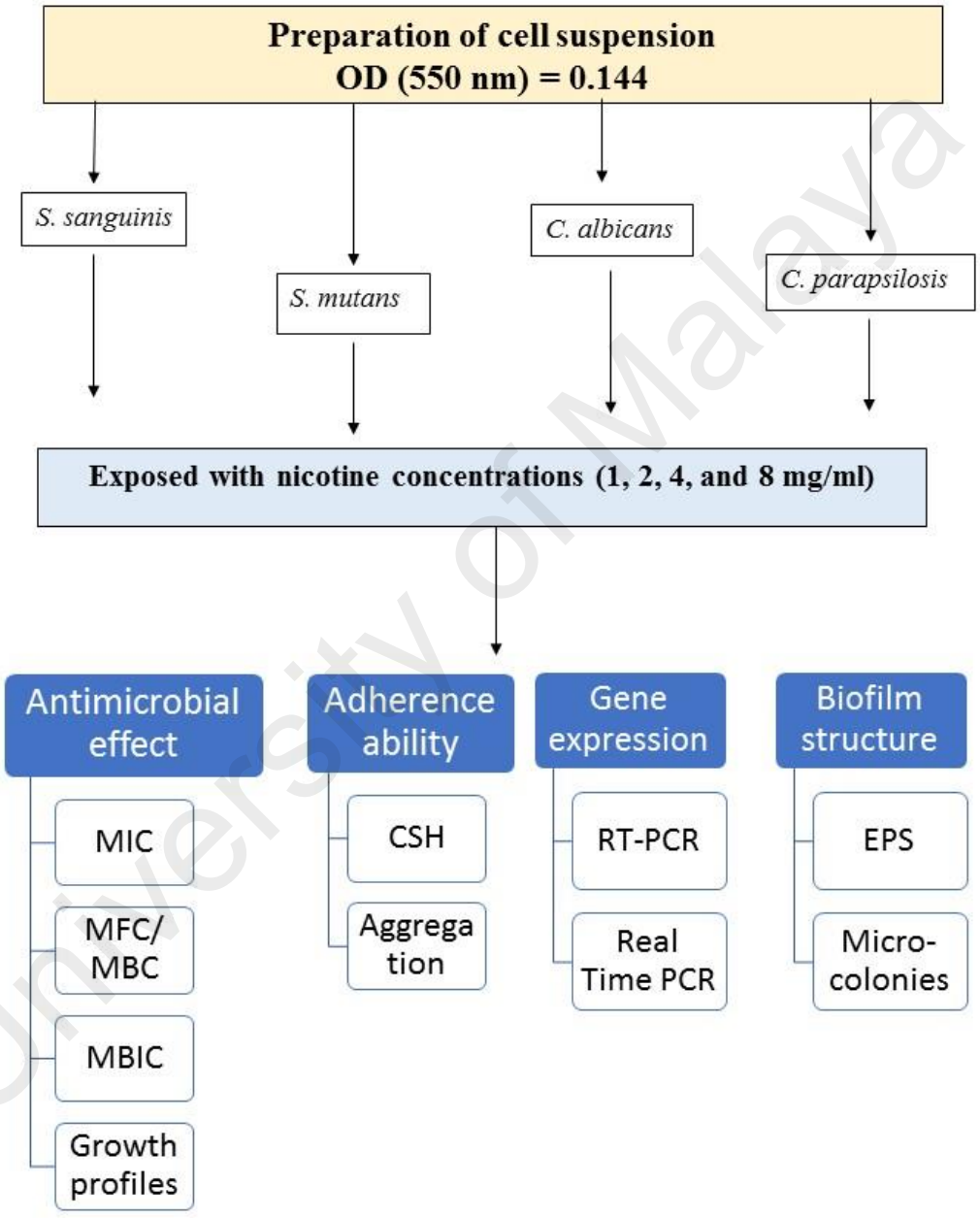



Figure 3.1. The outline of research methodology applied in this study

### 3.2.2 Preparation of culture media

Yeast Peptone Dextrose (YPD) and Brain Heart Infusion (BHI) agar and broth were used as growth media for the *Candida* and *Streptococcus* strains, respectively throughout the study. The powder form of the stock media was initially dissolved in distilled water before further sterilized at 121ºC for 15 min in an autoclave (Appendix 1). The agar media was poured into 15 mm agar plates while broth media was kept in Schott bottle. The agar plates and broth were stored at 4ºC for later use.

### 3.2.3 Preparation of stock culture *Candida* and *Streptococcus* species

*Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Candida albicans* ATCC 14053 and *Candida parapsilosis* ATCC 22019 were purchased from American Type Culture Collection (ATCC). The samples were delivered in lyophilized forms in vials. The lyophilized cells were diluted with sterile distilled water. To initiate the cultures, *Candida* species were revived in YPD broth while *Streptococcus* species in BHI broth followed by overnight incubation at 37°C (35°C for *Candida parapsilosis*).

#### 3.2.3.1 Short term storage on agar slants

A loopful colonies of microbial species from the agar were sub-cultured onto fresh YPD or BHI agar slants individually and incubated overnight at 37ºC. The slants were then kept at 4ºC prior to use in the experiment.

#### 3.2.3.2 Long term storage in 20% glycerol

Glycerol stock media is required to maintain cells' viability for long term storage. A loopful of microbial colonies from the slant was inoculated individually into 5 ml of broth and incubated overnight at 37ºC. Following incubation, 800 µl of the growth suspension was transferred into sterile micro centrifuge tube containing 200 µl of 20% v/v glycerol. The glycerol stock culture was then stored at -80°C for later use.

### 3.2.4 Preparation of microbial cell suspension

Briefly, a few single colonies from fresh cultured agar were inoculated into 5 ml of respective broth and mixed thoroughly by vortexing. For nicotine-exposed microbes, a loopful of single colonies of each respective species was inoculated into 5 ml of BHI or YPD broth containing different nicotine concentrations (1, 2, 4 and 8 mg/ml). The inoculated broth was incubated overnight at 37ºC (35ºC for *C. parapsilosis*). The cells were harvested by centrifugation at 12000 × g. Then, the cell pellet was washed twice with phosphate-buffered saline (PBS, pH 7.2) and re-suspended in fresh buffer. Following which the turbidity of bacterial (Huang *et al.,* 2012; Krzysciak *et al.,* 2014) and candidal (Himratul *et al.,* 2013) suspension was adjusted and standardized spectrophotometrically to an optical density ($OD_{550}$) of 0.144 which is equivalent to $1 \times 10^6$ cells/ml. Routine sub-culturing was carried out before being used in any experiment to ensure the viability of cells.

### 3.2.5 Preparation of nicotine suspension

Nicotine (>99% (GC), liquid) was purchased from Merck Millipore, United States. The concentration of nicotine stock provided by the manufacturer was 1010 mg/ml. To make 16 mg/ml, 0.158 ml of stock concentration was diluted in 10 ml of sterile distilled water. The concentration of nicotine was further diluted with distilled water to the desired working concentrations. Throughout the experiment, four different working concentrations of nicotine (1, 2, 4 and 8 mg/ml) were used.

### 3.2.6. Preparation of chemical buffers

To make 20% w/v glycerol, 1 ml of 100% glycerol was diluted in 4 ml of distilled water to give final volume of 5 ml. While preparation for 1 litre of 1X phosphate buffered saline, was done by diluting 5 tablets of PBS in 800 ml of deionised water and mixed thoroughly. Then, 200 ml of distilled water was added to make 1000 ml PBS solution (137 Mm NaCl, 10 Mm Phosphate, 2.7 Mm KCl, and a pH OF 7.4).

### 3.2.7 Antimicrobial effect of nicotine on *Streptococcus* and *Candida* species

#### 3.2.7.1 Minimum inhibitory concentration (MIC) and minimum fungicidal / bactericidal concentration (MFC/MBC) assay

The MIC and MFC/MBC were carried out according to the Clinical and Laboratory Standards Institute (CLSI) modified method M27-A3 (Clinical and Laboratory Standard Institute 2008). The minimum inhibitory concentration (MIC) is the lowest concentration of nicotine that inhibit the growth of a microorganisms. Minimum

fungicidal / bactericidal concentration (MFC/MBC) is the lowest concentration of nicotine required to kill a microorganism.

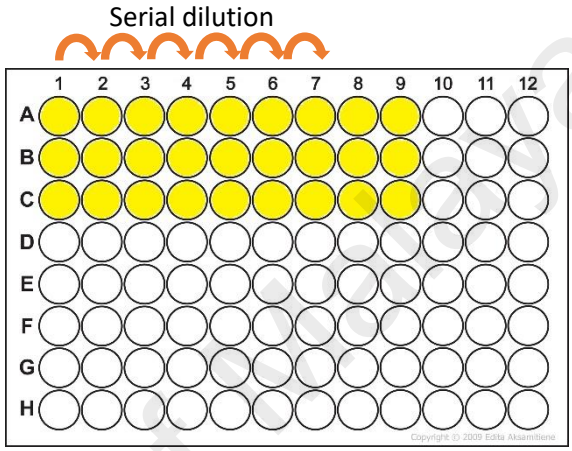
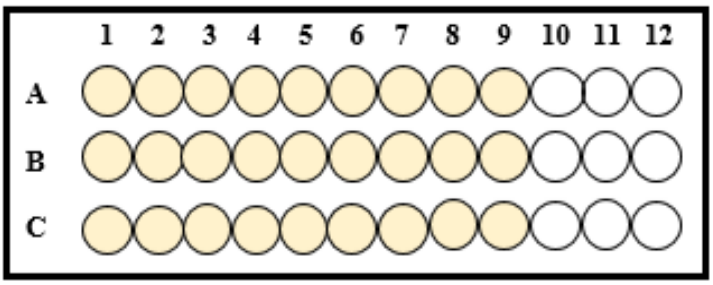
<table>
<tr><td colspan="8">(1) 50 µL of broth was dispersed into Well 1 (W1) to Well 9 (W9).</td></tr>
<tr><td colspan="8">(2) 50 µL of the nicotine (16 mg/ml) was dispensed into W1</td></tr>
<tr><td colspan="8">(3) Two-fold serial dilution was performed in W1 to W7. W8 is negative control and W9 is blank</td></tr>
<tr><td></td><td colspan="7"> 
</td></tr>
<tr><td rowspan="2">Final concentration of nicotine (mg/ml)</td><td>W1</td><td>W2</td><td>W3</td><td>W4</td><td>W5</td><td>W6</td><td>W7</td></tr>
<tr><td>8</td><td>4</td><td>2</td><td>1</td><td>0.5</td><td>0.25</td><td>0.125</td></tr>
<tr><td colspan="8">(4) 50 µL of Candida or Streptococcus cell suspension (1 × $10^6$ cells/ml) was added to W1 through W8.</td></tr>
<tr><td colspan="8">(5) The plate was incubated overnight at 37°C.</td></tr>
<tr><td colspan="8">(6) Turbidity of cell suspension following the exposure to nicotine was measured by $OD_{550nm}$ using microplate reader.</td></tr>
</table>

Figure 3.2. An illustration of microdilution broth assay protocol that has been determined in the experiment for determination of MIC. Symbols meaning  indicated serial dilution.

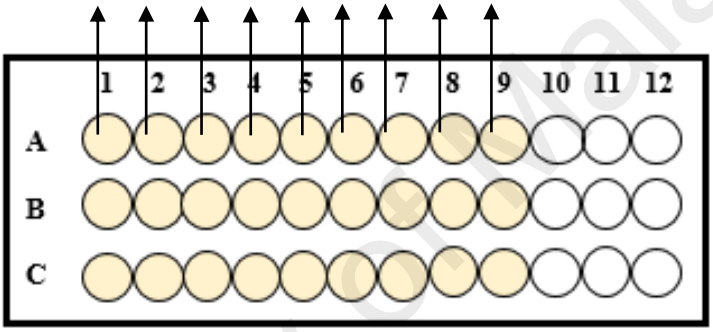
### 3.2.7.2 Minimum biofilm inhibitory concentration (MBIC) assay

MBIC is defined as the lowest concentration of an antimicrobial agent required to inhibit the formation of biofilms (Huang et al., 2012). The biomass of oral microorganisms was determined by measuring the colour intensity of crystal violet in the microplate wells. This assay is one of the common method used for the indirect quantification of cell death and to determine differences in proliferation upon stimulation with adherent-inducing agents. The attached cells were stained with crystal violet dye, which binds to proteins and DNA. Cells that undergo cell death lose their adherence and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture or vice versa. The reading was presented in reflective light unit (RLU). Thus, the higher readings of RLU indicate higher biofilm formed.

Briefly, the cells were cultured in broth (*Streptococcus* sp. in BHI broth while *Candida* sp. in YPD broth) at 37°C overnight. The cultures were then harvested by centrifugation at 12 000 × g and rinsed with PBS. The washed cells were then re-suspended in BHI or YPD broth. The turbidity of the cell suspension was adjusted and standardized spectrophotometrically to an optical density ($OD_{550}$) of 0.144 which is equivalent to $1 \times 10^6$ cells/ml.

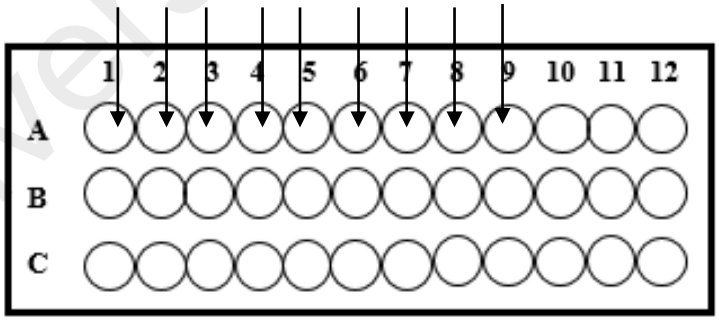



1) An amount of 20 µl of cell cultures was respectively added to the different nicotine concentrations starting from Well 2 through Well 9 (0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/ml) which have been pre-loaded in a microtiter plate wells. Cell suspension in the absence of nicotine (Well 1) was used as negative control.

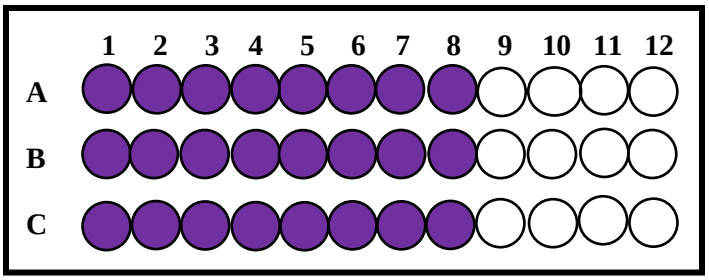



2) After 24 h of incubation at 37°C, the suspension was discarded from each well

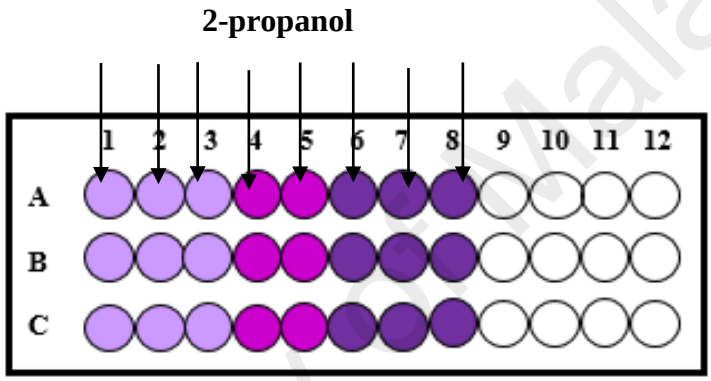
**10% formaldehyde**




3) The biofilm was gently washed twice with PBS and fixed with 10% formaldehyde for 10 min.




4) Then, the plates were washed twice with PBS and stained with 0.5% crystal violet for 1 h followed by washing three times with PBS.




5) Following that, 200 µl of 2-propanol was added to the wells with biofilm layer and incubated for an hour.

6) Finally, the intensity of crystal violet was measured by $OD_{550nm}$ using ELISA microplate reader

Figure 3.3. Procedure for determination of biofilm agianst nicotine. Symbols meaning = ↓ dispenses and ↑ = withdraw

### 3.2.7.3 Effect of nicotine on growth profiles determination of *Streptococcus* sp. and *Candida* sp.

The growth rates of *Streptococcus* sp. and *Candida* sp. to nicotine was investigated using a microdilution broth system based on continuous monitoring of changes in the optical density of cell growth over time. Some environmental factors such as concentration of cell suspension as well as incubation temperature and time was kept constant throughout the experiment. The suspension of microbes was fixed at $10^6$ cells/ml. While the incubation temperature was kept constant at 37ºC for all microbial species (35ºC for *C. parapsilosis*) and incubation time at 24 hours.

To determine the growth curve of cells under the influence of nicotine, 5 ml of cell suspension ($1 \times 10^6$ cells/ml) was added to 45 ml of broth containing various nicotine concentrations (1, 2, 4 and 8 mg/ml) in a sterile conical flask. A sterile flask containing fresh broth and cell suspension without nicotine concentration served as the negative control. The flasks were placed in a shaking water bath incubator set at 35°C to 37°C with continuous agitation. The growth of each microbial species was elucidated by viable cell counts (CFU enumeration) which were estimated at 2, 6, 10, 14 and 18 h interval. The cell suspension was diluted by serial dilution in PBS before plating. Continuous monitoring of changes in the optical density of cell growth was carried out using spectrophotometric assay. One millilitre of the respective cell suspension was taken periodically for turbidity measurement at hourly intervals over a period of 18 h. Turbidity was read using a spectrophotometer at a wavelength of 550 nm. The OD readings were plotted against time,

and the effect of nicotine was also determined based on viable cell counts. The specific growth rate (μ) and doubling time (g) were calculated using the formula below.

(i) Specific growth rate, $\mu = \text{In} (N_t / N_o) / (t_2 - t_1)$

(ii) Doubling time, $g = \log_{10} (N_t / N_o) / \log_{10} 2$

where $N_t$ represented the number of cells at log phase, $N_o$ represented the number of cells at zero time, $t_2$ was the time taken to reach a plateau, and $t_1$ zero time when the cells enter the log phase.

### 3.2.8 Determination of the effect of nicotine on adherence ability of *Streptococcus* sp. and *Candida* sp.

#### 3.2.8.1 CSH assay

The microbial cell suspensions were prepared as described in section 3.2.4. However, the turbidity of cell suspensions was adjusted and standardized spectrophotometrically to an optical density ($OD_{550}$) of 0.450 which is equivalent to $1 \times 10^7$ cells/ml (Klotz *et al.*, 1985).

Non-specific adhesion of nicotine-exposed oral microbes was carried out by exposing 2 ml of cell suspension ($1 \times 10^7$ cells/ml) to 2 ml of 1, 2, 4, and 8 mg/ml nicotine respectively. Two ml of 85% PBS were added to the respective exposed cells and the absorbance value at optimum density of 550 nm was measured. The absorbance of each tube represents the initial absorption in the absence of hexadecane ($C_{16}H_{34}$; MW: 226.448 g/mol) ($A_t$). Two hundred µl of hexadecane was added to each tube and vortex vigorously. The tubes were left for 20 min at room temperature to separate the hexadecane from the aqueous phase. Then, the lower aqueous phase was carefully aliquoted into a cuvette and the absorbance value ($A_u$) was measured. As for control, 2 ml of each cell suspension was mixed with 2 ml of sterile saline in a sterile test tube without the addition of nicotine. The CSH was calculated using the formula below;

$$\text{Percentage changes in } A_{550} = [(A_t - A_u) / A_t] \times 100$$

where $A_t$ is the absorbance value of total cell suspension in the absence of hexadecane and $A_u$ is the absorbance value of total cell suspension in the presence of hexadecane.

Hydrophobicity was expressed as a percentage of adsorption of the bacterial and candidal cells to hexadecane for each of the nicotine-exposed bacteria and candida. Elevation in these percentages when compared to the CSH determined in the absence of nicotine was taken as the effect of the nicotine on the CSH.

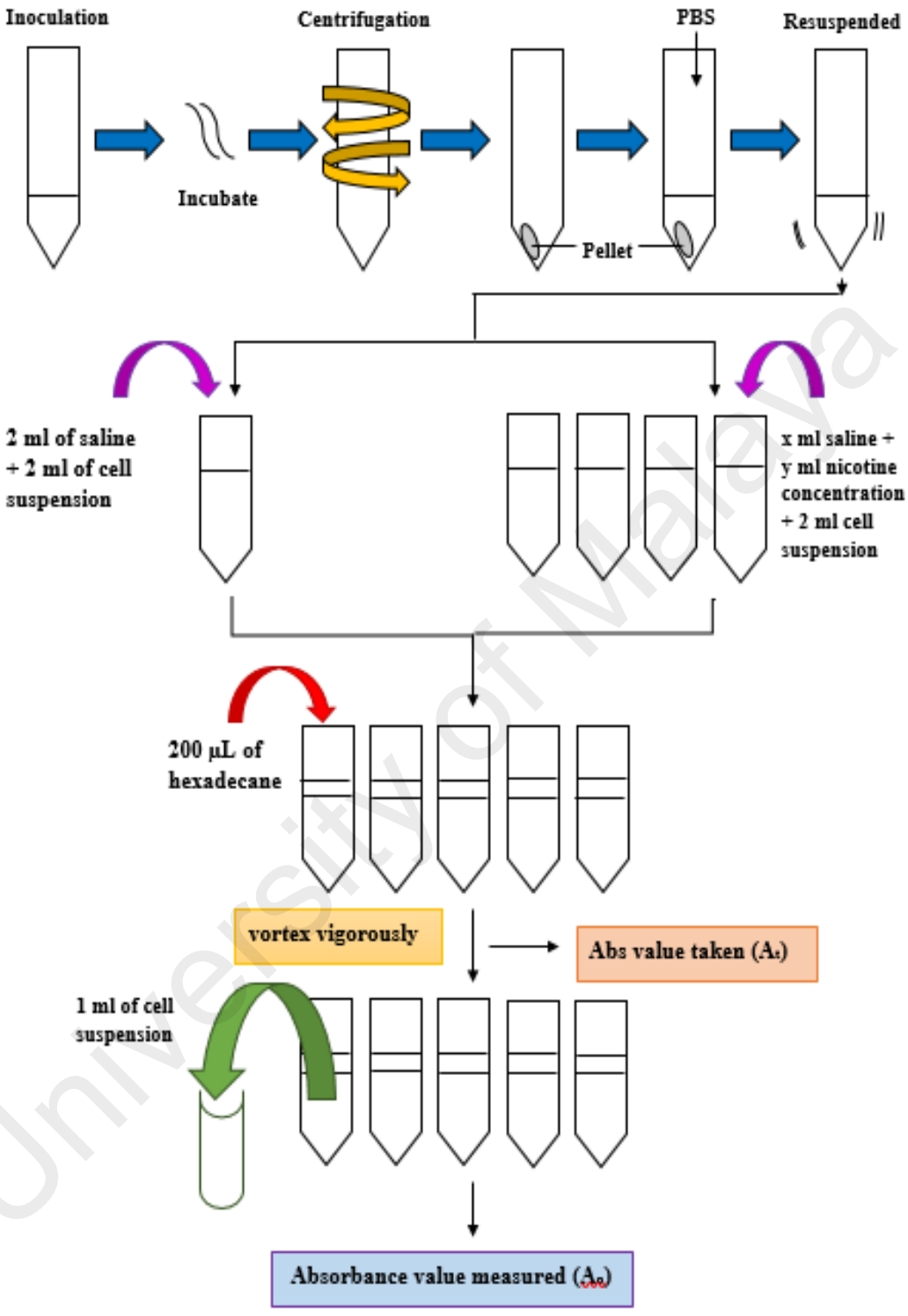



Figure 3.4. Methodology for determination of cell surface hydrophobicity of *Streptococcus* sp. and *Candida* sp. after exposed with nicotine.

### 3.2.8.2 Aggregation assay

One millilitre of each microbial suspension ($1 \times 10^6$ cells/ml) was added to 10 ml of PBS as described by (Joe *et al.,* 2013) consisting of final concentration of nicotine (1, 2, 4 and 8 mg/ml) in sterile test tubes. For positive control, 1 ml of cell suspension was dispensed into 10 ml PBS without addition of nicotine. Uninoculated buffer was served as negative control. The mixtures were mixed vigorously and left at room temperature for 24 h. At every 6 h interval, 1 ml of the suspension was transferred into a fresh cuvette and the absorbance was measured at 550 nm. The time taken for the cells to sediment to the bottom of tube and the percentage of aggregation with different concentration of nicotine were calculated using the formula described below;

$$\text{Aggregation } \% = 1 - (A_t / A_o) \times 100$$

where $A_t$ is absorbance of value at time t and $A_o$ is absorbance of value at time t=0.

### 3.2.9 Effect of nicotine on the expression of the adherence-associated genes

#### 3.2.9.1 Preparation of microbial cell suspension

One ml of $1 \times 10^6$ cells/ml suspension (described in section 3.2.4) for each species was respectively added with 1, 2, 4 and 8 mg/ml nicotine and incubated for 18 hours. The cell pellet was harvested by centrifugation at 15000 × g and washed three times with saline.

#### 3.2.9.2 Total RNA Extraction

*S. sanguinis*, *S. mutans*, *C. albicans* and *C. parapsilosis* were freshly cultured overnight and washed in phosphate buffered saline (PBS). Total RNA from the cells was extracted using an easy-RED BYF Total RNA Extraction Kit (Intron Biotechnology Inc.) following the manufacturer's instruction.

Following the washing step, 250 µL of pre-lysis buffer was added and resuspended thoroughly. Then, 750 µL of easy-RED solution was added; mix in room temperature for 15 s, and left to stand at room temperature for 5 min. Following this, 200 µL of chloroform was added; mix in room temperature for 15 s by vigorous vortex, and left to stand at room temperature for another 5 min.

Following a centrifugation at 8,000 x g (4ºC) for 15 min, the colourless aqueous phase was carefully transferred to a new microcentrifuge tube. An equal volume of isopropanol (2-propanol) was added and mixed well by inverting the tube for 6-7 times

and left aside at room temperature for 10 min. The tube was centrifuged at 8,000 x g (4ºC) for 10 min, and the supernatant was discarded without disturbing the pellet. One ml of 70% ethanol was added and the solution was mix by inverting the tube several times. The mixture was centrifuged at 8,000 x g (4ºC) for 5 min. The supernatant was discarded and the remaining RNA pellet was left to dry. The total RNA was dissolved in 50 µL of RNase free water, and stored at -80ºC.

### 3.2.9.3 RNA Quantification

Total RNA concentrations and purity ratios (260/280 and 260/230) were determined by NanoDrop 2000 UV-vis Spectrophotometer (Thermo Fisher Scientific Inc., USA). Finally, the concentration of total RNA was standardized to 5 µg (Appendix 2) before use in cDNA synthesis step.

### 3.2.9.4 cDNA synthesis

First strand of cDNA synthesis was performed using Prime RT-PCR Premix 2X (GENET BIO). Briefly, 2 µl of total RNA (5 µg) was mixed with 10 µl of RT-PCR Premix 2X mix, 1 µl of selected forward and reverse primers and 6 µl of RNase-free water in a sterile PCR tube to obtain total volume of 20 µl. Reverse transcription was carried out at 42°C for 30 min to synthesize the cDNA followed by denaturation at 94°C for 10 min to deactivate the reverse transcriptase and activating the HS *Prime Taq* DNA polymerase. The synthesized cDNA was kept in -20°C prior to use.

### 3.2.9.5 Determination of Primers

Genes that are related to cell adhesion; *HWP1* and *ALS3* for *Candida* species while *spaP*, *gtfB* and *gbpB* for *Streptococcus* species were selected for gene expression analysis. Housekeeping genes, *actin1* (*ACT1*) and 16sRNA were used as a control for normalization in real time PCR experiments and to ensure *Candida* sp. and *Streptococcus* sp. have the specific genes respectively. The properties of each selected genes were well described in Table 3.1. The cDNA samples were subjected to 30 cycles of denaturation (94°C), annealing (45°C to 65°C), and extension (72°C), each for 30 s followed by the final extension at 72°C for 5 min. Six µl of PCR product was used for separation by electrophoresis in 1% (w/v) of agarose gel and stained with ethidium bromide. The standard percentage of agarose used to run a DNA gel is 1.0%. The mRNAs of the *Candida* species were loaded individually into the agarose wells. Expressions of the selected genes were visualized using ultraviolet (UV) illumination (Alphaimager 2200, Alpha Innotech).

### 3.2.9.6 Real time-polymerase chain reaction (qPCR)

RNA samples were incubated at 65ºC for 5 min and chill on ice for at least 1 min with appropriate primers and RNase-free water followed by brief centrifugation. Then, 10 µl of SuPrimeScript RT Premix (2X) was added and mixed thoroughly before incubated at 50ºC for 60 min according to the instruction provided by manufacturer. The reaction was stopped by heating at 70ºC for 10 min and chilled on ice. Twenty µg RNA was used to synthesize cDNA by a high-capacity cDNA reserve transcription kit with specific primers (Applied Biosystem, Life Technologies Corp., CA, USA). The final product of 2

g of cDNA samples and specific primers were processed with EvaGreen master mix (Applied Biosystems). The qPCR amplification was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems) with default program settings as described by (Li *et al.*, 2013) and (Staniszewska *et al.*, 2014). Unexposed nicotine RNA and distilled water were loaded with Titan EvaGreen master mix to serve as an operative system control. The value of $2^{-\Delta CT}$ instead of $2^{-\Delta\Delta CT}$ of each sample was recorded (where CT represents the qPCR amplifying cycle number once the threshold is reached), and the value for housekeeping genes (*ACT1* for *Candida* sp. while 16sRNA for *Streptococcus* sp.) data equals 1. All samples were compared to the calibrator. The differences between exposed genes were compared using $2^{-\Delta\Delta CT}$.

### 3.2.10 Determination of the effect of nicotine on the structure of EPS and micro-colonies formation of *Streptococcus* and *Candida* sp.

#### 3.2.10.1 Collection of saliva

A single healthy donor was used for saliva collection to minimize any variations that may arise between individuals. The donor is 25 years old, non-smoker, and practising good oral hygiene with healthy oral cavity. The donor was first asked to rinse the mouth with distilled water to reduce microbe carriage before collecting the saliva into ice-chilled sterile test tubes. The saliva was centrifuged at 15,000 × g for 30 min to remove the debris. The supernatant was then filter-sterilized through a disposable 0.22 µm low-protein-binding filter (Cellulose acetate syringe filters Sartorius, USA) into sterile centrifuge tubes and stored at - 20 ºC until further use.

#### 3.2.10.2 Confocal laser scanning microscopy (CLSM)

CLSM has been widely used to study biofilm structure, composition and metabolism in several different microorganisms (Cerca *et al.,* 2012; Luo *et al.,* 2015). One of the big advantages of CLSM is that it allows in-depth analysis of biological structures, without killing or damaging the biological structure (Neu & Lawrence, 1999). Live/dead staining has been used as indicator of cell viability, as determined by the integrity of the cell wall membrane in many bacterial populations, including biofilms. Alexa Fluor_647 dye has been used to stain EPS which may include polysaccharides, proteins, nucleic acids, and amphiphilic polymeric compounds. Thus, in biofilm systems, one can expect

two types of polymeric carbohydrate structures: (1) those located on cell surfaces and (2) those located extracellularly throughout the biofilm matrix.

One ml of each single species of $1 \times 10^6$ cell/ml was respectively diluted 1:8 with YPD or BHI broth in sterile test tubes containing final nicotine concentration of 1 and 2 mg/ml. The tubes were then incubated for approximately 3 hours to reach mid-log phase. Five hundred microliter from prepared cell suspensions were pipetted into each well of Lab-Tek Chamber Slides and incubated for 24 hours at 37ºC (35ºC for *C. parapsilosis*). After overnight incubation, the planktonic cells were aliquoted from each well by pipetting out and washed twice with sterile deionized water. Biofilms formed on the slide were stained in the dark with 0.1 µl SYTO_ 9 nucleic acid stain (Molecular Probes Inc.) to pigment the microbe cells for 20 minutes followed by 0.1 µl of extracellular polysaccharide (EPS) dye Alexa Fluor_647 (10 000 MW; Molecular Probes Inc.) to label the formed EPS. Then, the solution was removed from the each well and washed twice with sterile deionized water to remove excess dyes. The slide was allowed to dry completely and the plastic chamber piece was removed by using the slide separator. Finally, the slides were stained with Calcofluor White (Sigma Chemical Co., St. Louis, Mo.) at 37ºC for 30 min. Samples were viewed using a Leica confocal microscope (USA) with 100x-oil objective, using excitation/emission wavelengths of 358/461 nm for blue fluorescence, 485/498 nm for green fluorescence and 650/498 nm for red fluorescence. Pictures and the intensity of different colour were analysed using ZEN 2 (Carl Zeiss) and 3-D images were made using Leica AF TCS SP5 (Leica, USA).

### 3.3 Statistical analysis

All data were computed and expressed as mean ± standard deviation (SD) from three independent tests performed in triplicate (n = 9). Independent t-test was carried out to compare two groups. Statistical analysis was performed using SPSS software (version 23.0). A $\rho$-value of $< 0.05$ was considered as statistically significant.

Table 3.1. Oligonucleotides and its sequences used in this study

| Set | Gene | Direction | Primers (5' – 3') | Melting point (°C) | Product size (bp) |
|---|---|---|---|---|---|
| 1 | *ACT1* | F | GGCTGGTAGAGACTTGACCAACCATTTG | 61.0 | 95 |
| | | R | GGAGTTGAAAGTGGTTTGGTCAATAC | 56.3 | |
| 2 | 16sRNA | F | AGCGTTGTCCGGATTTATTG | 53.2 | 464 |
| | | R | CTACGCATTTCACCGCTACA | 55.2 | |
| 3 | *HWP1* | F | CCATGTGATGATTACCCACA | 52.3 | 572 |
| | | R | GCTGGAACAGAAGATTCAGG | 53.3 | |
| 4 | *ALS3* | F | CCACAGCTGCTTCCACTTCT | 57.6 | 340 |
| | | R | TGCAGATGGAGCATTACCACC | 57.4 | |
| 5 | *SpaP* | F | TCCGCTTATACAGGTCAAGTTG | 54.5 | 121 |
| | | R | GAGAAGCTACTGATAGAAGGGC | 54.6 | |
| 6 | *GtfB* | F | AGCAATGCAGCCATCTACAAAT | 55.3 | 98 |
| | | R | ACGAACTTTGCCGTTATTGTCA | 55.2 | |
| 7 | *GbpB* | F | CGTGTTTCGGCTATTCGTGAAG | 56.3 | 108 |
| | | R | TGCTGCTTGATTTTCTTGTTGC | 54.9 | |

# CHAPTER 4: RESULTS

## 4.1. Antimicrobial effect of nicotine against *Streptococcus* sp. and *Candida* sp.

### 4.1.1. MIC, MFC/MBC and MBIC of *Candida* and *Streptococcus* species

The MIC, MBC or MFC, and MBIC of nicotine against *S. sanguinis, S. mutans, C. albicans* and *C. parapsilosis* were given in Table 4.1. From the data, nicotine has increased the activity of all strains. *S. sanguinis* exhibited the highest MIC value of 8 mg/ml while both *Candida* sp. and *S. mutans* were found more susceptible to nicotine with MIC of 4 mg/ml.

The MFC or MBC of nicotine against *Candida* sp. and *Streptococcus* sp. were determined within the range of 16 to 32 mg/ml. Out of four, both *S. sanguinis* and *S. mutans* showed the highest MBC value for nicotine of 32 mg/ml. While the MFC values towards *C. albicans* and *C. parapsilosis* were 16 mg/ml. The data indicated that *S. sanguinis* and *S. mutans* are more resistant to nicotine because higher concentration of nicotine is required to kill the cells.

The MBIC values for nicotine of both *C. albicans* and *C. parapsilosis* were 4 mg/ml while *S. mutans* and *S. sanguinis* was 8 mg/ml. From the data, we found that nicotine has exhibited similar inhibition effect on planktonic cells and biofilm of *C. albicans, C. parapsilosis* and *S. sanguinis.* However, *S. mutans* has showed higher

inhibition effect on planktonic cells than biofilm cells. Overall, the results have concluded that Streptococci biofilms are more resistant to nicotine effect than candidal biofilms.

Table 4.1. The MIC, MBC/MFC, MBIC of nicotine against *Streptococcus* sp. and *Candida* sp.

| Oral Microbes | MIC (mg/ml) | MFC / MBC (mg/ml) | MBIC (mg/ml) |
|---|---|---|---|
| ***S. sanguinis*** | 8 | 32 | 8 |
| ***S. mutans*** | 4 | 32 | 8 |
| ***C. albicans*** | 4 | 16 | 4 |
| ***C. parapsilosis*** | 4 | 16 | 4 |

MIC = Minimum inhibitory concentration
MFC or MBC = Minimum fungicidal concentration or minimum bactericidal concentration
MBIC = Minimum biofilm inhibitory concentration

### 4.1.2 Inhibitory activity of nicotine on biofilm formation

The biomass of biofilm was measured using microplate based crystal violet assay. The reading was presented in reflective light unit (RLU). Thus, the higher readings of RLU indicate higher biofilm formed or vice versa. Figure 4.1 summarizes the influences of nicotine on the development of biofilm of *Candida* sp. and *Streptococcus* sp. In the absence of nicotine, the formation of biofilm of *S. sanguinis* was 0.502 ± 0.104 RLU which is higher than *S. mutans* (0.289 ± 0.213 RLU). The biofilm mass of *C. albicans* without nicotine exposure was 0.105 ± 0.002 RLU which was higher compared to *C. parapsilosis* that demonstrated only 0.09 ± 0.003 RLU. Thus, this indicated that *S. sanguinis* produced more biofilm than *S. mutans*, *C. albicans*, and *C. parapsilosis*.

The formation of biofilm increases in ascending order as the concentration of nicotine increases to 0.5 mg/ml. At 0.25 mg/ml nicotine, 26.7% (0.133 ± 0.002 RLU) of biofilm build up in *C. albicans* was reported compared to 18.6% (0.115 ± 0.004 RLU) in *C. parapsilosis* while, the biomass in *S. sanguinis* was reported to be 0.706 ± 0.002 RLU. Among all, the highest biofilm formation was observed in *S. mutans* (1.102 ± 0.006) when exposed with 0.25 mg/ml nicotine concentration. In total, 37.1% (0.144 ± 0.001 RLU) and 20.6% (0.117 ± 0.006 RLU) of *C. albicans* and *C. parapsilosis* biofilm at 0.5 mg/ml respectively was found to produce the highest biomass compared to other nicotine concentrations. *S. sanguinis* was also found to have the highest formation of biofilm (0.720 ± 0.004 RLU) when exposed with 0.5 mg/ml nicotine concentration. A significant

difference in biofilm formation at of 0.25 and 0.5 mg/ml nicotine concentration were observed in both *Streptococcus* sp. and *Candida* sp.

However, we found that the biofilm mass was reduced at nicotine concentration higher than 0.5 mg/ml. Exposing 1 mg/ml nicotine has led to 21% (0.127 ± 0.001 RLU) reduction in the biofilm mass of *C. albicans* followed by another reduction of 13.4% (0.113 ± 0.001 RLU) at 2 mg/ml. The biofilm mass of *C. parapsilosis* was reduced to 9.28% (0.106 ± 0.003 RLU) at 1 mg/ml and remain unchanged when exposed with 2 mg/ml (0.106 ± 0.002 RLU) compared to unexposed. Similar pattern of result was observed in both *Streptococcus* species. Approximately 20% to 50% reduction in biofilm formation of *S. sanguinis* (0.710 ± 0.014) and *S. mutans* (0.587 ± 0.032) were observed when the cells exposed with 1 mg/ml nicotine concentration respectively and further reduced upon exposure to 2 mg/ml nicotine concentration. The biofilm formation of *S. mutans* was continuously reduced to 0.192 ± 0.036 RLU at 4 mg/ml and 0.146 ± 0.028 RLU at 8 mg/ml. We observed the same results from *S. sanguinis* when exposed to 4 mg/ml (0.646 ± 0.013 RLU) and 8 mg/ml (0.494 ± 0.020 RLU) overnight. When *C. albicans* was exposed with 4 mg/ml nicotine, the biofilm formation was found to reduce to 4% (0.082 ± 0.002 RLU) and further reduced to 3.8% (0.081 ± 0.001 RLU) at 8 mg/ml compared to unexposed. Further reduction in biofilm mass was observed when the cells were exposed with 4 mg/ml (0.086 ± 0.001 RLU) and 8 mg/ml (0.083 ± 0.002 RLU). The data showed that any further increment of nicotine concentration resulted in unchanged biofilm mass.

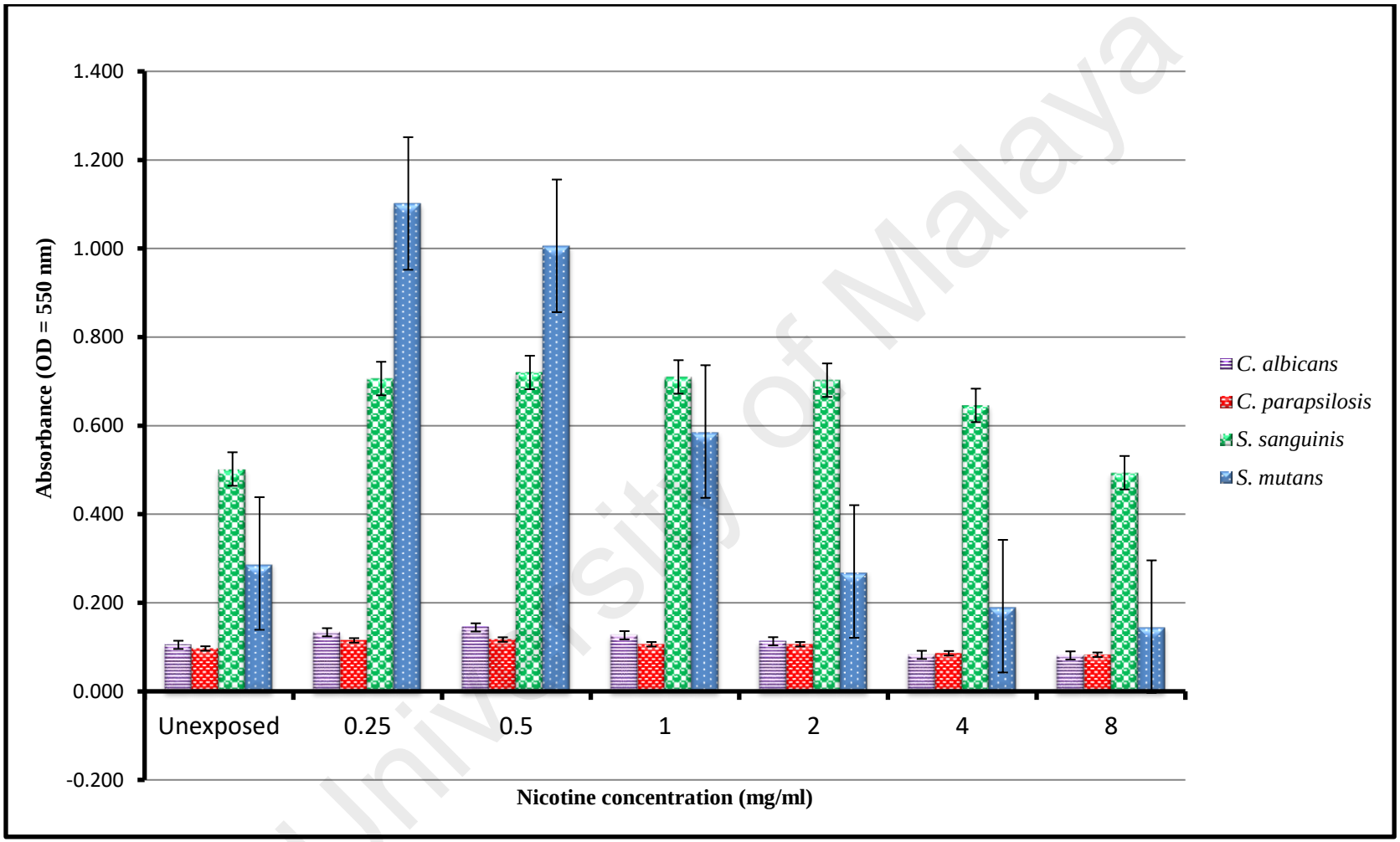



Figure 4.1. Biofilm formation of *Candida* sp. and *Streptococcus* sp. after 24 h. The absorbance at 550 nm of crystal violet stained biofilm at different nicotine concentrations is shown with mean and standard deviation after three independent experiment.

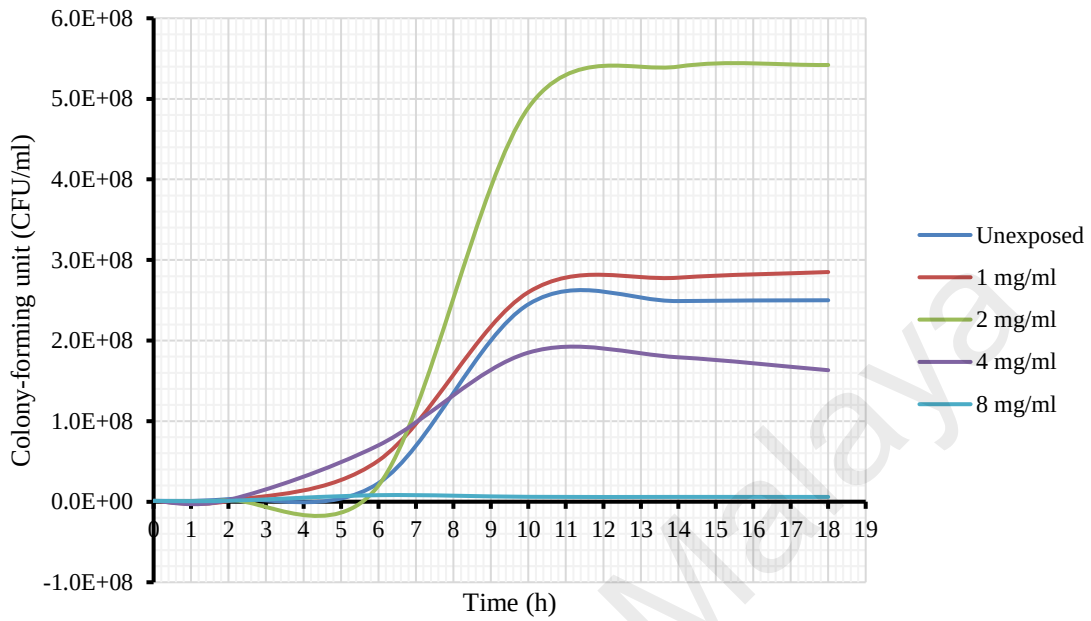
### 4.1.3 The effect of nicotine on growth profiles of *Streptococcus* sp. and *Candida* sp.

The effects of nicotine on planktonic cell growth of *Candida* sp. and *Streptococcus* sp. were studied. The growths of *S. mutans*, *S. sanguinis*, *C. albicans* and *C. parapsilosis* were increased drastically at logarithmic phase when the nicotine concentration was 0 to 0.5 mg/ml and slightly influenced when exposed to nicotine concentration of 1 and 2 mg/ml. For example, the count of *S. sanguinis* was increased from $1 \times 10^6$ cells/ml to 5.4 $\times 10^8$ cells /ml at 2 mg/ml. The data also have showed that approximately 12.5% cells from $1 \times 10^6$ cells/ml to $1.8 \times 10^8$ cells/ml were increased when *S. mutans* was exposed to nicotine concentration of 2 mg/ml. Whereas, the count of *C. albicans* and *C. parapsilosis* showed an increment from $1 \times 10^6$ cells/ml to $7.0 \times 10^8$ cells/ml and to $5.0 \times 10^8$ cells/ml respectively at nicotine concentration of 2 mg/ml. Among all oral microbes used in the study, *C. albicans* multiplied rapidly followed by *S. sanguinis*. The significant increases in viable cells have clearly shown that the populations of both *Streptococcus* sp. and *Candida* sp. multiplied rapidly as the nicotine concentration increases. However, at concentration above 2 mg/ml, the growth rate of all microbes was slowed down. The candidal count of *C. albicans* and *C. parapsilosis* and bacterial count of *S. mutans* and *S. sanguinis* were reported to reduce about 30% to 75% than unexposed cells at 4 mg/ml of nicotine. At 8 mg/ml of nicotine, the growth curves of both species were completely constrained where about 85% to 90% of the cells were reduced compared to unexposed cells (Figure 4.2). Based on CFU enumeration, it is clearly shown that the population of all species were gradually increased upon exposed to 1 and 2 mg/ml of nicotine. However,

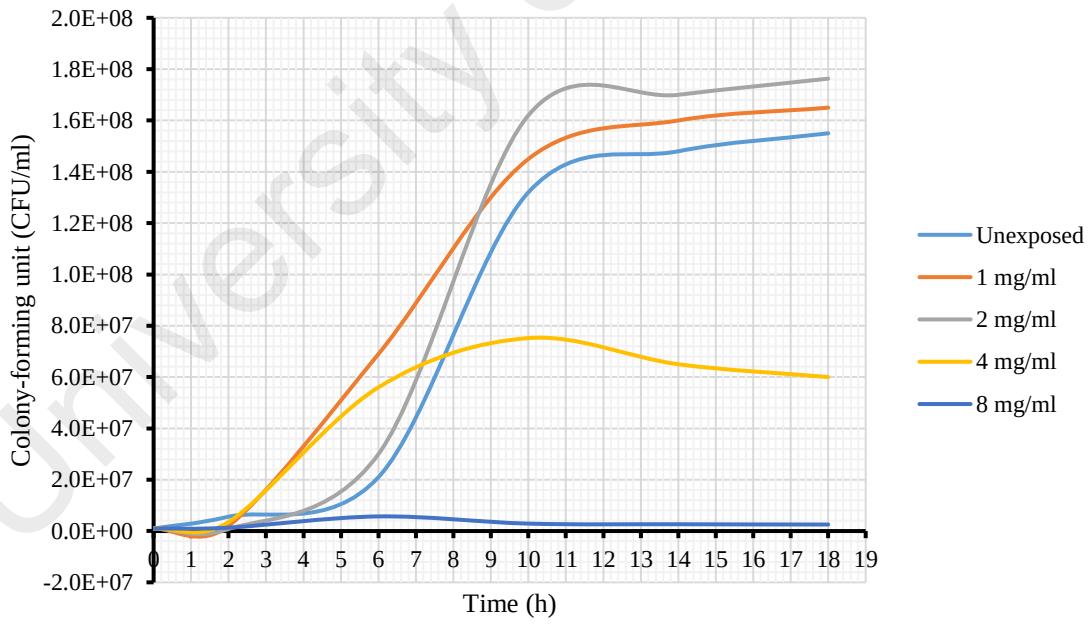
at higher concentration of 4 mg/ml and 8 mg/ml, the population of *Streptococcus* sp. and *Candida* sp. were reduced drastically (Figure 4.3).

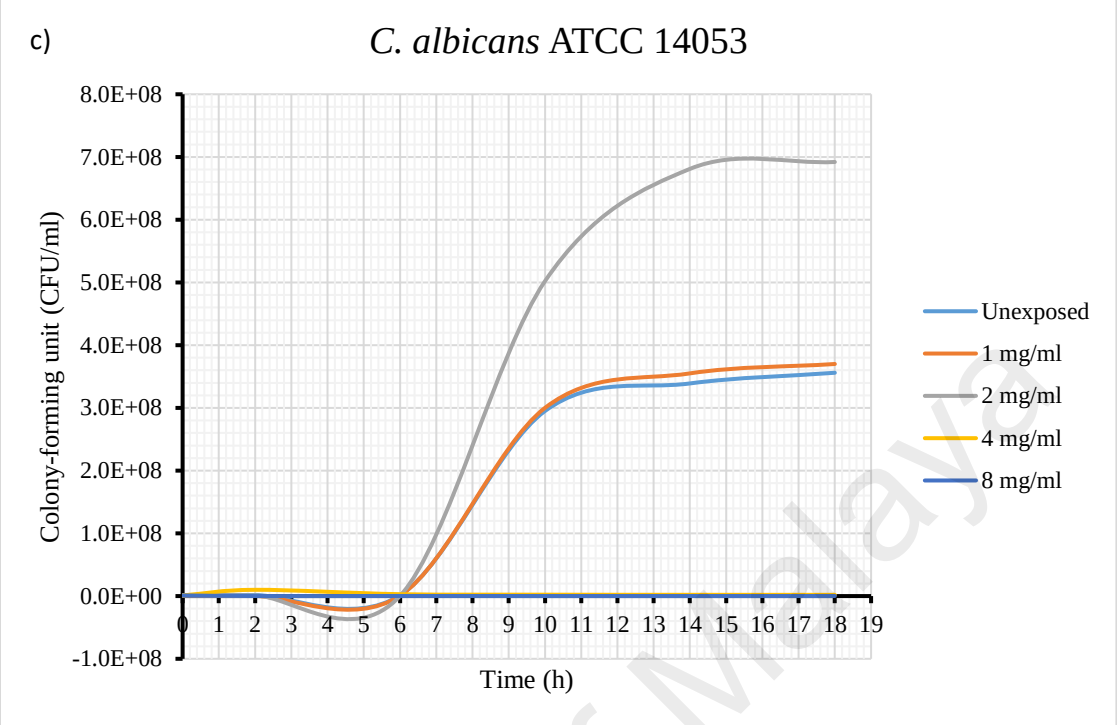
Besides that, the growth kinetics of microbes was also elucidated based on the enumeration of colony forming units (CFU). From the data, the growth rates of *C. albicans* (0.503 ± 0.008) and *C. parapsilosis* (0.777 ± 0.014) were observed to be highest at 2 mg/ml nicotine when compared with other concentrations. *C. parapsilosis* showed the highest growth rates (0.680 ± 0.006) indicating high proliferation compared to *C. albicans* (0.453 ± 0.012). However, the doubling time of *C. parapsilosis* (7.872 ± 0.003) was observed to be slightly shorter than *C. albicans* (8.493 ± 0.004; Table 4.2). *S. mutans* showed the highest growth rates (0.508 ± 0.002) indicating high proliferation when compared with *S. sanguinis*. The doubling time of *S. sanguinis* (7.967 ± 0.005) was observed to be slightly longer than *S. mutans*. The growth rates of *S. mutans* (7.493 ± 0.012) and *S. sanguinis* (9.078 ± 0.009) were higher at 2 mg/ml of nicotine when compared with unexposed and 1 mg/ml (Table 4.2). While at 4 mg/ml, the bacterial count of *S. sanguinis* was reduced more than 30% and further reduced up to 90% at nicotine concentration of 8 mg/ml. Similar pattern of reduction was observed in *S. mutans*. The bacterial count was drastically reduced 50% at nicotine concentration of 4 mg/ml and further reduced 87.5% at 8 mg/ml.

a)




b)





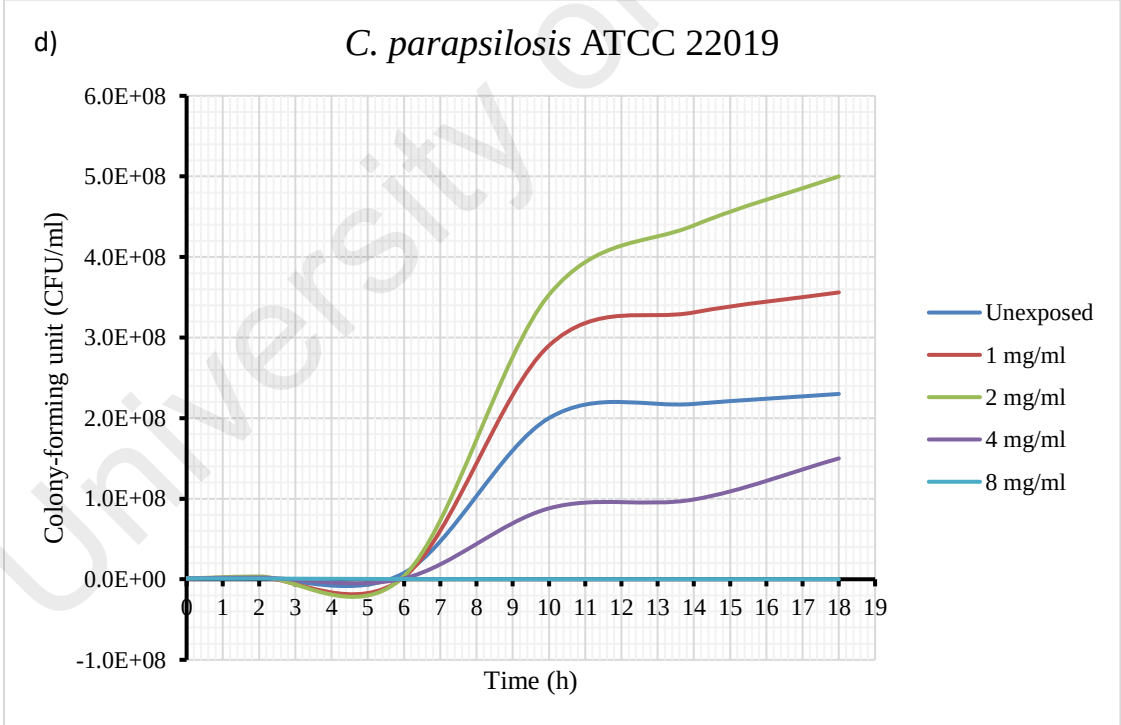
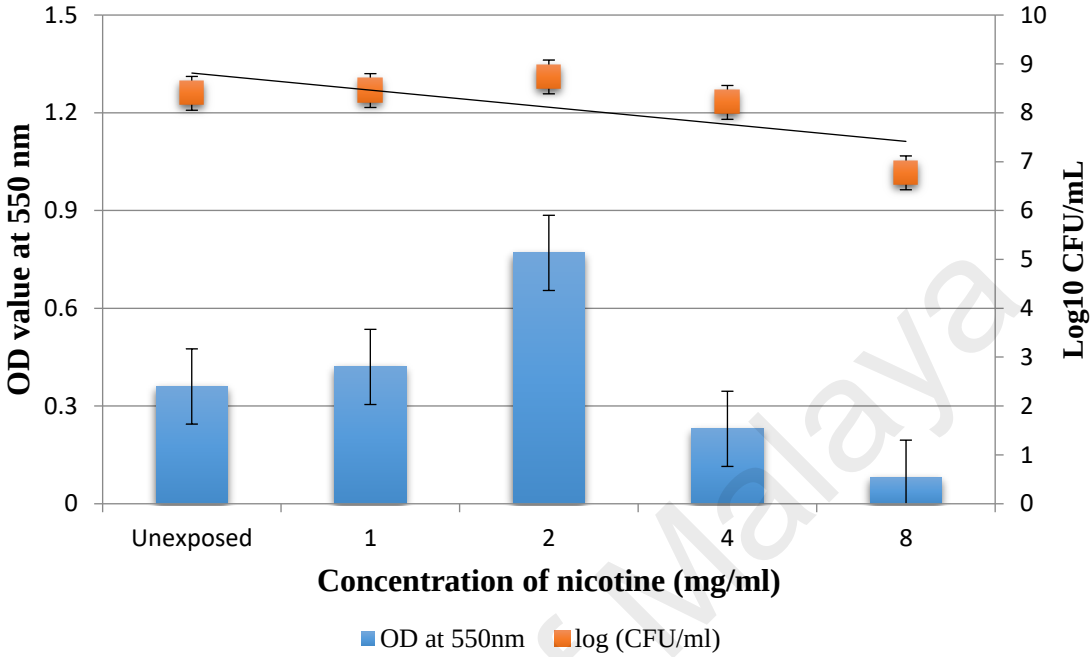




Figure 4.2 The effect of nicotine on growth rate of (a) *Streptococcus sanguinis* BAA 4155, (b) *Streptococcus mutans* ATCC 25175, (c) *Candida albicans* ATCC 14053, (d) *Candida parapsilosis* ATCC 22019

a)

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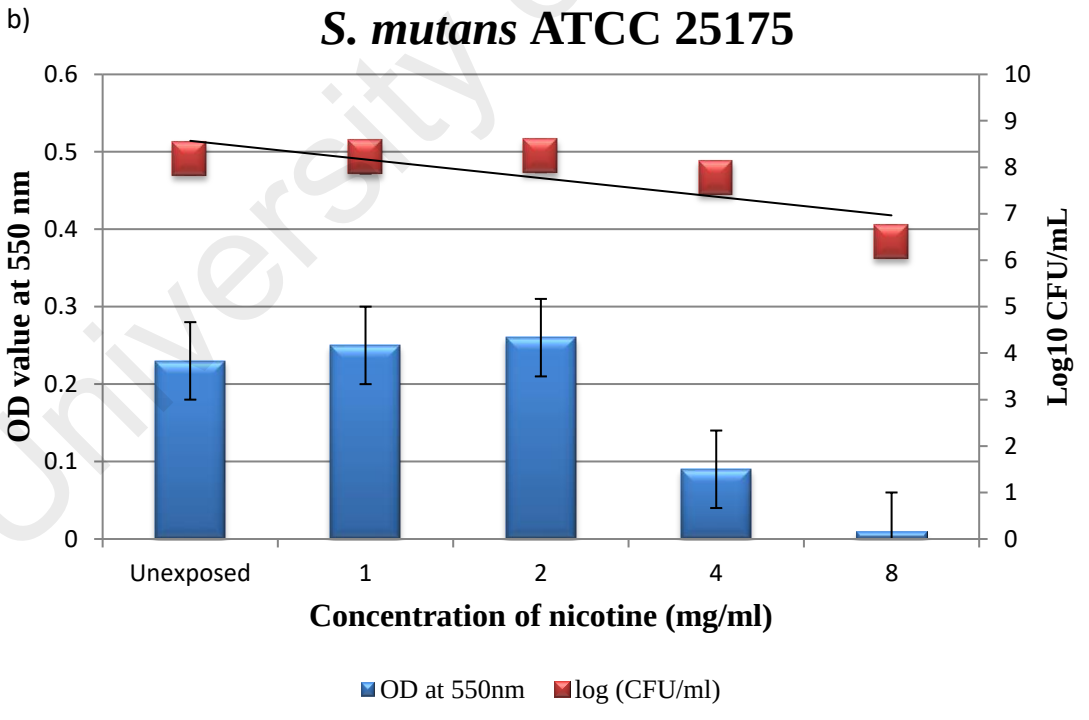
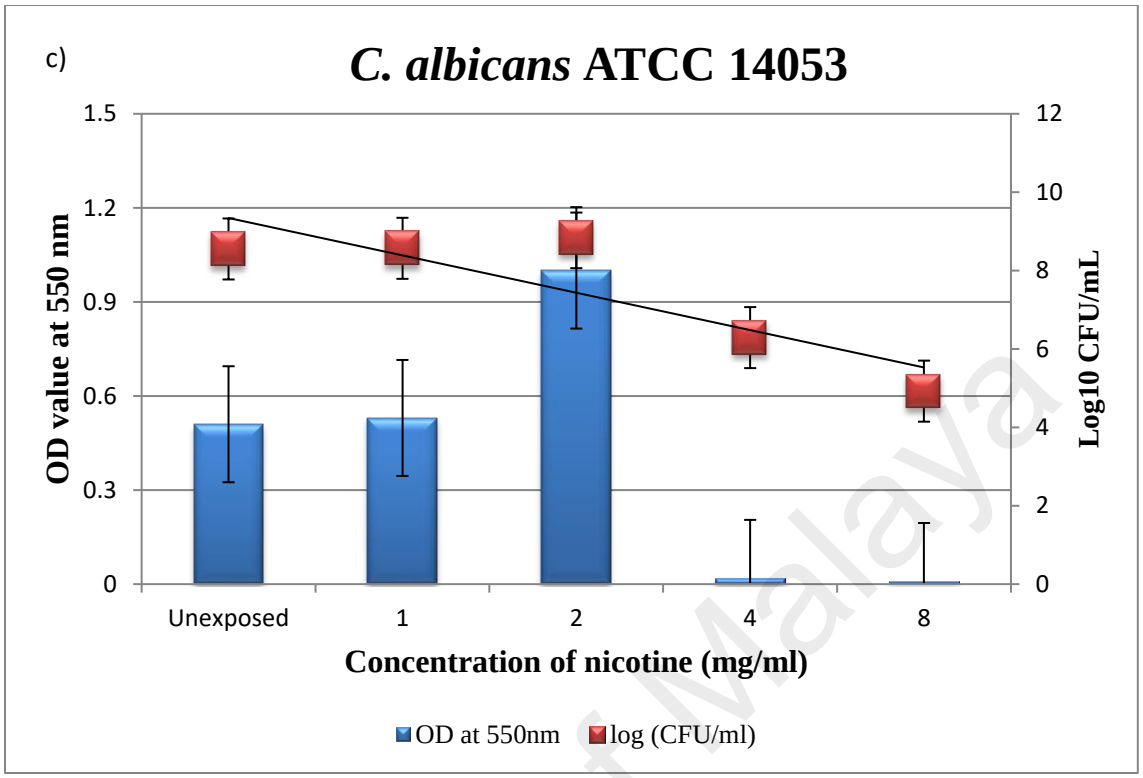




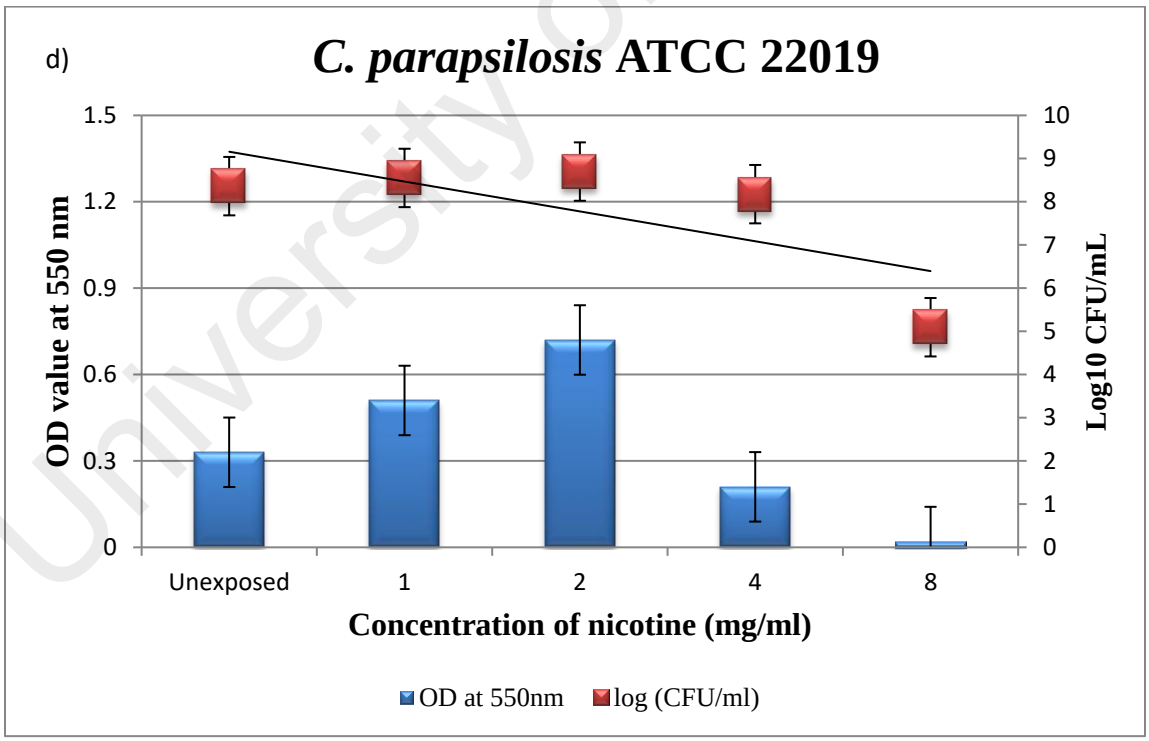




Figure 4.3 The effect of nicotine on OD and CFU of (a) *Streptococcus sanguinis* BAA 4155, (b) *Streptococcus mutans* ATCC 25175, (c) *Candida albicans* ATCC 14053, (d) *Candida parapsilosis* ATCC 22019

**Table 4.2.** Changes in doubling time (g) and specific growth rates (μ) of *Candida* sp. and *Streptococcus* sp. in the absence (unexposed) and presence of nicotine

| Oral Microbes | Doubling time (g) & Specific growth rate (μ) | Unexposed | Nicotine concentration | |
|---|---|---|---|---|
| | | | 1 mg/ml | 2 mg/ml |
| ***S. sanguinis*** | μ ($h^{-1}$) | 0.460 | 0.472 | 0.524 |
| | g (h) | 7.967 | 8.181 | 9.078 |
| | Increase in μ | - | 0.012 | 0.064 |
| | Increase in g | - | 0.214 | 1.111 |
| | | | | |
| ***S. mutans*** | μ ($h^{-1}$) | 0.508 | 0.514 | 0.519 |
| | g (h) | 7.323 | 7.410 | 7.493 |
| | Increase in μ | - | 0.006 | 0.011 |
| | Increase in g | - | 0.087 | 0.170 |
| | | | | |
| ***C. albicans*** | μ ($h^{-1}$) | 0.453 | 0.455 | 0.503 |
| | g (h) | 8.493 | 8.532 | 9.431 |
| | Increase in μ | - | 0.002 | 0.050 |
| | Increase in g | - | 0.039 | 0.938 |
| | | | | |
| ***C. parapsilosis*** | μ ($h^{-1}$) | 0.680 | 0.736 | 0.777 |
| | g (h) | 7.872 | 8.521 | 8.997 |
| | Increase in μ | - | 0.056 | 0.097 |
| | Increase in g | - | 0.649 | 1.125 |

## 4.2 Effect of nicotine on non-specific adherence ability of *Streptococcus* sp. and *Candida* sp.

### 4.2.1 CSH of *Streptococcus* sp. and *Candida* sp. upon Exposure to Nicotine

*S. sanguinis, S. mutans, C. albicans* and *C. parapsilosis* are categorized as highly hydrophobic species ($P < 0.05$). The cell surface hydrophobicity (CSH) of *Candida* species was found to increase ($P< 0.05$) upon exposure to nicotine concentration of 2 mg/ml and below. Compared to unexposed cells, 1 mg/ml nicotine has drastically increased the CSH of *C. albicans* by more than 28.35% while 2 mg/ml could further increase the CSH as much as 85.39%. However, at nicotine concentration of more than 2 mg/ml the CSH was found to cause a gradual reduction in *C. albicans*. A similar pattern of reduction in CSH was also observed in *C. parapsilosis*. Following exposure to 1 mg/ml nicotine, *C. parapsilosis* showed highest CSH effect which is 104.57%. However, at 2 mg/ml nicotine, a slight reduction of CSH was observed compared to CSH of 1 mg/ml nicotine. Approximately 25% reduction was observed at higher concentration of 4 and 8 mg/ml nicotine (Figure 4.4).

Nicotine was also found to provide up surging effect on the CSH of *S. sanguinis* and *S. mutans* ($P < 0.05$). Compared to unexposed cells, 1 mg/ml nicotine has increased the CSH of *S. sanguinis* (62.69%) and *S. mutans* (17.53%). CSH was found to cause continuous increasing in *S. sanguinis* (5.8%) and *S. mutans* (22.05%) after exposed with 2 mg/ml nicotine. Interestingly, exposing the bacterial to higher concentrations of nicotine

has further increase the CSH by more than 90% ($P < 0.05$) indicating the acceleration effect was concentration dependent.

From our study, it was shown that nicotine has influences the CSH of *S. sanguinis*, *S. mutans*, *C. albicans* and *C. parapsilosis*. Among all oral microbes, independent t-test demonstrated that the means percentage adsorption of nicotine-exposed *S. mutans* to hexadecane was significantly higher ($P > 0.05$). This data indicated that *S. mutans* was more hydrophobic followed by *S. sanguinis* than *C. albicans* and *C. parapsilosis* after exposed to nicotine.

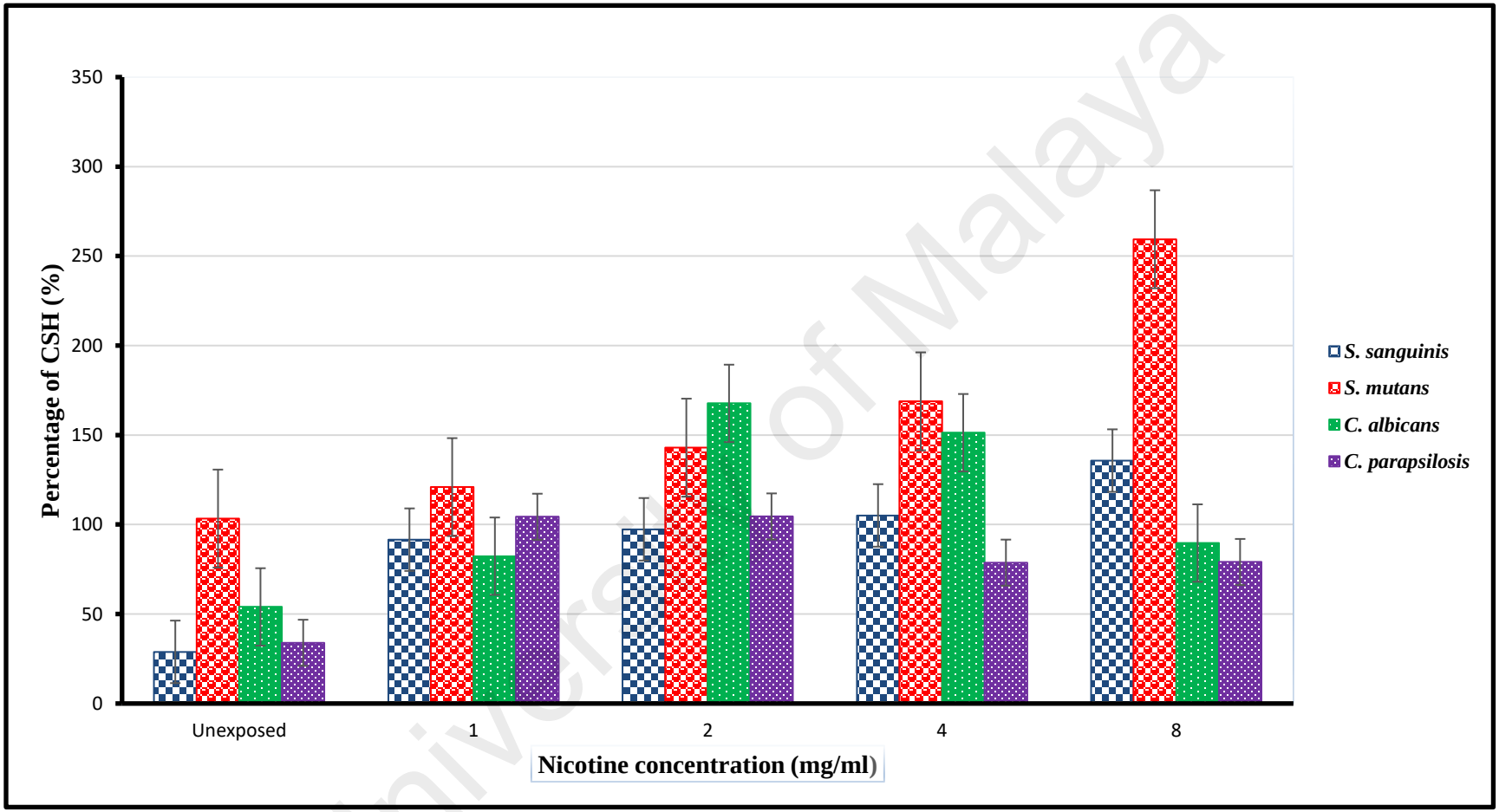



Figure 4.4 The effect of nicotine on the CSH of *Streptococcus* and *Candida* cells. The data represents the average and standard deviation of three independent experiments (n = 9).

### 4.2.2 Aggregation effect of nicotine on *Streptococcus* sp. and *Candida* sp.

To determine the effect of smoking on cell aggregation, *Streptococcus* sp. and *Candida* sp. strains were exposed to nicotine. In our study, we have observed that cells tended to aggregate in an ascending order to the period of exposure to nicotine.

It was observed that by 6 h incubation in various concentrations of nicotine (1, 2, 4 and 8 mg/ml), about 10-40% of *Candida* sp. and *Streptococcus* sp. were aggregated. The cell aggregation was increased to 25-80% at 12 h incubation and further increased in cell aggregation to about 70-90% at 24 h. Thus, exposing the cells with nicotine for 24-h has caused more microbes to aggregate compared to 12-h (Figure 4.5.1 and Figure 4.5.2).

Nicotine exposure was found to have high influence on the facilitation of aggregation in both species up to 80%. At 12 h of treatment, 33.94% of *C. albicans* were aggregated compared to 70.72% in *C. parapsilosis* when exposed to 1 mg/ml nicotine. The candidal cells have achieved the maximum aggregation at this concentration of nicotine. Cell aggregation inclined by 28.46% and 70.01% of *C. albicans* and *C. parapsilosis* at nicotine concentration of 2 mg/ml. Nicotine has further aggregated *C. albicans* up to 32.84% at 4 mg/ml. However, at 8 mg/ml of nicotine, the aggregation level has been reduced to 25.84%. While we noticed the cell aggregation in *C. parapsilosis* has been reduced to 66.14% at 4 mg/ml nicotine followed by 62.25% at 8 mg/ml nicotine concentration than unexposed (Figure 4.5.1). Highest increment of 69.39% in aggregation by nicotine at 1 mg/ml was observed in *S. sanguinis* while for *S. mutans*, the highest

increment (29%) was observed at 4 mg/ml. Enhancement of 17.36% and 7% of aggregation by nicotine concentration of 2 mg/ml was observed in *S. sanguinis* and *S. mutans* (Figure 4.5.2)

The data showed that *C. albicans* and *S. mutans* could aggregate more than *S. sanguinis* and *C. parapsilosis.* Nicotine has somehow induced the aggregation of *C. albicans, C. parapsilosis, S. mutans* and *S. sanguinis* in a dose- and time-dependent manner.

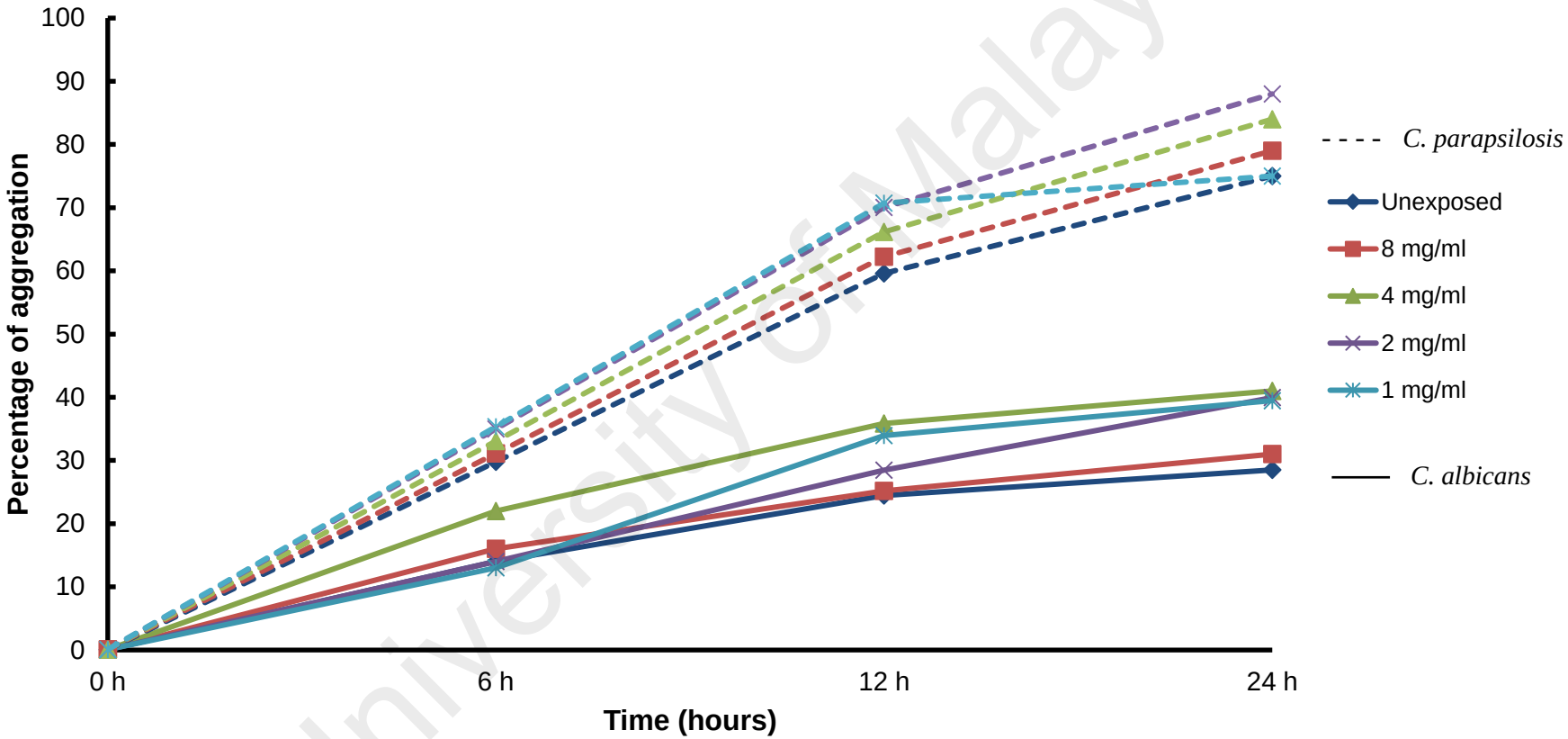



Figure 4.5.1 The effect of nicotine on aggregation of *Candida* cells at different time periods. The solid line represents *C. albicans* and the dotted line represents *C. parapsilosis*. The data displayed were the average of three independent experiments (n = 9).

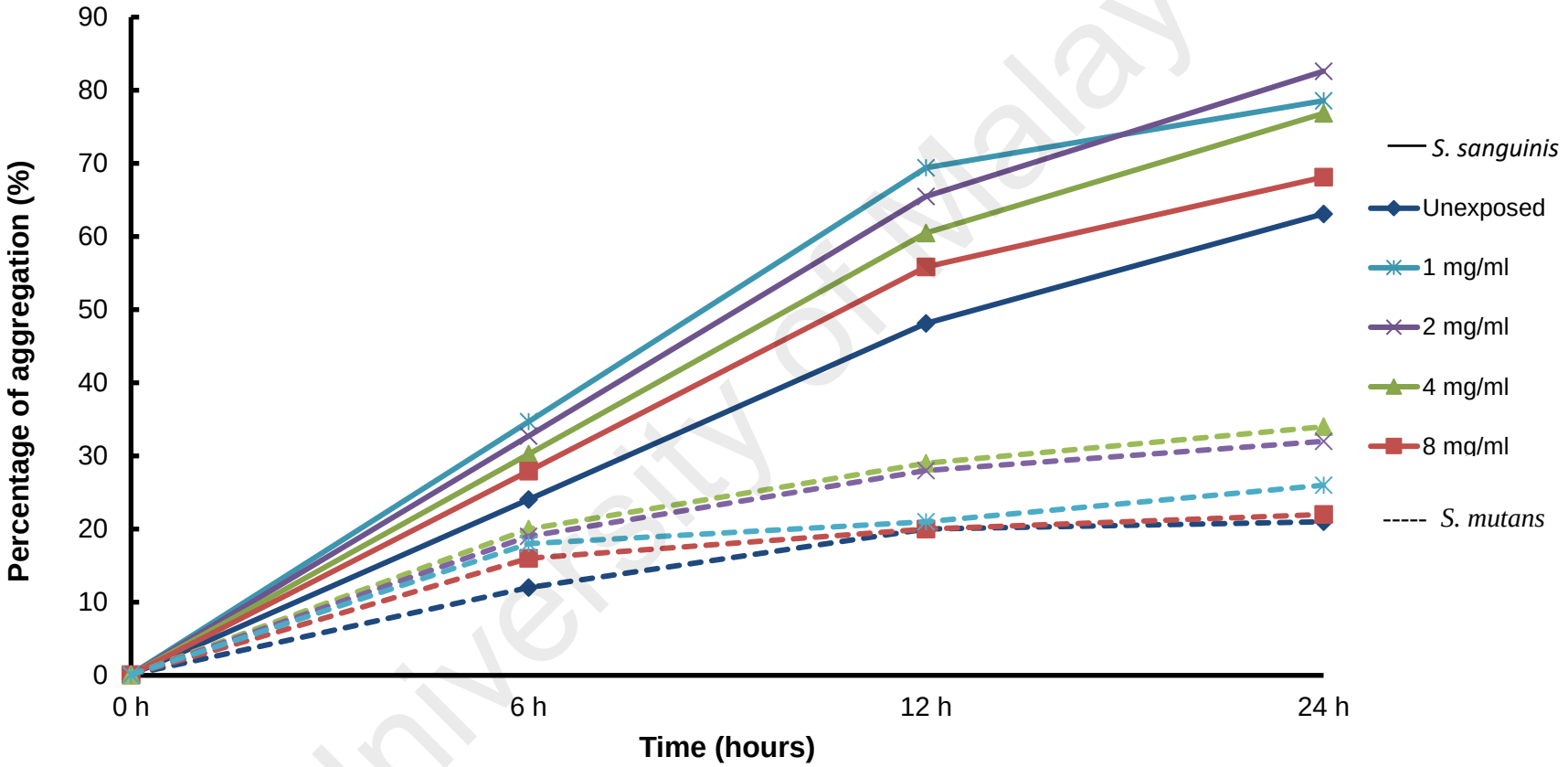



Figure 4.5.2 The effect of nicotine on aggregation of *Streptococcus* cells at different time periods. The solid line represents *S. sanguinis* and the dotted line represents *S. mutans*. The data displayed were the average of three independent experiments (n = 9).

### 4.3 Effect of nicotine on the expression of the adherence-associated Genes

#### 4.3.1 Qualitative gene expression via RT-PCR

##### 4.3.1.1 *Candida* species

The expression of *HWP1* and *ALS3* were investigated in two *Candida* species; *Candida albicans* and *Candida parapsilosis* cultured overnight in YPD broth. The results of gel electrophoresis after RT-PCR are shown in Figure 4.6. *ACT1* was used as the positive control while 100 bp marker was used to identify the approximate size of a molecule run on a gel during electrophoresis.

Based on the data, *C. albicans* and *C. parapsilosis* were shown to have *HWP1* and *ALS3* transcript regulation. However, the expression of *HWP1* and *ALS3* in *C. albicans* and *C. parapsilosis* were influenced by nicotine. Figure 4.6 showed *C. parapsilosis* have broader band for *HWP1* than *C. albicans* whereas for *ALS3*, *C. albicans* have broader band than *C. parapsilosis*. Different intensity of mRNAs was identified in both *Candida* species illustrated that the expression of *HWP1* and *ALS3* were increased in a dose-dependent manner. The addition of nicotine could possibly have disturbed the biological system of the cells and the transcription of the genes (Li *et al.*, 2014; Huang *et al.*, 2015).

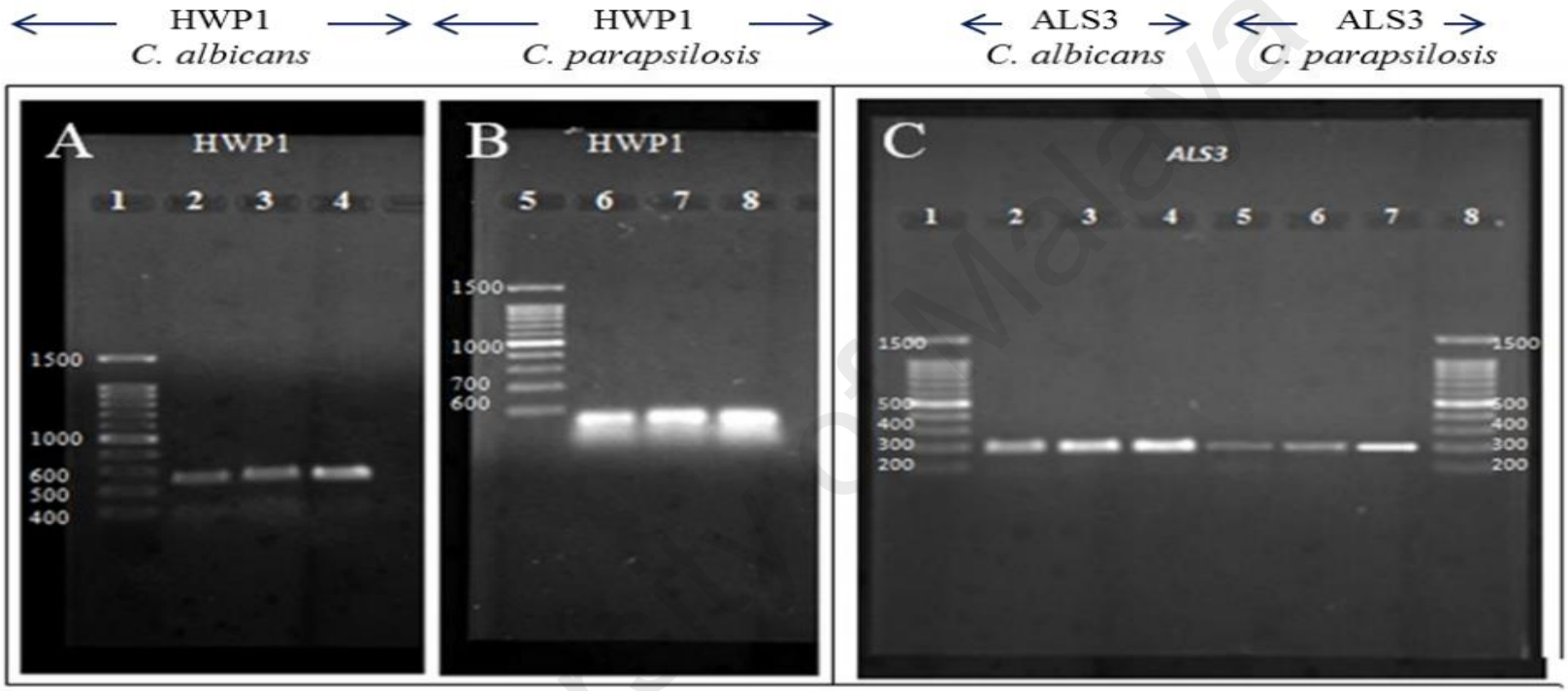



Figure 4.6 (A) Expression of *HWP1* in *C. albicans* at nicotine concentration of 1 and 2 mg/ml. Lane 1: 100bp DNA marker; Lane 2: Unexposed Ca; Lane 3: Ca-exposed to 1 mg/ml nicotine and Lane 4: Ca-exposed to 2 mg/ml nicotine. (B) Expression of *HWP1* in *C. parapsilosis* at 1-2 mg/ml. Lane 5: 100bp DNA marker; Lane 6: Unexposed Cp; Lane 7: Cp-exposed to 1 mg/ml nicotine and Lane 8: Cp-exposed to 2 mg/ml nicotine. (C) Expression of *ALS3* mRNA in *C. albicans* and *C. parapsilosis* following exposure of nicotine at 1 mg/ml and 2 mg/ml. Lane 1 and 8: 100bp DNA marker; Lane 2: Unexposed *C. albicans*; Lane 3: Ca-exposed to 1 mg/ml nicotine; Lane 4: Ca-exposed to 2 mg/ml nicotine; Lane 5: Unexposed *C. parapsilosis*; Lane 6: Cp-exposed to 1 mg/ml nicotine: Lane 7: Cp-exposed to 2 mg/ml nicotine.

#### 4.3.1.2 *Streptococcus* species

The regulation of three binding associated genes; *spaP*, *gtfB* and *gbpB* were studied in *S. sanguinis* and *S. mutans* cultured in BHI for 24 hours. The quantification of *Streptococcus* species mRNAs using RT-PCR were carried out. The results were shown in Figure 4.7. 16sRNA was used as positive control.

The expression of *spaP, gtfB* and *gbpB* transcript were present in both *S. sanguinis* and *S. mutans*. From our observation, we found that *S. mutans* have high regulation of selected transcript than *S. sanguinis*. Thus, explained why *S. mutans* have thicker EPS than *S. sanguinis*. Our findings showed that exposing the bacterial cells to nicotine has apparently influenced the expression of *spaP, gtfB* and *gpbB* transcripts. This showed the requisite of the gene in biofilm formation of *S. sanguinis* and *S. mutans*. The expression level of selected transcript was increased upon the addition of higher concentration of nicotine. In contrast, the DNA band of *S. sanguinis* and *S. mutans* at 2 mg/ml nicotine concentration was thicker and brighter than unexposed and 1 mg/ml nicotine concentration.

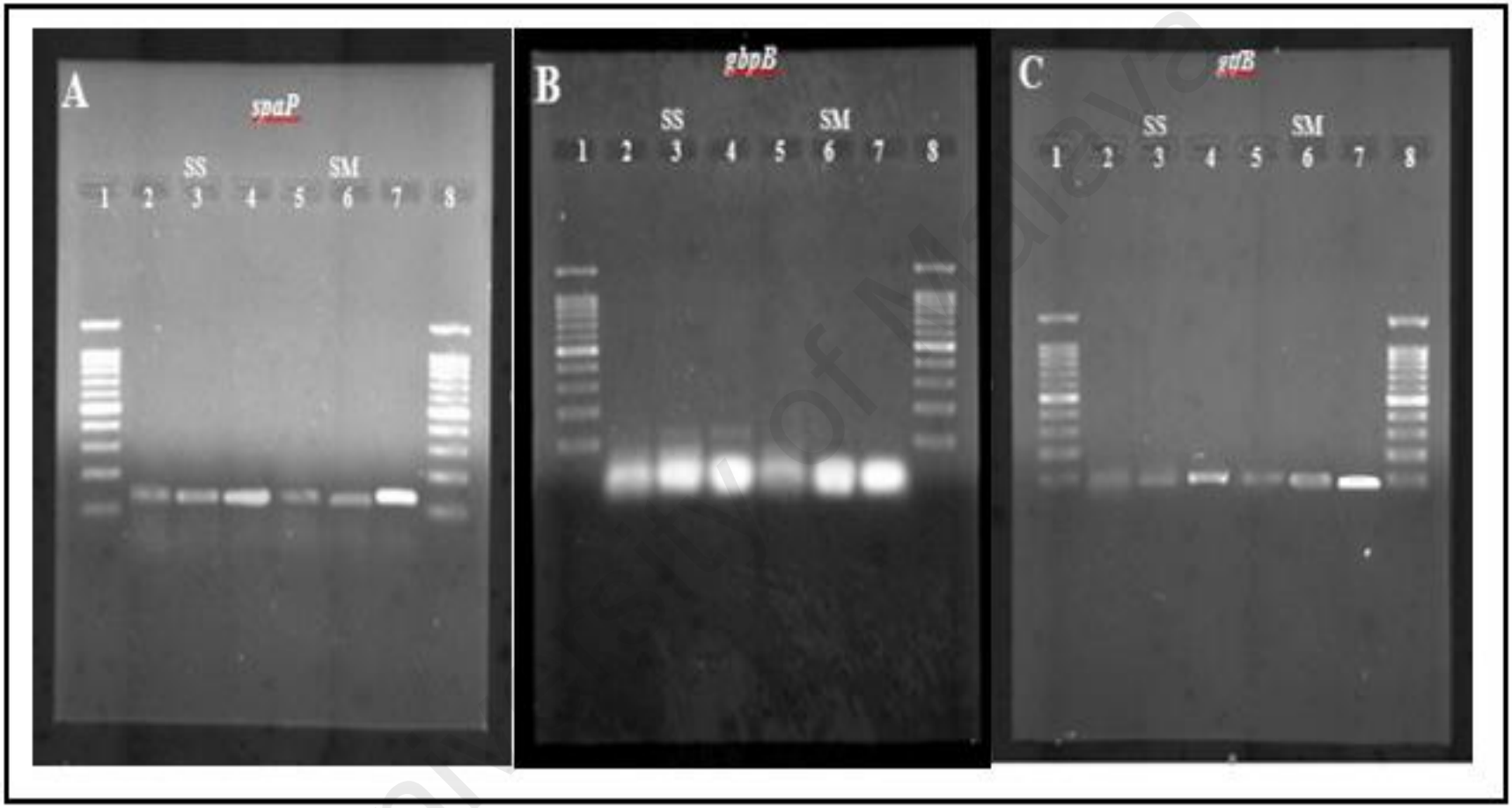



Figure 4.7 (A) Expression of *spaP* gene; (B) Expression of *gbpB* (C) Expression of *gtfB* in *S. sanguinis* and *S. mutans* following nicotine exposure at 1 and 2 mg/ml. Lane 1 and 8: 100bp DNA ladder; Lane 2 and 5: Unexposed bacterial sp; Lane 3 and 6: 1 mg/ml nicotine exposure; Lane 4 and 7: 2 mg/ml nicotine exposure.

### 4.3.2 Quantitative gene expression via real time PCR

#### 4.3.2.1 *Candida* species

Total RNA of *C. albicans* and *C. parapsilosis* were successfully extracted. The concentration and purity of the RNA were also determined. cDNA was successfully synthesized from the standardized total RNA. PCR reaction for *ACT1*, *HWP1* and *ALS3* was optimized using the selected reaction parameters. Real-time PCR (qPCR) was performed and gene expression analysis was shown in Figure 4.8.

Figure 4.6 showed the relative expression of *HWP1* and *ALS3* of *C. albicans* and *C. parapsilosis* upon exposure to 1 mg/ml and 2 mg/ml nicotine concentration. Expression of these genes was found to be significantly changed ($P \leq 0.05$ and $P \leq 0.01$) after being exposed to 1 mg/ml nicotine. This finding showed the requisite of the gene in biofilm formation of *C. albicans* and *C. parapsilosis*. Expression of *HWP1* was significantly upregulated ($P \leq 0.05$) with 1.37-fold changes at nicotine concentration of 1 mg/ml and upregulated by 1.42-fold changes when the cells were exposed with 2 mg/ml nicotine. Expression of *ALS3* was significantly upregulated ($P \leq 0.05$) with 1.78 and 2.11-fold changes both upon exposure to 1 mg/ml and 2 mg/ml nicotine respectively.

The expression of *HWP1* of *C. parapsilosis* was upregulated with 2.14-fold changes at 1 mg/ml nicotine concentration and further upregulated with 2.22-fold changes after being exposed to 2 mg/ml nicotine concentration. In contrast, the expression of *ALS3*

of *C. parapsilosis* at 1 mg/ml nicotine concentration was upregulated with 1.5-fold changes and upregulated further with 1.86-fold changes at 2 mg/ml.

#### 4.3.2.2 *Streptococcus* species

RNA quantification was performed and the purity of the extracted RNA was checked. The cDNA was successfully synthesized from the standardized total RNA. PCR reaction for 16sRNA, *spaP*, *gtfB* and *gbpB* was optimized using the selected reaction parameters. Real-time PCR (qPCR) was performed and gene expression analysis was shown in Figure 4.9.

Figure 4.9 showed the relative expression of *spaP*, *gtfB* and *gbpB* of *S. sanguinis* and *S. mutans* upon exposure to 1 mg/ml and 2 mg/ml nicotine concentration. Expression of these genes was found to be significantly changed ($P \leq 0.05$ and $P \leq 0.01$) after being exposed to 1 mg/ml nicotine. Expression of *spaP* was significantly upregulated ($P \leq 0.05$) with 1.45-fold changes at nicotine concentration of 1 mg/ml and was further upregulated to 1.55-fold changes when the cells were exposed with 2 mg/ml nicotine. Expression of *gtfB* was significantly upregulated ($P \leq 0.05$) with 1.34 and 1.65-fold changes both upon exposure to 1 mg/ml and 2 mg/ml nicotine respectively. Meanwhile, the expression of *gbpB* at 1 mg/ml and 2 mg/ml were upregulated with 1.50 and 1.67-fold changes respectively.

The expression of *spaP* of *S. mutans* was upregulated with 1.57-fold changes at 1 mg/ml nicotine concentration and further upregulated when exposed to 2 mg/ml nicotine concentration. In contrast, expression of *gtfB* of *S. mutans* at 1 mg/ml nicotine concentration was upregulated with 1.46-fold changes and begins to be upregulated further with 1.78-fold changes at 2 mg/ml. Similar pattern of increment was observed in *gbpB* expression of nicotine-exposed *S. mutans*. At 1 mg/ml, the expression of *gbpB* was upregulated with 1.69-fold changes. Upon exposing the cells to 2 mg/ml, the expression of *gbpB* was found to be further upregulated with 1.94-fold changes.

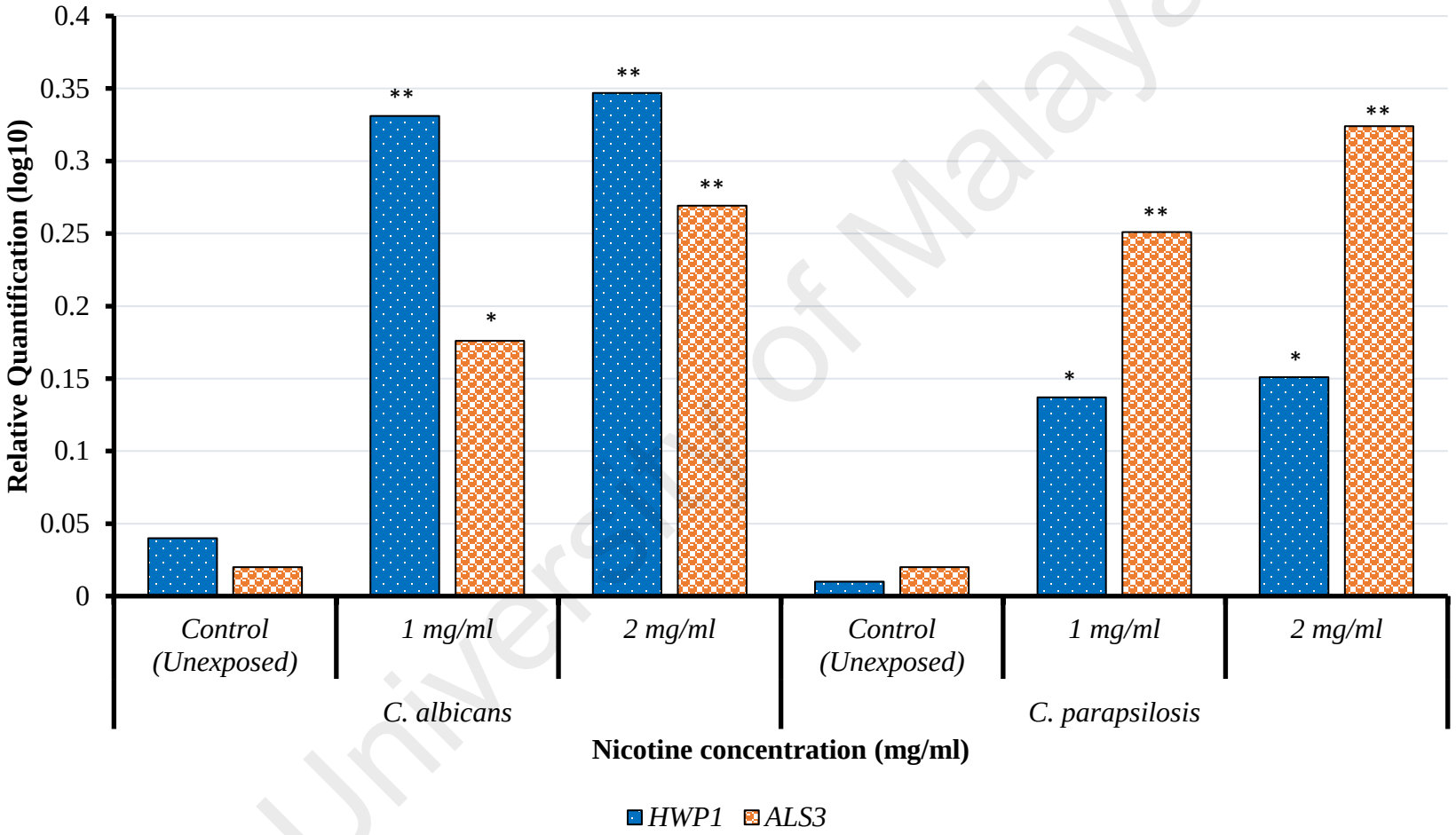



Figure 4.8 Relative expression of *HWP1* and *ALS3* of *C. albicans* and *C. parapsilosis* after exposed with nicotine. Each data point represents the mean ± SD (n = 3). Asterisks (*) and (**) label indicate p-value of < 0.05 and < 0.001, respectively.

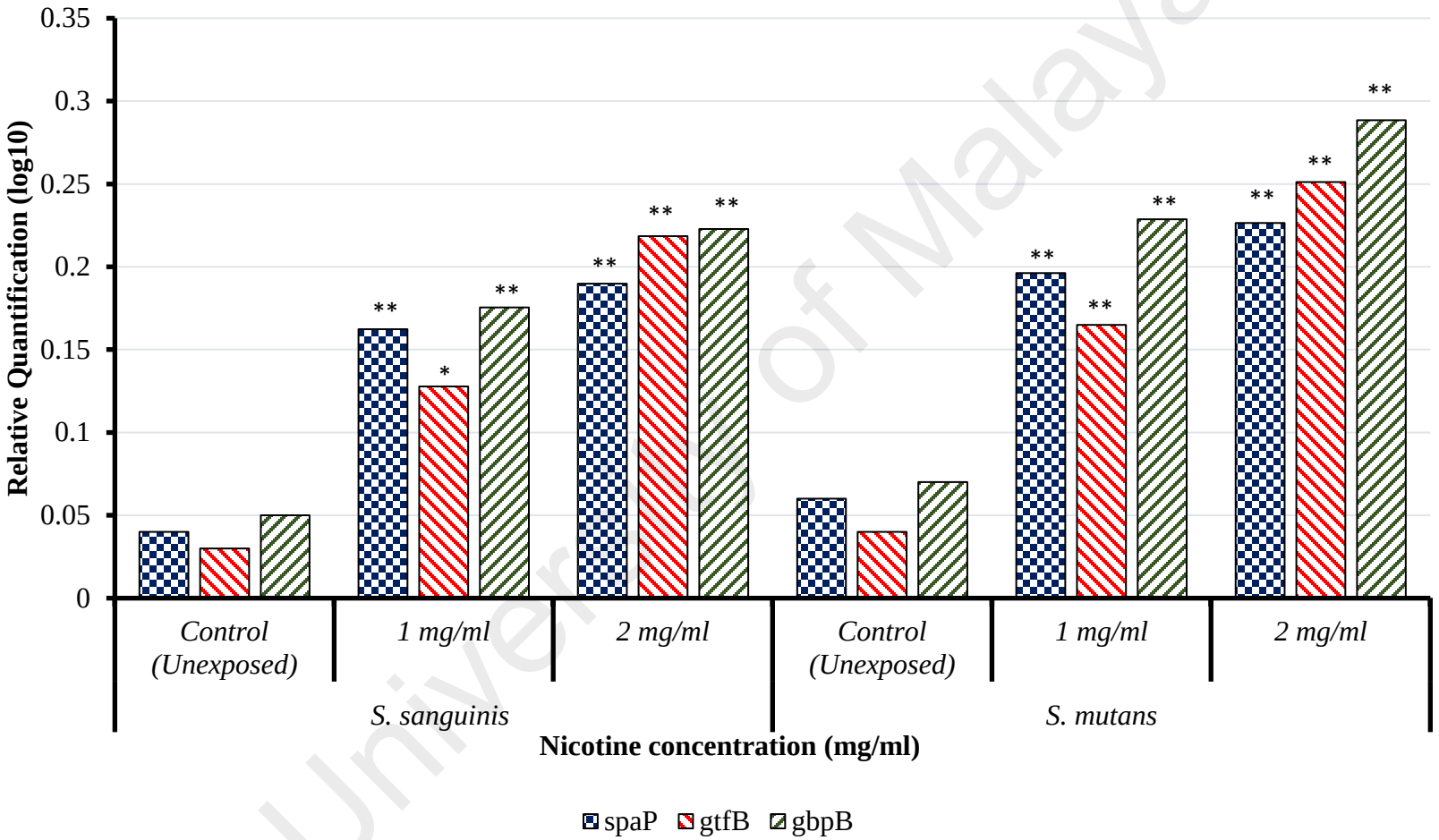



Figure 4.9 Relative expression of *spaP*, *gtfB* and *gbpB* of *S. sanguinis* and *S. mutans* after exposed with nicotine. Each data point represents the mean ± SD (n = 3). Asterisks (*) and (**) label indicate p-value of < 0.05 and < 0.001, respectively.

## 4.4 Effect of nicotine on the structure of extracellular polysaccharide and micro-colonies formation by *Candida* sp. and *Streptococcus* sp.

We formed the biofilms on Lab-Tek Slide Chamber and visualised them using CLSM to analyse their morphology, structure and architecture characteristics and the differences between biofilms developed by *S. sanguinis, S. mutans, C. albicans,* and *C. parapsilosis*. Confocal laser scanning microscopy observations have given a more detailed characterization of the main structure and three-dimensional characteristics of the *Streptococcus* sp. and *Candida* sp. biofilms under the influenced of nicotine. Live cell staining (syto_9) has been used as indicator of cell viability as determined by the integrity of cell wall membrane in many bacterial and candidal populations, including biofilm. The thickness of extracellular polymeric material is a crucial factor characterizing mature biofilms. Alexa fluor_647 conjugate selectively binds to polysaccharide including α-mannopyranosyl and α-glucopyranosyl residues and gives red fluorescence. The Calcofluor White Staining was used as non-specific fluorochrome to bind to the cellulose and chitin found in the cell walls of *Candida* sp. which gives blue fluorescence.

Based on the Z-value, the biofilms formed by *C. albicans* and *C. parapsilosis* upon exposure of nicotine stained with Syto_9 (green) were thicker than in *Candida* sp. without exposure to nicotine (Figure 4.16). Confocal images revealed that *Candida* sp. biofilms were embedded in EPS, as shown by red fluorescence. Biofilms formed in the presence of 1 mg/ml nicotine concentration demonstrated a higher cellular density along with high production of EPS seen in red. The *C. albicans* biofilms are thicker than those formed by

control group. Moreover, we found that the architecture and composition of the *Candida* sp. biofilm formed after being exposed with 2 mg/ml nicotine concentration was denser and heavier than unexposed. The distribution of candidal cells and EPS stained with Ruby EPS (red) of the 2 mg/ml nicotine group was significantly different from the control and 1 mg/ml nicotine groups. The gap between the biofilms were occupied with abundant of candidal cells and the biofilm was composed of a few layers of yeast cells. Figure 4.10 and Figure 4.11 shows confocal laser scanning microscopy images of *Candida albicans* ATCC 14053 and *C. parapsilosis* ATCC 22019 biofilms. Live bacteria were labelled green (Syto_9) and EPS was labelled red (Alexa Flour 647). Images were obtained at ×60 magnification. The structure of *C. albicans* and *C. parapsilosis* biofilm of unexposed (control) and exposed with 1, and 2 mg/ml nicotine in 24 h. As shown in confocal micrographs, following a 24-h attachment phase of the yeast cells at 2 mg/ml nicotine concentration, we observed that *C. albicans* had switched to hyphal form (elongated tube-like blastoconidia were seen) (Figure 4.14). We noted the blue fluorescence intensity readings were higher in 2 mg/ml nicotine exposed- *C. albicans* than unexposed, thus potentially indicating high metabolic activity of *C. albicans* cells despite their thickness and density. The results of the confocal laser scanning microscopy (CLSM) show the total fluorescence intensity of biofilms had significantly increased in 1 mg/ml and 2 mg/ml groups and the candidal cells as well as EPS were more abundant after being exposed with nicotine (Figure 4.15).

There was an obvious increased in *S. mutans* biofilm mass (stained with green) exposed with 1 and 2 mg/ml of nicotine compared to the biofilm without nicotine. The

biofilm structure of *S. mutans* was much denser and the bacterial density was heavier as the nicotine increases. In addition, nicotine also enhanced the production of EPS (stained with red) of *S. mutans*. Similar pattern of results was observed in *S. sanguinis*. The biofilm structure of *S. sanguinis* was much denser than unexposed biofilm as the concentration of nicotine increased. The EPS production of *S. sanguinis* biofilm was increased as well with the increasing nicotine concentration. Figure 4.12 and Figure 4.13 shows confocal laser scanning microscopy images of *Streptococcus mutans* ATCC 25175 and *Streptococcus sanguinis* BAA 4155 biofilms. Live bacteria were labelled green (Syto_9) and EPS was labelled red (Alexa Flour 647). Images were obtained at ×60 magnification. The structure of *S. mutans* and *S. sanguinis* biofilm of unexposed (control) and exposed with 1, and 2 mg/ml nicotine in 24 hours.

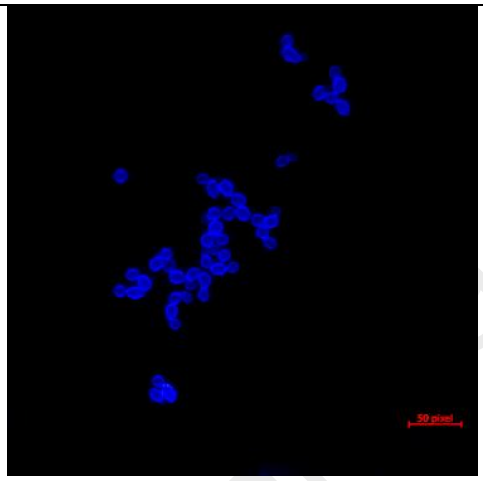
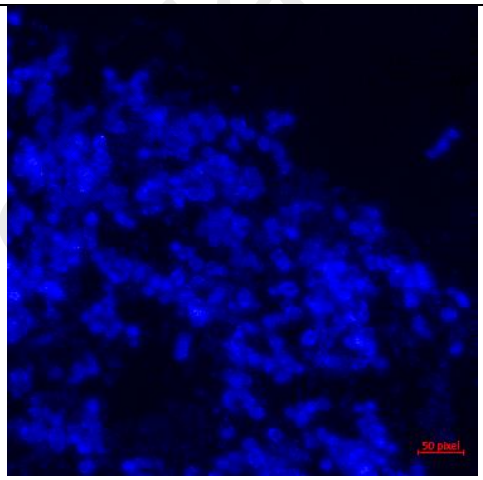
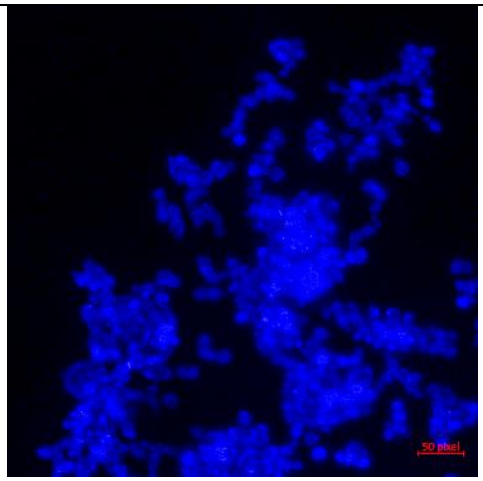
| *Candida albicans* | Membrane |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.10.1** CLSM *C. albicans* cell wall (Scale: 50 µm)

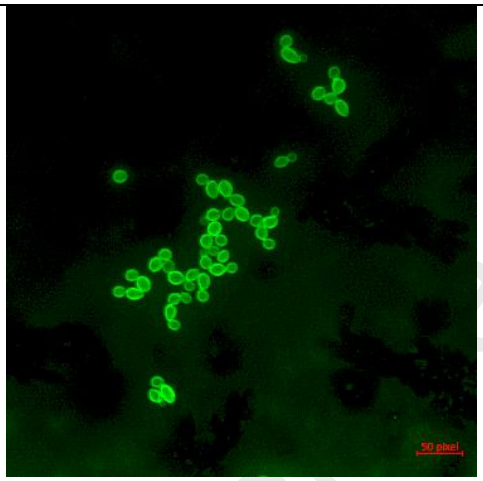
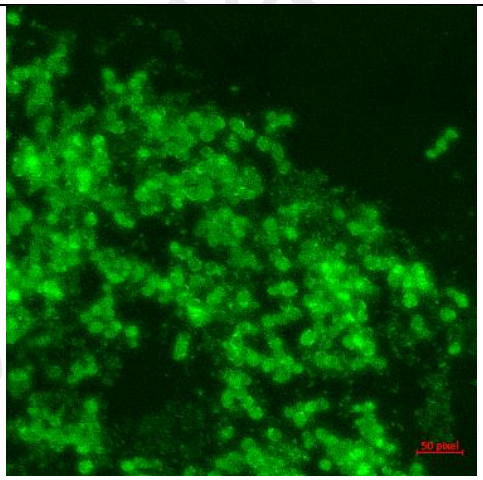
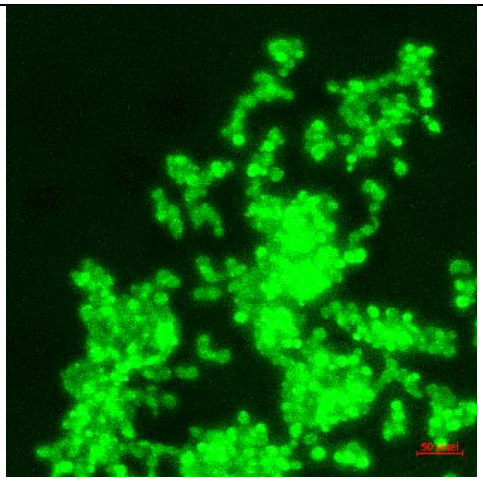
| *Candida albicans* | Live cells |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.10.2** CLSM *C. albicans* live cells (Scale: 50 µm)

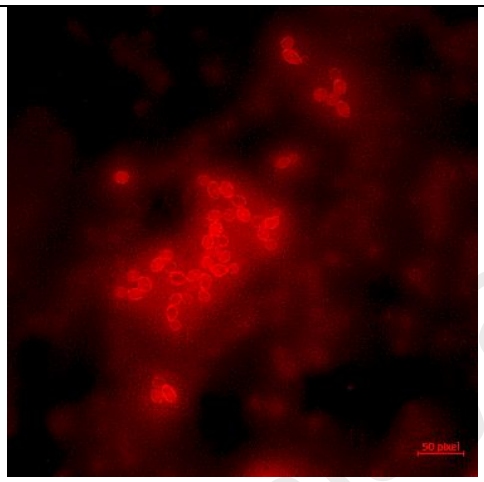
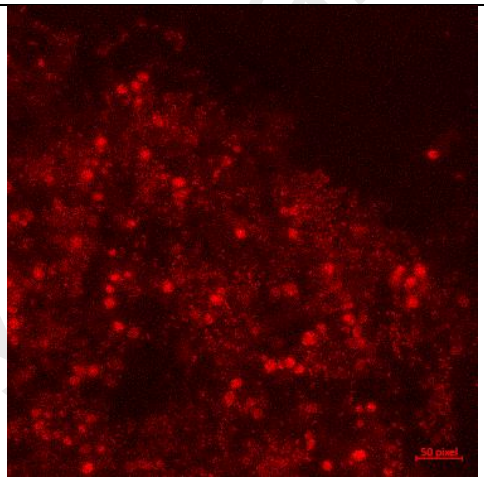
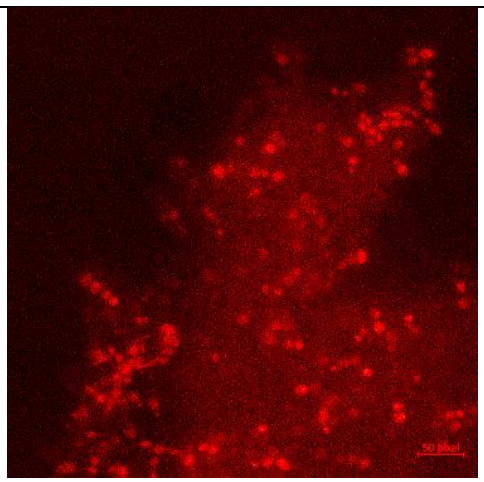
| *Candida albicans* | EPS |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.10.3** CSLM *C. albicans* EPS (Scale: 50 µm)

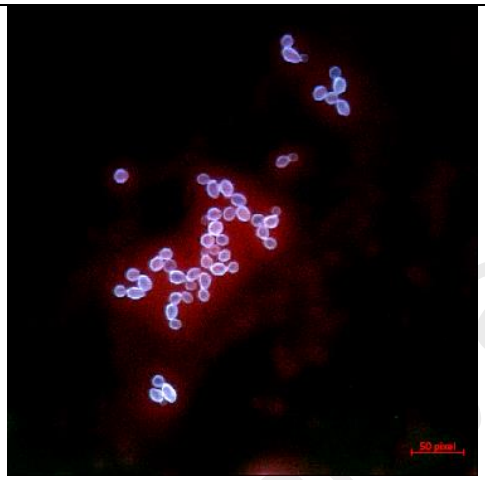
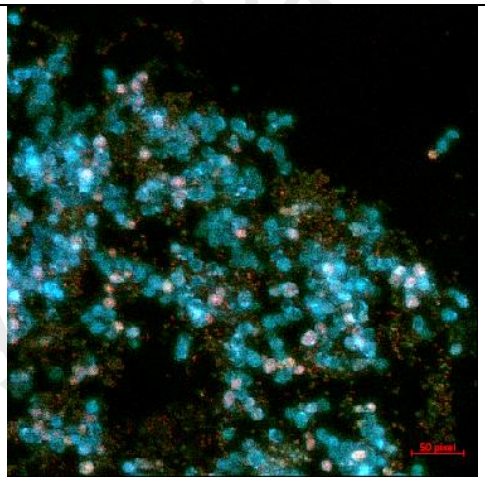
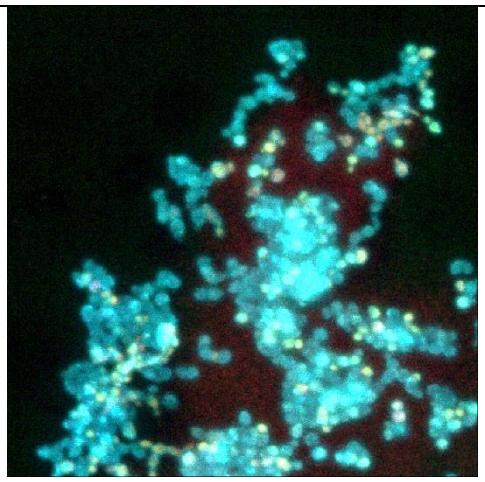
| *Candida albicans* | Overlay |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.10.4** CLSM *C. albicans* Overlay (Scale: 50 µm)

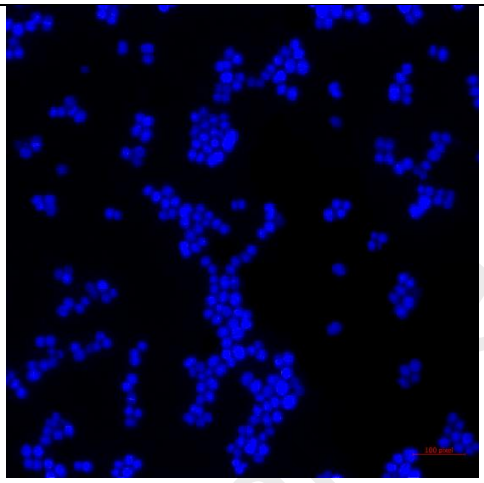
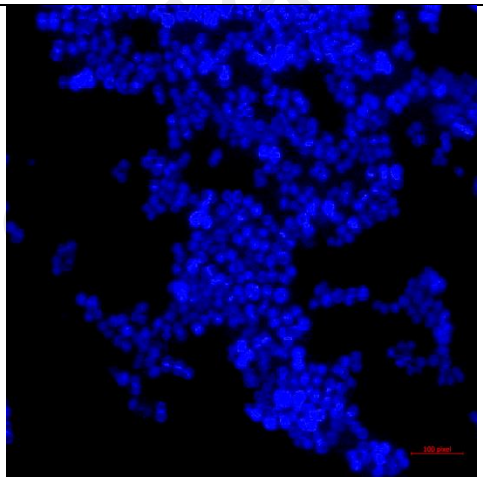
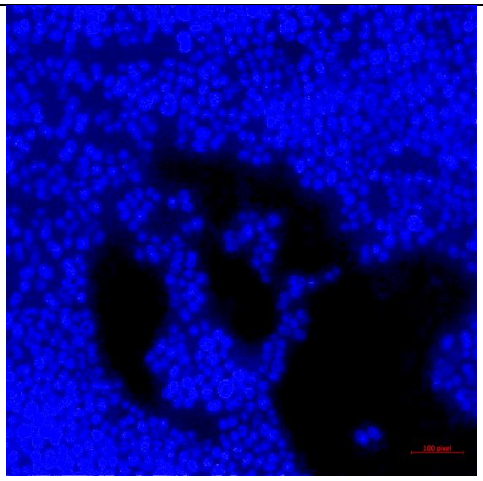
| *Candida parapsilosis* | **Membrane** |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.11.1** CLSM *C. parapsilosis* cell wall (Scale: 50 µm)

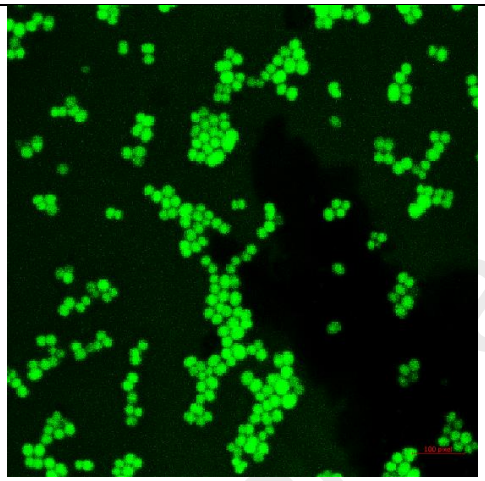
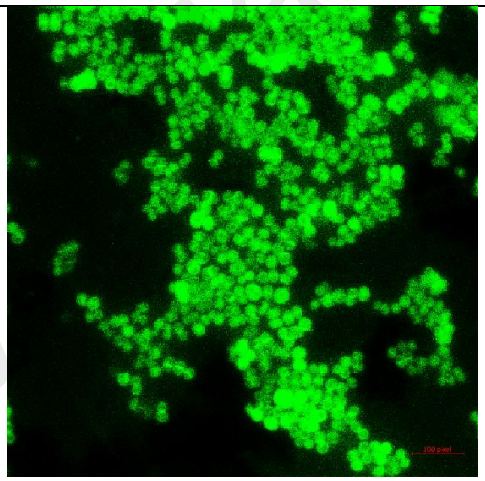
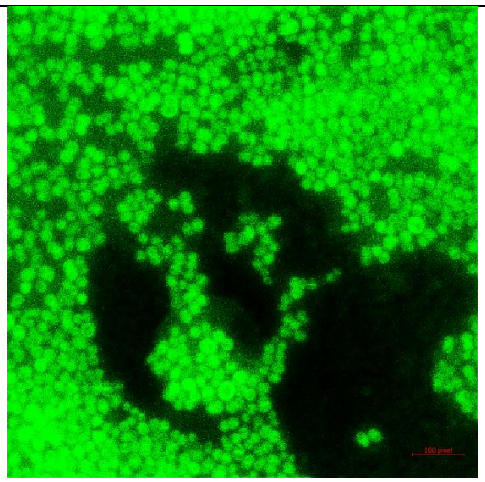
| *Candida parapsilosis* | **Live cells** |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.11.2** CLSM *C. parapsilosis* live cells (Scale: 50 µm)

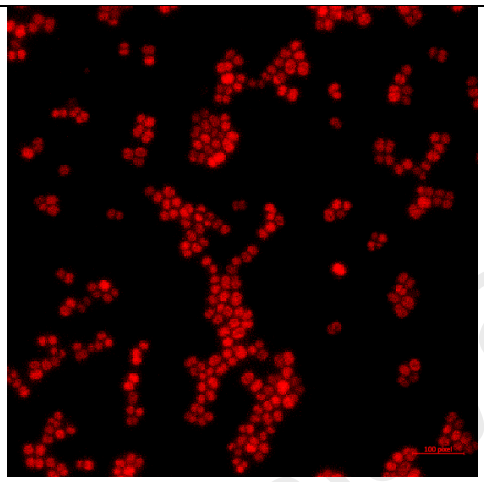
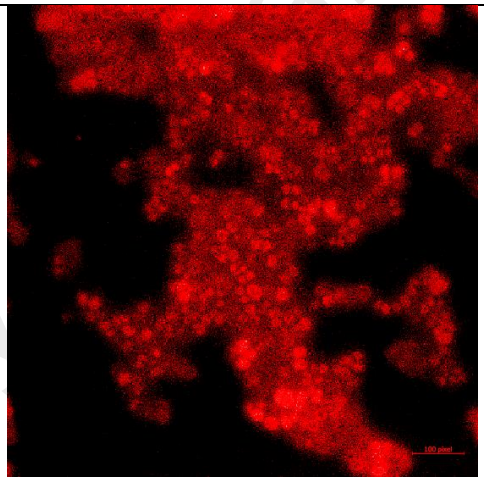
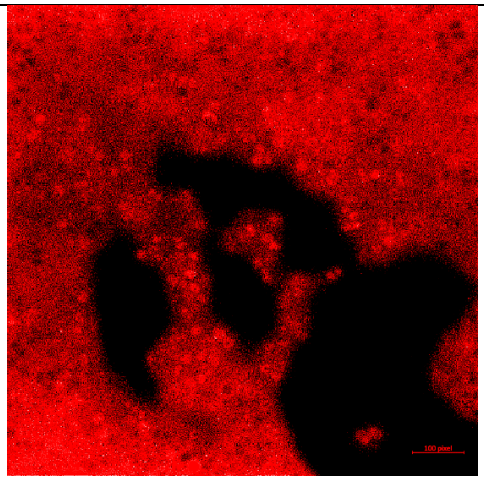
| *Candida parapsilosis* | EPS |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.11.3** CLSM *C. parapsilosis* EPS (Scale: 50 µm)

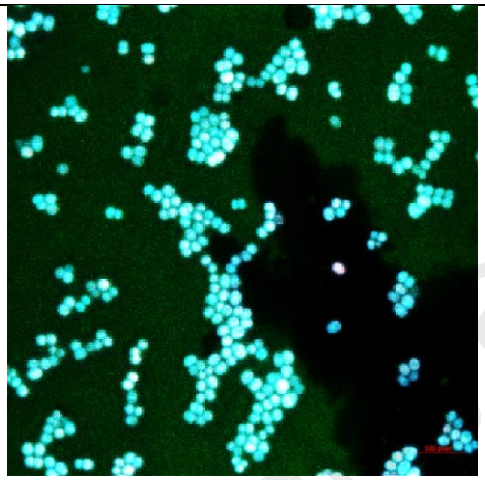
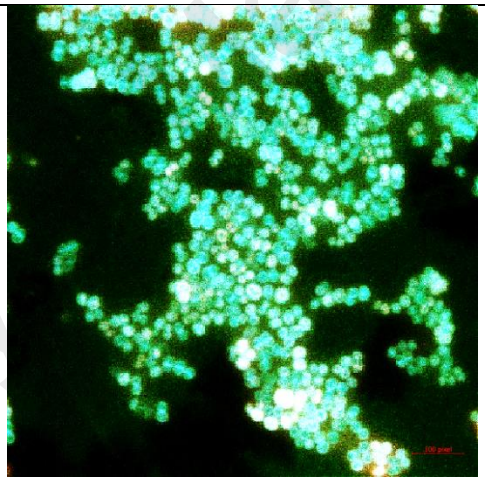
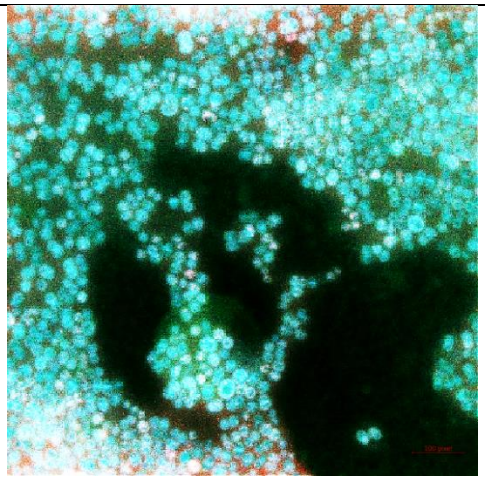
| *Candida parapsilosis* | Overlay |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.11.4** CLSM *C. parapsilosis* Overlay (Scale: 50 µm)

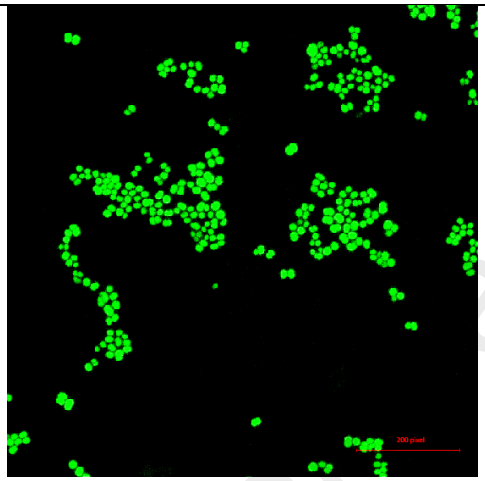
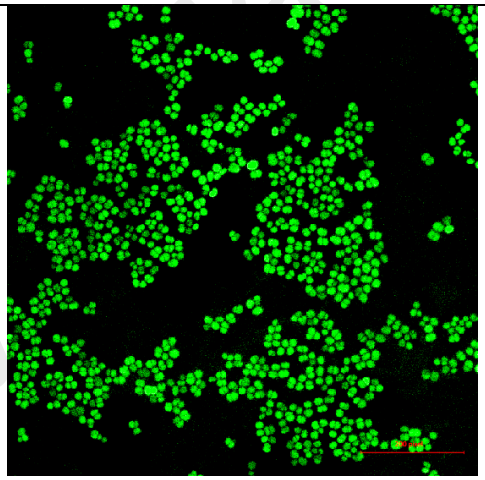
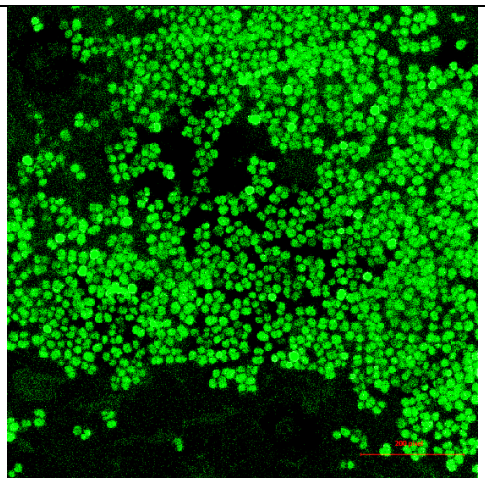
| *Streptococcus mutans* | **Live cells** |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.12.1** CLSM *S. mutans* live cells (Scale: 50 µm)

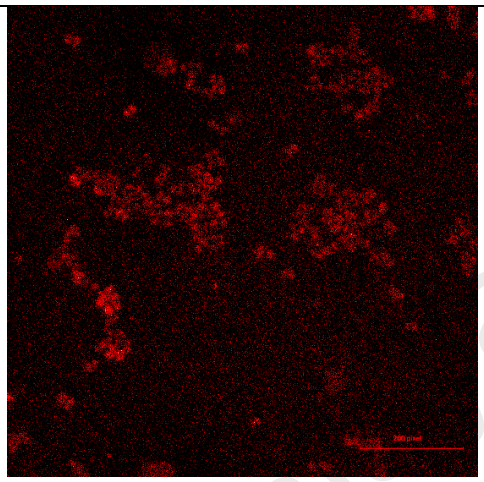
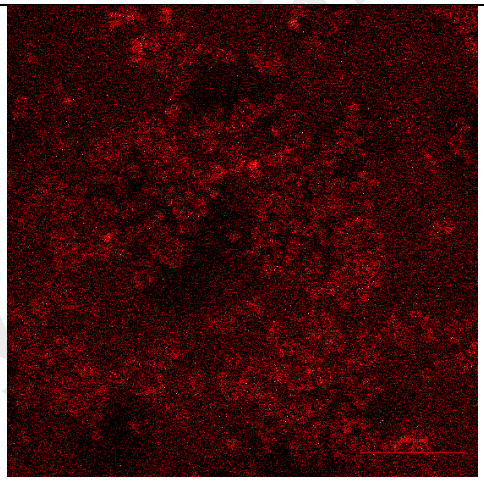
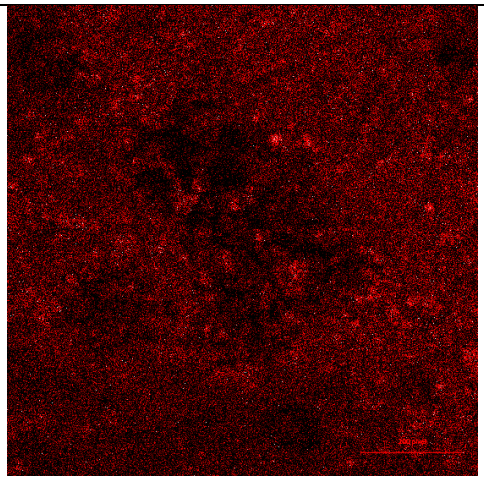
| *Streptococcus mutans* | EPS |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.12.2** CLSM *S. mutans* EPS (Scale: 50 µm)

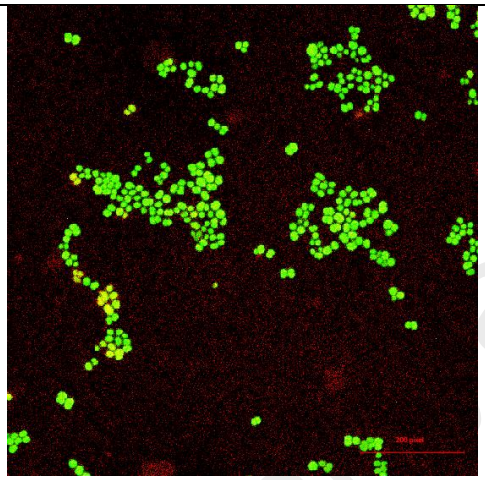
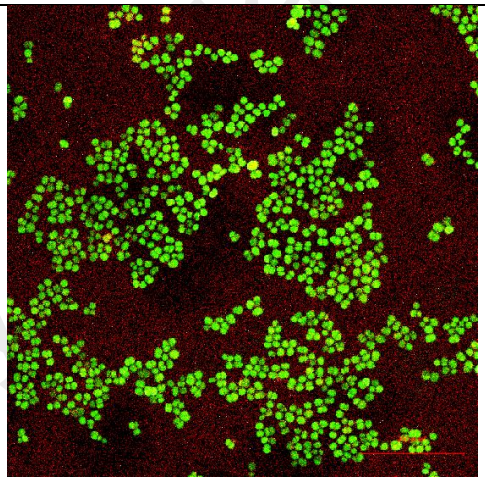
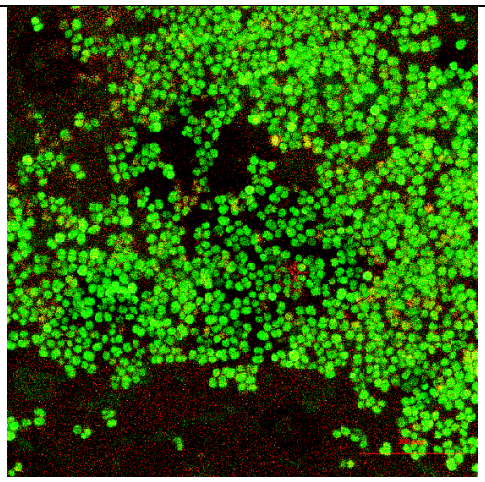
| *Streptococcus mutans* | Overlay |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.12.3** CLSM *S. mutans* Overlay (Scale: 50 µm)

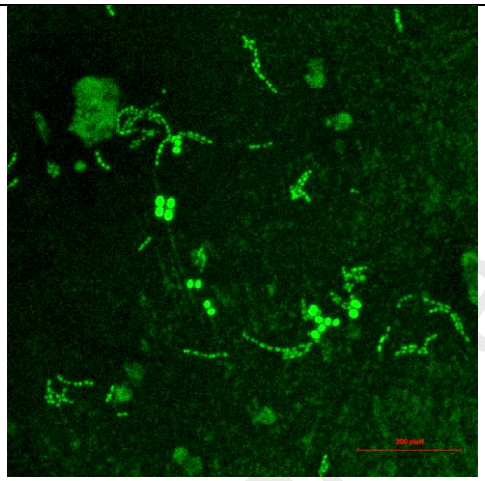
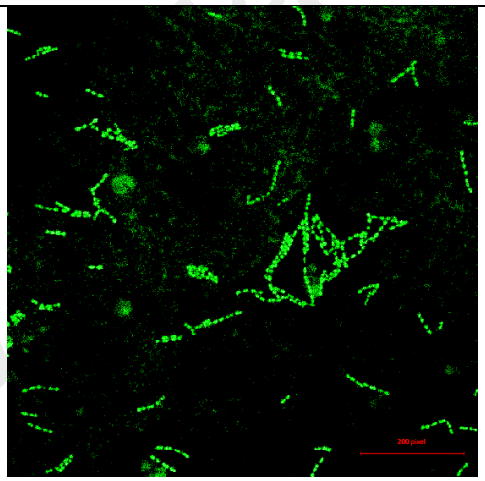
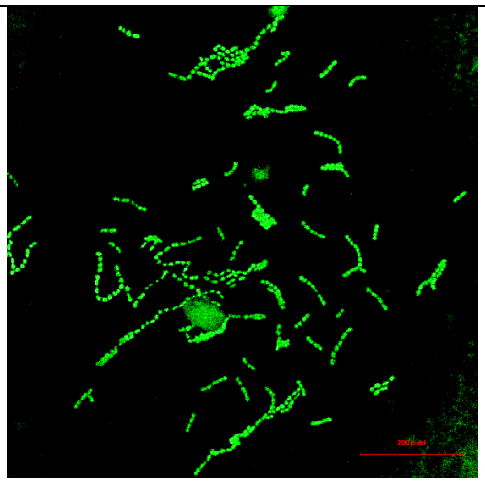
| *Streptococcus sanguinis* | **Live cells** |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.13.1** CLSM *S. sanguinis* live cells (Scale: 50 µm)

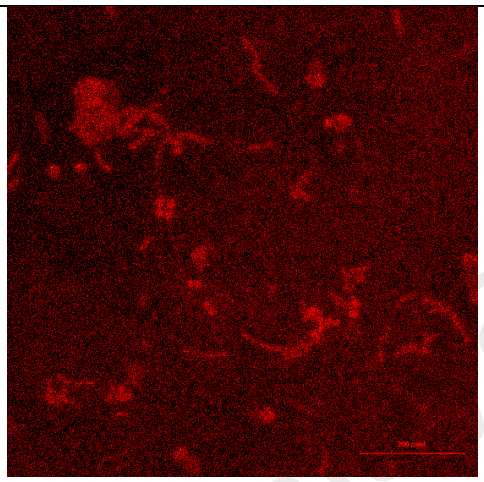
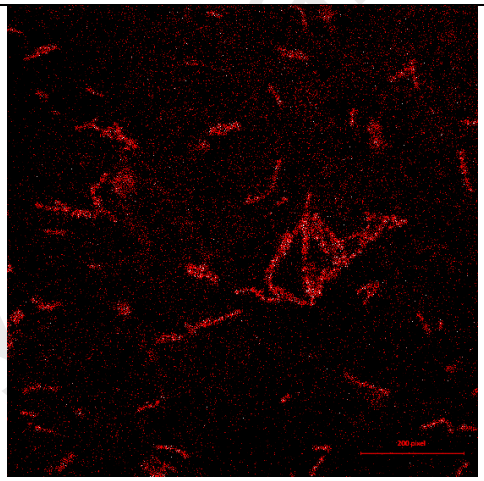
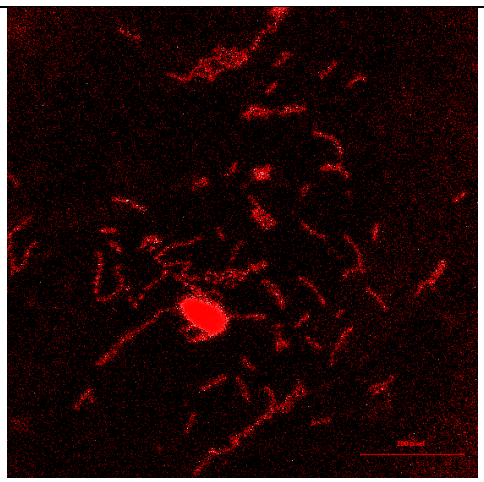
| *Streptococcus sanguinis* | EPS |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.13.2** CLSM *S. sanguinis* EPS (Scale: 50 µm)

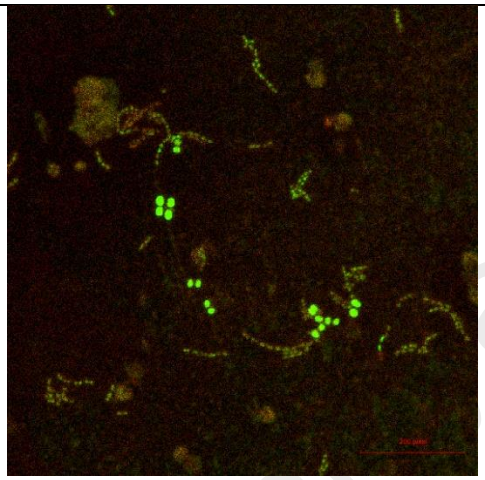
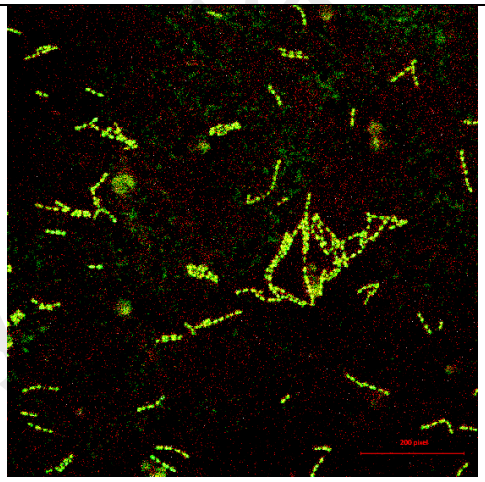
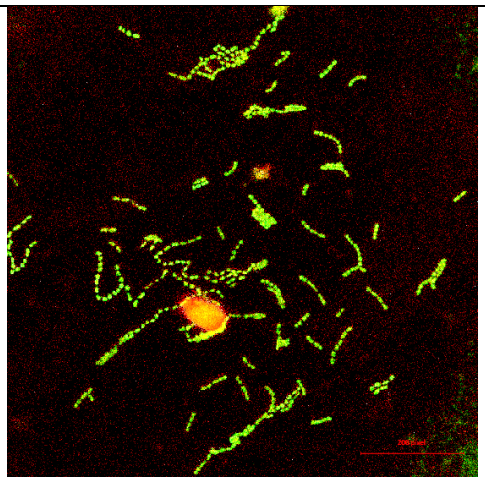
| *Streptococcus sanguinis* | Overlay |
|---|---|
| **Control (unexposed)** |  |
| **1 mg/ml Nicotine** |  |
| **2 mg/ml Nicotine** |  |

**Figure 4.13.3** CLSM *S. sanguinis* Overlay (Scale: 50 µm)

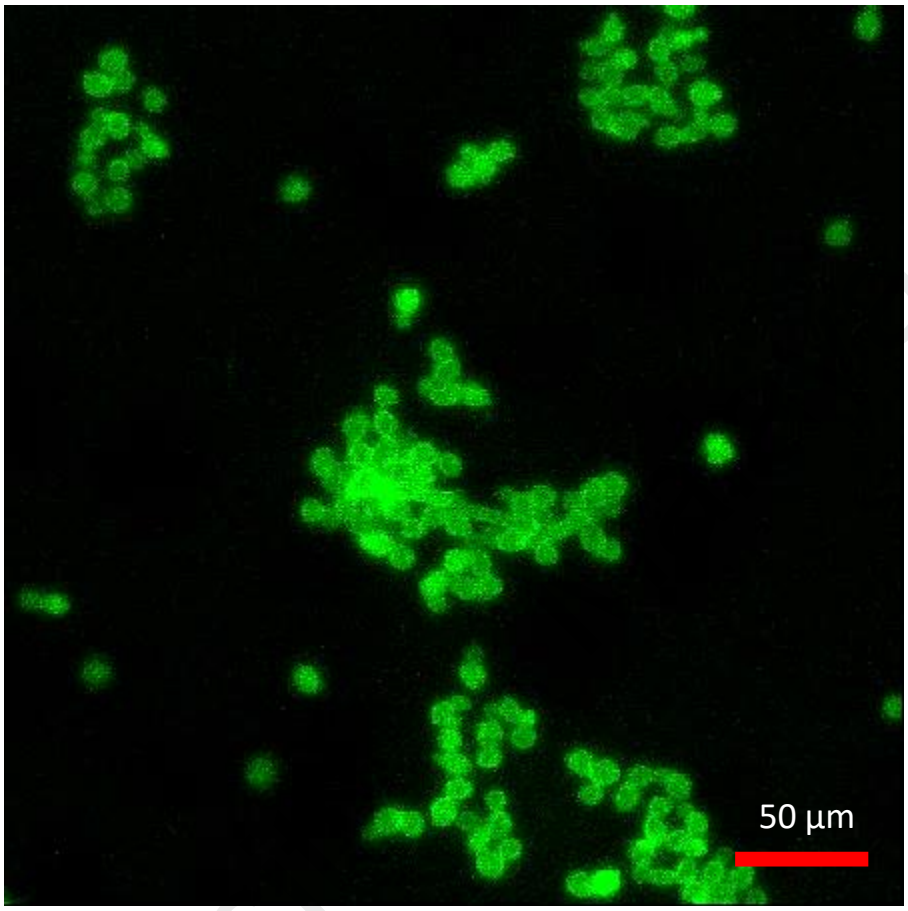



**Figure 4.14** Hyphal form (elongated tube-like blastoconidia) was observed under CLSM when *C. albicans* cells were exposed to 2 mg/ml nicotine (50 µm scale)

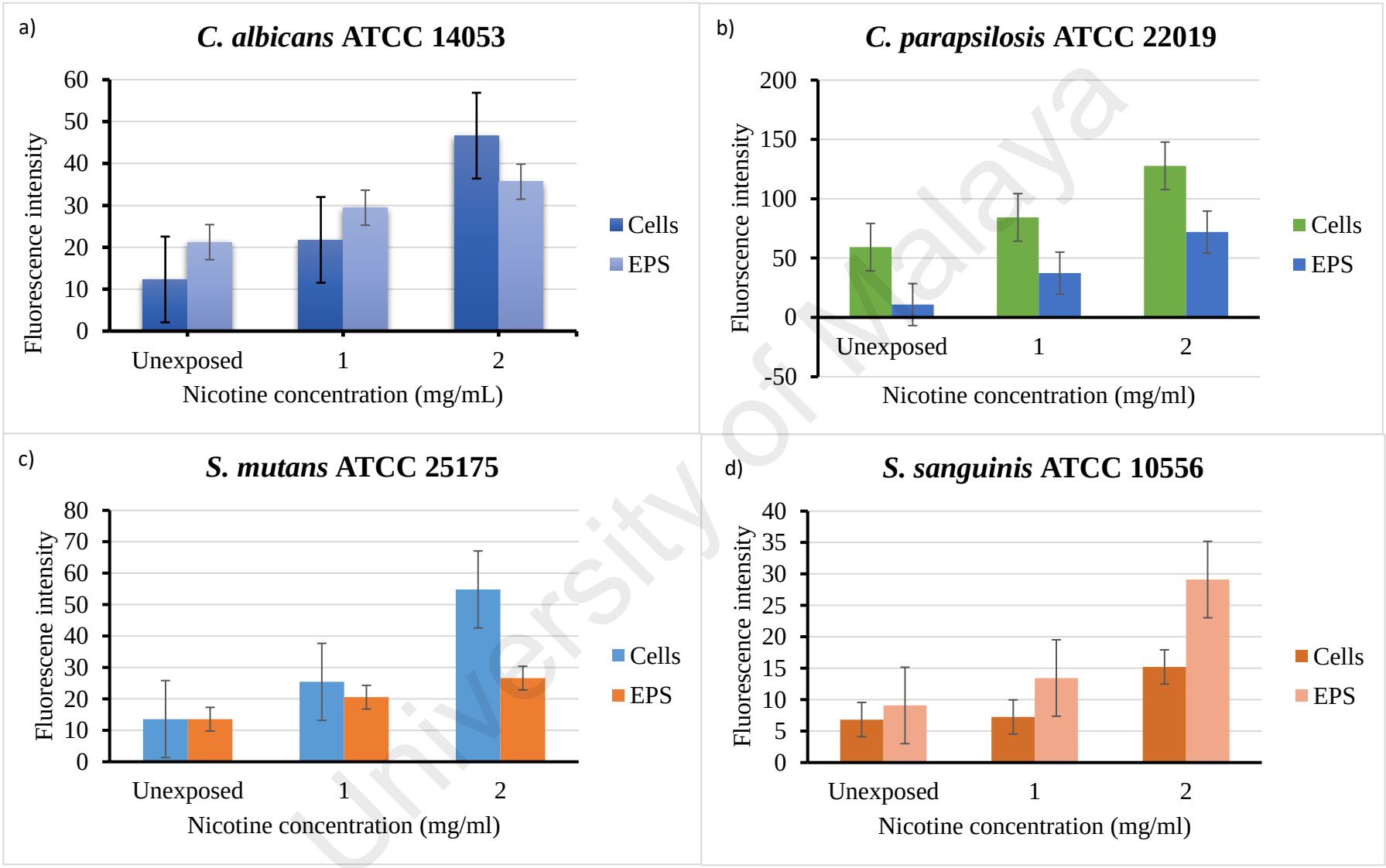





**Figure 4.15** The effect of nicotine on the fluorescence intensity of cells and EPS of (a) *C. albicans* (b) *C. parapsilosis* (c) *S. mutans* and (d) *S. sanguinis*

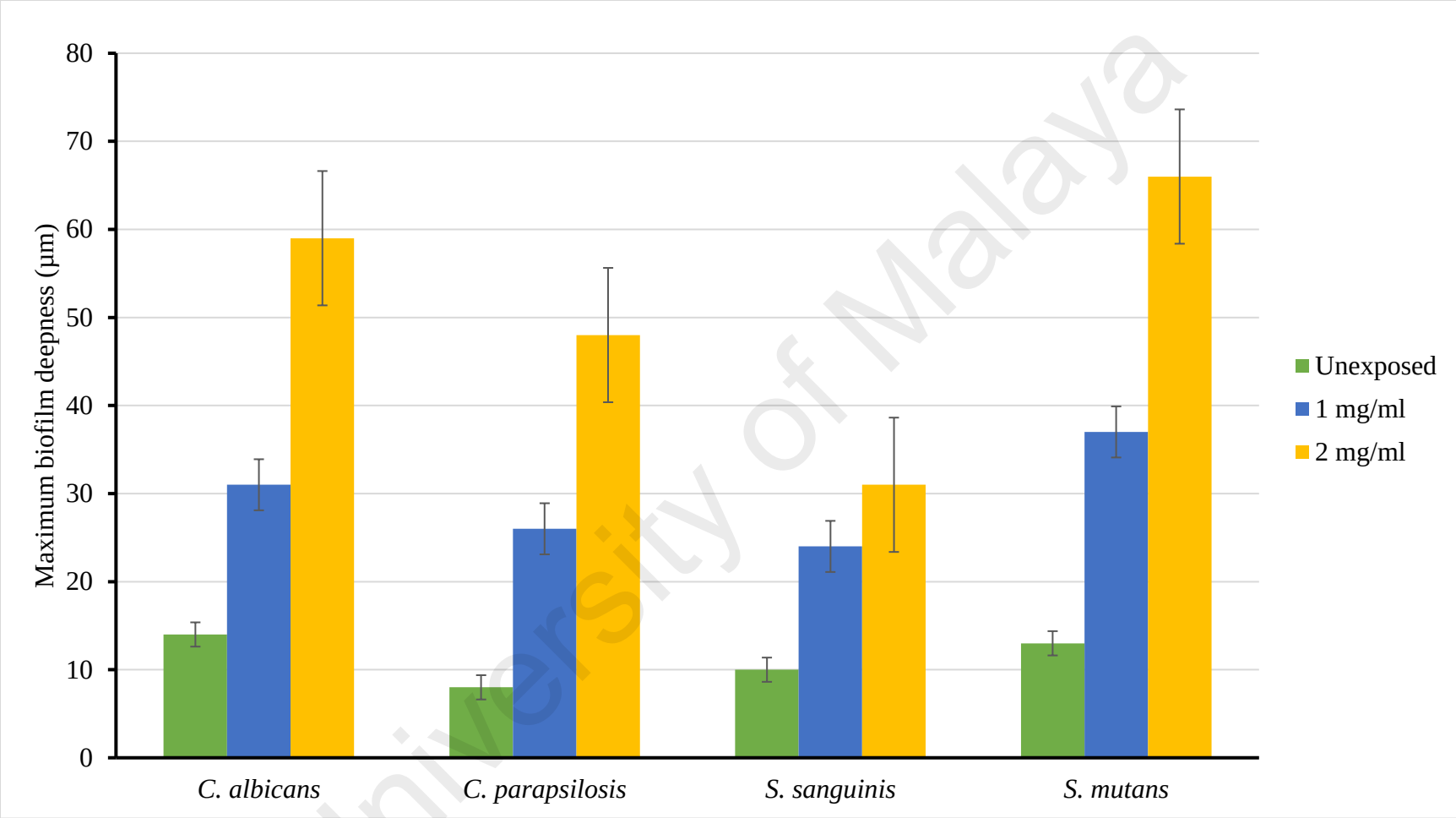





Figure 4.16 Effect of nicotine on biofilm maximum depth of *C. albicans*, *C. parapsilosis*, *S. mutans* and *S. sanguinis*

# CHAPTER 5: DISCUSSION

## 5.1 Antimicrobial effect of nicotine on *Streptococcus* sp. and *Candida* sp.

It is estimated that tobacco smoke contains more than 4000 different compounds such as tar, carbon monoxide, toxic substances, additive cholinomimetic drug such as nicotine. An average cigarette consists of 10 to 14 mg of nicotine, and it is proven that about 1 to 1.5 mg of nicotine is absorbed by our respiratory system during smoking (Institute for Public Health, 2011). Some studies have discussed the presence of nicotine in saliva and gingival crevicular fluid (McGuire *et al.,* 1989; Robson *et al.,* 2010; Curvall *et al.,* 1990). The prevalence of bacterial and fungal infections in the oral cavity have been changed due to the presence of nicotine in the oral fluids. Therefore, we began to embark on serious efforts to investigate how nicotine, a major component of cigarette alters the expression of specific microbial genes that are associated with the adherence of those microbes to the surfaces of oral tissues and influence the adhesion of *C. albicans, C. parapsilosis, S. sanguinis* and *S. mutans*.

The physiological concentration of nicotine in the saliva of smokers was reported in the range of 0.40 to 2.27 mg/ml (Feyerabend *et al.,* 1982; Robson *et al.,* 2010). The concentration is highly variable and depends upon the volume of saliva secreted in oral cavity, nicotine exposure duration, and the technique used for measurement (Curvall *et al.,* 1990). Besides that, other factors such as amount of cigarette consumed, properties of saliva either stimulated or unstimulated and the types of tobacco may have influenced the levels of nicotine in the saliva (Robson *et al.,* 2010; Hoffmann and Adams, 1981). Interestingly, even non-smokers had some detectable amount of nicotine in their urine or

saliva and some of them have similar concentration as light smokers (Feyerabend *et al.*, 1982).

In previous studies, nicotine has been reported to increase the activity of *P. gingivalis* (Baek *et al.*, 2012) as well as *S. aureus* (McEachern *et al.*, 2015) in the oral cavity. Despite the many biological activities of nicotine, to our knowledge, there is little information with regards to their effect on the molecular and structure of oral biofilm consisting of *Streptococcus* sp. and *Candida* sp. In the present study, the mechanisms of pathogenesis involving biofilms of both species under the influence of nicotine were studied. The effect of nicotine on the growth rates and biofilm formation of *Streptococcus* sp. and *Candida* sp., and the expression of the interest genes of the microbial species in normal and stressed environment were investigated to provide better healthcare for the heavy smokers to reduce dental biofilm. Besides that, the influence of nicotine on the adherence mechanisms of oral microbes and EPS were studies as well. In addition, the outcomes of this research in overall may applied to smoking cessation measures in smokers and may aid in producing guidelines for control and prevention of biofilm-associated oral diseases such as dental caries and candidiasis.

The concentration of nicotine used in the study was based from previous reported findings. Thus, in our study, nicotine was added to the cell culture suspension to give a final concentration of 1, 2, 4, and 8 mg/ml. Based on the MIC and MBC including MFC determinations, the antimicrobial effect of nicotine on *Candida* sp. were greater than *Streptococcus* sp. *Candida* cells have a larger surface area than *Streptococcus* sp. and have

led to a higher amount of nicotine required to provide the killing effect which was observed from an increase in nicotine level.

In normal environment, the duration of the log phase of *Streptococcus* sp. and *Candida* sp. was observed at range of 7 to 8 h indicating that different genus have various replication time when exposed to different growth environment (Table 4.2). Among the four microorganisms, *C. albicans* and *S. sanguinis* showed the longest log phase whereas *C. parapsilosis* and *S. mutans* showed the shortest in normal and exposed conditions (Figure 4.2).

It was found that nicotine has influenced the activity of *C. albicans*, *C. parapsilosis, S. sanguinis* and *S. mutans*. The growth of *Candida* sp. and *Streptococcus* sp. were rapidly increased at nicotine concentration of 1 to 2 mg/ml compared to cells without exposure of nicotine. Two parameters; the doubling time (g) and specific-growth rates (μ) of four microbial sp. were studied together with growth rates. Specific growth rate and doubling time were calculated to compare the frequency distribution of the growth rate variables in relation to the growth rate of candidal and bacterial cells without nicotine exposure. Both parameters under the influence of nicotine were increased compared to unexposed. It was shown that the higher the concentration of nicotine used, the longer the log phase of its growth curves. The presence of nicotine has increased the multiplication of microbial cells resulting in longer log phases. When the microbial cells were exposed to nicotine, the log phases of *Streptococcus* sp. and *Candida* sp. were increased too. These may favour the transition of normal microorganisms; *C. albicans, C.*

*parapsilosis, S. mutans* and *S. sanguinis* to opportunistic microorganisms in the oral cavity. This effect was previously observed where nicotine enhances the growth of *Candida* sp. (Arendorf and Walker, 1980). However, the growth was totally constrained at 8 mg/ml nicotine. A higher concentration of nicotine may be harmful and could inhibit the cell metabolism that can decrease the growth rate of bacterial and candidal cells.

Biofilm is defined as the mass of microorganisms in which cells are frequently lodged within a self-produced matrix of extracellular polymeric substance (EPS) which adhere to each other or to a solid surface. Many recent studies have indicated that biofilm is the preferred form of growth of most microbes in oral cavity (Huang *et al.,* 2012; Calderone & Fonzi, 2001; Hazen & Hazen, 1992). Biofilms have significantly developed resistance against chemicals, antibodies, and various antimicrobials than planktonic cells. From our data, we established that nicotine could highly influence the growth of microorganisms in biofilm environment than planktonic cells (Figure 4.1).

## 5.2 Non-specific adherence capacity; CSH and aggregation effect of nicotine on *Streptococcus* and *Candida* sp.

The initiation of plaque formation on tooth surfaces in oral cavity entails the strong adherence of oral microbes such as *Streptococcus* and *Candida* species to the acquired pellicle (Van Houte, 1982). Cell surface hydrophobicity (CSH) plays an important role in the attachment and detachment of microbes on the tooth surface. The influence of CSH on adherence is considered a crucial virulence factor in bacteria or yeast because colonization and invasion of oral tissues are dependent on their adherence capacity. There

are two types of mechanisms on how the oral microbes adhere to tooth surfaces; either by specific or non-specific adhesion. Specific adhesion involves the connection between the receptors on the acquired pellicle and adhesins on the cell wall while non-specific adhesion involves the acquired pellicle and the surface proteins of the bacterial and fungal cells (Busscher *et al.,* 1992; Busscher & Weerkhamp, 1987).

The presence of hydrophobic proteins in cell wall of microorganisms can increase their proclivity to adhere. Cells which are more hydrophobic can adhere strongly to oral tissue surfaces. The involvement of CSH in the adhesion of other microorganisms was also reported in previous studies which found positive correlation between hydrophobicity and adhesion to host cells by using hexadecane (Stanley & Lazazzera, 2004; Razak *et al.,* 2006; Nordin *et al.,* 2013). Hexadecane is a hydrocarbon which mimic the hydrophobic surfaces of teeth or denture in oral cavity. Thus, increased in CSH may affect virulence of *Streptococcus* sp. and *Candida* sp. and may contribute the adherence mechanisms of the cells.

In this study, all four microorganisms were found to be highly hydrophobic ($P \leq 0.05$). Nevertheless, *S. sanguinis* and *S. mutans* were found to be significantly more hydrophobic compared to *C. albicans* and *C. parapsilosis* ($P \leq 0.05$). It was reported that each species exhibited different hydrophobicity characteristics which explained the differences of CSH level between *Candida* and *Streptococcus* sp. Many studies have been carried out to describe the involvement of CSH in adherence of bacteria. However, very

few researches were conducted on *Candida* species. In previous work, *S. sanguinis* was found to be highly hydrophobic (Razak *et al.,* 2006). Similarly, *Candida* species has also been reported to exhibit hydrophobicity characteristics (Nordin *et al.,* 2013). This finding suggested that *Streptococcus* species could adhere strongly to oral surfaces than *Candida* species. This may be due to the hydrophobic nature of *Streptococcus* species. They have a higher molecular mass of cell surface protein in the cell wall polypeptide compared to *Candida* species (Chaffin *et al.,* 1998; Ruiz-Herrera *et al.,* 2006; Razak *et al.,* 2006). Other factors such as the presence of lipoteichoic acids (LTA) may contribute to the various degree of CSH. Scientists suggested that the presence of external appendages gives a solid grip between the cells and surfaces which eventually increases the percentage of hydrophobic interaction (Krasowska & Sigler, 2014).

It was shown that the hydrophobic properties of *S. sanguinis* and *S. mutans* that had been exposed with nicotine increased uniformly with increased concentrations of nicotine and hence, the effect is concentration dependent. However, the hydrophobic of *C. albicans* and *C. parapsilosis* was found to increase at 1 and 2 mg/ml nicotine only (Figure 4.4). Therefore, the stress induced on *S. sanguinis, S. mutans, C. albicans* and *C. parapsilosis* by cigarette smoke has induced the hydrophobicity leading to increased adherences. This may imply that nicotine can alter the surface characteristics of the microorganisms as demonstrated and thus increased the adsorption to the hydrocarbon hexadecane. It has been reported that, the presence of hydrophobic proteins in the polysaccharide matrix of cell wall contributes to the strength of adhesion receptor and increases the pathogenesis of oral microbes (Sardi *et al.,* 2010).

Besides the extracellular polysaccharides and salivary components, direct cell-to-cell binding may also contribute to the formation of dental plaque (Van Houte, 1982). To determine the nature of surface components involved in cell-to-cell interaction leading to aggregation, the selected oral microorganisms were exposed with nicotine. Our data demonstrated that the level of aggregation in *Streptococcus* and *Candida* species without exposure of nicotine were high. However, the aggregation level of oral microbes was found to be higher after being exposed to nicotine especially at the range of 0.25 to 2 mg/ml. Similarly to the studies conducted by Muzurovic et al (2013) who reported that nicotine could escalate cell aggregation in *Candida* species. An increased aggregation indicated more microbes are able to accumulate and aggregate with each other by forming strong bonds between proteins (Joe *et al.,* 2013). Thus, to justify this finding, we carried out molecular analysis to find the influence of *spaP, gftB, gbpB, HWP1* and *ALS3* genes associated with binding ability of oral microorganisms when under the influence of nicotine.

### 5.3 Expression of *HWP1, ALS3, spaP, gtfB* and *gbpB* of oral microbes following exposure to nicotine

The regulation of the expression of genes gives a clear picture of the different metabolic pathways of cell which is a factor of oral infection. In this study, we focus on the genes that are responsible for the cell adherence and biofilm formation. Based on a study conducted by Huang et al (2014), planktonic cells expressed at least 11 surface-binding-related proteins compared to biofilm cells. Interaction between the host and pathogens are typically mediated by molecules that are either secreted or displayed on the

cell wall. Also, the cell wall proteins play important roles in the pathogenesis of oral microbes.

Extracellular polysaccharide is the major components of thick and mature biofilm on tooth surface. EPS is important for cell adhesion-cohesion and provides binding sites for bacterial to accumulate on the teeth. EPS are synthesized from sucrose via the glucosyltransferases (GTFs) which may leads to caries development (Yoshida & Kuramitsu, 2002). The Glucosyltransferases in combination with glucan-binding proteins (GBPs) play important roles in dental plaque development and formation of glucans from sucrose. Therefore, there are three antigen groups related to bacterial attachment including surface protein antigen I/II, glucosyltrasnferases (Gtf) and glucan-binding protein (Gbp) that are responsible for biofilm formation in oral cavity.

To date, this is the first study to investigate the influences of nicotine specifically on the regulation of *spaP*. The multi-functional adhesion *spaP* also known as PAc1 or P1 is necessary to produce the protein named antigen I/II (Ag I/II) during the initial adherence of *S. mutans* to the tooth. In our research, the presence of *spaP* gene was examined in *S. mutans* and *S. sanguinis* species. The results showed that *spaP* mRNA transcript was found in both *Streptococcus* species. In contrast, Ono et al (1994) has reported *spaP* was strongly expressed in *S. mutans* compared to *gtf*s and *gbp*s. The data suggested that a part of *spaP* gene of *S.mutans* region may exist in *S. sanguinis* (Ma *et al.*, 1991). The expression of *spaP* in *S. mutans* and *S. sanguinis* was elevated as the nicotine concentration increases. The presence of *spaP* genes in bacterial strains are associated

with adherence; therefore, it is possible that *S. mutans* and *S. sanguinis* creates a fixation to the tooth by means of the interaction of the Ag I/II with the proteins of the acquired pellicle.

Gtfs are enzymes essential for the conversion of glucosyl groups from one chemical compound to another which is responsible for the formation of dental plaque by *S. mutans* and *S. sanguinis* and are expressed in three different transcripts; *gtfB*, *gtfC* and *gtfD*. It has been reported that the expression of *gtfB* was demonstrated by an upregulation trend with nicotine treatment in both bacteria species, *gtfB* could rapidly synthesize α-1,3-rich water-insoluble polysaccharide (Yoshida and Kuramitsu, 2002) and thus enable more *S. mutans* and *S. sanguinis* to accumulate on the teeth. This agrees with a recent study which showed that expression of *gtfB* increased significantly following exposure to nicotine (Huang *et al.*, 2015). Gbps are well known as non-gtf glucan binding protein. It is essential in bacterial cell wall synthesis mainly *gbpB. GbpB* expression in *S. mutans* and *S. sanguinis* planktonic cells were significantly increased with nicotine exposure. Hence, the high expression of *gbpB* gene demonstrated that *gbpB* facilitates more bacterial cells to aggregate on the teeth.

In *C. albicans* and *C. parapsilosis*, cell wall proteins (CWP) are generally covalently linked to the polysaccharides on the cell envelope. However, some proteins are not covalently attached (Sundstrom *et al.*, 2002). The roles of CWPs can be very diverse ranging from enzymatic activity and antigenicity in maintaining the structure or morphology of the cell. Adhesins is one of the CWPs class in *Candida* that is important for attachment and communication to biotic and abiotic surfaces. These are generally large

proteins with two distinct domains: N-terminus, which mediates the interaction with other molecules, and the C-terminal domain, which attaches the protein indirectly to the glucan moiety of the cell wall (Snide & Sundstrom, 2006). The two most studied adhesin gene families are the *ALS,* and *HWP* (Hoyer & Cota, 2016; Himratul *et al.*, 2013; Snide & Sundstrom, 2006; Sundstrom *et al.,* 2002).

Hyphal Wall Protein 1 (*HWP1*) and agglutinin-like sequence (*ALS3*) have been reported to be involved in adhesions of *Candida* species on hard and soft tissues in the oral cavity. *HWP1* has unique adherence characteristics where it forms covalent bonds to proteins on tooth surface and human buccal epithelial cells in host tissue (Sundstrom *et al.*, 2002). Hwp proteins also function in different possess such as biofilm formation and adhesion. Both *C. albicans* and *C. parapsilosis* have expressed *HWP1* mRNA transcripts in normal growth condition (Himratul *et al.*, 2013). While in the presence of increasing concentration of nicotine, the expression of *HWP1* in both *Candida* species has been highly regulated. It probably forms more covalent bonds to the protein on the tooth surface and human epithelial cells which enable more candidal cells to attach to host tissues. This could maximize the chances for the cells to propagate and sustain their existence in the oral cavity.

The *ALS* family contain eight Als (Agglutinin-like sequence) proteins: *ALS1, ALS2, ALS3, ALS4, ALS5, ALS6, ALS7,* and *ALS9*. Although these are all adhesins important for biofilm formation and involved in attachment to abiotic surfaces, their functions differ and range from cell–cell interactions and invasion of host tissue to iron

acquisition, in which *ALS3* is the only one involved as a transferrin receptor (Liu & Filler 2011). *ALS3* also involved in invasion of *Candida* into the host tissue by inducing their own endocytosis. It binds to host cell receptors such as E-cadherin and N-cadherin on oral epithelial cells to induce host cell to endocytose the organism (Phan *et al.*, 2007). In this study, both *C. albicans* and *C. parapsilosis* expressed *ALS3* which is responsible for mediating attachment of candidal cells to the hard and soft tissue in the oral cavity. Our results corresponded with earlier findings of the presence of *ALS3* gene in *C. albicans* (Phan *et al.*, 2007) and *C. parapsilosis* (Pryszcz *et al.*, 2013). We have also found that nicotine-exposed *C. albicans* and *C. parapsilosis* resulted in the up-regulation of *ALS3*. This explains the reason why growth rates of *Candida* cells increased as nicotine concentration increases.

### 5.4 Effect of nicotine on the structure of extracellular polysaccharide (EPS) and micro-colonies formation of *Streptococcus* and *Candida* sp.

Extracellular polysaccharides are composed of a wide variety of organic materials, including polysaccharides, proteins, nucleic acids, (phospho)lipids, and uronic acid. Researchers have only recently begun to understand the important roles of EPS in microbial communities which may include cell adhesion and aggregation, biofilm structure, cell-to-cell communication, granulation, organic chemicals degradation and many more. Therefore, CLSM have been used to study the biofilm structure, composition, and the formation of micro-colonies of oral microbes in this study (Lawrence & Neu, 1999). EPS is the main agent for cell-to-cell adhesion-cohesion and it plays a critical role in providing binding sites for cell attachment on tooth surface. The attachment of bacterial

and candidal cells on the surfaces and the formation of highly structured cell clusters (or known as microcolonies) are linked to the ability of microorganisms to survive and persist within the biofilm. When we analysed the biofilm structure and viability of cell by CLSM, some interesting results were found; despite the fact that nicotine was able to increase the growth rate of oral microbes, biofilm mass was strongly increased, as can be seen qualitatively in Figure 4.10 to Figure 4.13 or quantitatively in Figure 4.15. In fact, the biofilm maximum depth, an indirect measurement of biofilm was equally increased when exposed with 1 and 2 mg/ml nicotine concentration (Figure 4.16). The increased biofilm mass suggested that nicotine is able to induce cell attachment. Our data demonstrated that nicotine enhanced both cell numbers and EPS synthesis within biofilms. There are only two ways to multiply the number of cells in biofilms; either through rapid multiplication of cell inside the biofilm or attachment of cells from external sources. From the image analysis, we observed that both bacterial and candidal cells have increased in its volume and coverage in the biofilm environment as nicotine concentration increases. More cells were found to attach to the formed biofilm and higher volume of EPS was seen to be present in the biofilm environment when exposed with 2 mg/ml nicotine (Figure 4.10 and 4.11). However, some genetic regulation might be involved to potentially colonize the oral regions (Huang *et al.*, 2014; Li *et al.*, 2014).

Our data showed that the expression of *gtfB* in *S. mutans* and *S. sanguinis* after being exposed with nicotine markedly increase the capability of these strain to synthesis EPS matrix and the formation of micro-colonies on the saliva-coated well. The upregulation of *gtfB* and *gbpB* gene in *S. mutans* and *S. sanguinis* cell by nicotine

suggesting that nicotine enhanced the formation of glucans and established the structured micro-colonies. The images showed that there were polysaccharides surrounding and enmeshing the micro-colonies and filling the spaces of the micro-colonies. These observations indicated that the presence of functional *gtfB* gene is critical for clustering of bacterial cells and for further development of micro-colonies. The specific roles of *gtfB* in *Streptococcus* sp. biofilm formation could be associated with their specific localization, type of glucan product and the area of distribution (Krzysciak *et al.,* 2004). The glucans formed in the pellicle by the surface-absorbed *gtfB* have been shown to increase the adherence of *S. mutans* and *S. sanguinis*. The presence of α-1,6 linkages in conjunction with 1,3 linkages on the *gtfB*-derived glucan is critical for mediating the bacterial adhesion, possibly by conferring a specific structure that serves as a bacterial binding sites (Donlan, 2002). The upregulation of these genes by nicotine may increase the ability of *Streptococcus* sp. to attach and accumulate on the saliva-coated well, resulting in dental plaque. Furthermore, the polysaccharides formed by *gtfB* is responsible for the development of microcolony. *GtfB* binds strongly to the *S. mutans* cell surface than the other cells or enzymes and more importantly, in an enzymatically active form. Therefore, the carboxyl-terminal repeating units of *gtfB* plays a major role in the specific binding of the enzyme to the cell surface of oral streptococci (Krzysciak *et al.,* 2004; Donlan, 2002). The *gtfB* enhances the adherence of bacteria to each other, promoting the initial clumps of cells, resulting in micro-colonies. Exposing the cells to nicotine have increase the expression of *gtfB* which directly has increased the synthesis of glucans with elevated amounts of α-1,3-linkages, allowing the abundant growth of the micro-colonies and increasing the thickness of the biofilms.

In parallel, the composition and proportional of *Candida* cells and the amount of EPS in the biofilm matrix were profoundly altered by the presence of nicotine. After exposing the cells to 1 mg/ml and 2 mg/ml nicotine, *C. albicans* and *C. parapsilosis* became the major species in the biofilms and at the same time, the level of EPS has drastically increased. It is noteworthy that the microbiological and molecular data corresponded with confocal imaging analysis results. In *Candida* sp., *ALS3* and *HWP1* genes were responsible in increasing the formation of micro-colonies and the thickness of biofilms.

Thus, the structurally rigid insoluble glucans serve as a matrix that holds bacterial and candidal cells and mediates the initial assembly on the surface of saliva-coated wells and support the frame for further development of microcolonies, which results in stronger adhesion between the cells and to the surfaces. This observation could explain why upregulation of adherence-associated genes resulted in enhancement of the ability of the oral microorganisms to form biofilms.

# CHAPTER 6: SUMMARY AND CONCLUSIONS

In the oral environment, these findings showed the negative impact of nicotine on the oral health of smokers. This study has determined the cell growth and biofilm formation of *S. mutans, S. sanguinis, C. albicans* and *C. parapsilosis* under influence of nicotine as well as the adherence capacity to the hard and soft tissues in the oral cavity. The responses exhibited for unexposed and exposed-*Streptococcus* sp. and *Candida* sp. indicates that nicotine affects the adhesion of these microbes on the tooth surface. Thus, nicotine increases the pathogenesis property of *Streptococcus* sp. and *Candida* sp. in the oral cavity.

The conclusion to the findings of our study have answered the specific objectives outlined in the research as follows:

- Nicotine could increase the planktonic cell growth and biofilm formation of *C. albicans* and *C. parapsilosis, S. mutans* and *S. sanguinis*
- Nicotine could influence the non-specific adherence of *C. albicans, C. parapsilosis, S. mutans*, and *S. sanguinis*
- Nicotine speeds up the biofilm formation mediated by *Candida* and *Streptococcus* species by affecting the adherence-related gene expression.
- Nicotine could enhance EPS production and promoted micro-colonies formation by *C. albicans, C. parapsilosis, S. mutans* and *S. sanguinis*.

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**PP03**

### Effect of Nicotine on *Candida albicans* and *Candida parapsilosis*

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**Objectives:** Smoking is considered as one of the main factor of developing dental plaque. Along with other bacteria, yeasts have been described to contribute to the plaque community. The aim of this study was to investigate the influences of nicotine on growth of planktonic cells and biofilm mass of *Candida albicans* and *Candida parapsilosis*. **Methods:** *Candida albicans* ATCC 14053 and *Candida parapsilosis* ATCC 22019 were used in the present study. The minimum inhibitory concentration (MIC), minimum fungidical concentration (MFC), minimum biofilm inhibitory concentration (MBIC), planktonic cell growth (CFU enumeration), biofilm (crystal violet assay) and cell surface hydrophobicity (using hexadecane) of both candida species treated with different concentration of nicotine (1, 2, 4 and 8 mg/ml) were investigated. **Results:** The MIC, MFC and MBIC of both candida species were 4 mg/ml, 16 mg/ml and 4 mg/ml respectively. The results indicated that nicotine enhanced the growth of oral fungal in both planktonic and biofilm cells. Morever, the surface hydrophobicity of both candida species have been increased as the nicotine concentrations increased and thus encouraged higher colonisation on tooth surfaces. **Conclusions:** The data concluded that nicotine is able to enhance the growth of *Candida albicans* and *Candida parapsilosis* and influences its adherence on tooth surfaces.

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# Influence of nicotine on the adherence of *Candida albicans* ATCC 14053 and *Candida parapsilosis* ATCC 22019 and expression of selected binding-related genes

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**ABSTRACT**

Smoking is considered one of the main factors for development of dental plaque. Yeasts have been described to contribute to the plaque community. The aim of this study was to investigate the influence of nicotine on the growth of planktonic cells and biofilm, cell aggregation, surface hydrophobicity, cell adherence and binding-related genes expressed by *Candida albicans* and *Candida parapsilosis in vitro*. The relative number of viable fungal cells was determined based on viable plate counts. The biofilm growth was quantified using the crystal violet assay. Reverse transcription-polymerase chain reaction was used to evaluate the regulation of selected genes associated with adherence. The results indicated that nicotine enhanced the growth of both planktonic and biofilm oral fungal cells. Cell surface hydrophobicity and the expression of Hyphal Wall Protein 1 (*HWP1*) and agglutinin-like sequences 3 (*ALS3*) of *C. albicans* and *C. parapsilosis* were found to increase relative to the nicotine concentrations. The results suggest that nicotine can enhance the growth of *C. albicans* and *C. parapsilosis in vitro* and influence their adherence to the surface of microplate wells that mimic as the tooth surface.

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## Introduction

Cigarette smoke contains more than 4000 types of chemicals, at least 50 of them being very carcinogenic. Approximately 6%–30% of the cigarette content is constituted by nicotine, an alkaloid in cigarette smoke. A stick of cigarette contains 9–30 mg of nicotine; however, the nicotine absorbed in the body by inhalation through smoking is about 0.5–2 mg per cigarette [1]. Although this amount is low, it is sufficient to cause many serious illnesses (e.g. cancer), heart problems and abortion as well as other health problems. Nicotine has been reported to be able to alter certain cellular functions, such as cell growth, attachment, and matrix protein synthesis of oral microbes [2]. Nicotine can also stimulate oral biofilm formation and influence cell metabolism of biofilm microorganisms. Unfortunately, there are still questions about how nicotine increases the metabolic activity of microorganisms in the oral cavity [3].

It has been reported that *Candida* species are more prevalent in tobacco smokers than in non-smokers [4]. The *Candida* genus is comprised of about 150 species of yeast-like fungi. In general, they are classified as asexual diploid fungi that can exist in the form of yeasts or hyphae [5]. *Candida* species such as *C. albicans*, *C. tropicalis*, *C. krusei*, *C. lusitaniae*, *C. dubliniensis*, *C. kefyr*, *C. guilliermondii*, *C. parapsilosis* and *C. lipolytica* are present in the oral cavity as commensal microorganisms and these can act as opportunistic pathogens in cases of low immune system [6]. The most commonly isolated species from oral cavities is *C. albicans*, in up to 60% in the general population [7]. In addition, *C. albicans* and *Candida parapsilosis* are frequently reported to be present in oral biofilm [8]. Incubation temperature is one of the factors influencing the growth of *Candida* sp. from normal microbial flora to pathogenic state in the oral cavity. Most studies indicate that the optimal temperature for *C. parapsilosis* growth is 35 °C instead of 37 °C [9]. Specific adhesion between *Candida* and receptors on host tissues results in a stronger covalent attachment in oral biofilm [6]. It is linked with enhanced expression of some virulence genes, *HWP1* (Hyphal Wall Protein 1) [10] and *ALS* (agglutinin-like sequence) [11] which promotes the adhesion of *Candida* species to epithelial surfaces. In addition, interaction between *Candida* species has also been reported to favour biofilm formation in the oral cavity [6].

Thus, the aim of this study was to investigate the effect of nicotine on *C. albicans* and *C. parapsilosis*