# POLYMICROBIAL INTERACTIONS BETWEEN SELECTED MICROBES OF ORAL BIOFILM AND DIFFERENTIAL EXPRESSION OF *ALS* GENES AND THEIR RELATED PROTEINS

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**FACULTY OF DENTISTRY**
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**FACULTY OF DENTISTRY**
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## ABSTRACT

More than 700 different types of microorganisms dwell in human oral mucosal surfaces. The interactions between these microorganisms contribute to formation of polymicrobial communities on hard and soft tissues as well as on dentures and implants. *Candida albicans*, a pleomorphic fungus inhabiting oral and gastrointestinal (GIT) mucosa becomes pathogenic in favourable conditions causing local or systemic disease. Since streptococci are early colonisers, the ability of *Candida albicans* to adhere to streptococci paves the way for an additional surface for candidal colonization and propagation. This study was aimed to investigate polymicrobial interactions between *C. albicans*, *S. sanguinis* and *S. mitis* on *in-vitro* oral biofilms and to identify differential expression of agglutinin-like sequence *ALS1, ALS2, ALS3* genes and associated proteins. Single, dual and mixed species oral biofilms were formed *in-vitro* in static and dynamic flow conditions for 24 h. Biomass of each biofilm was determined using crystal violet (CV) stain and the cellular metabolic activity within these biofilms was assessed using tetrazolium XTT assay. Microbial growth of biofilms was calculated by counting colony forming units (CFU) on continuous flow model. The morphology of organisms in these biofilms was viewed under scanning electron microscope (SEM) and analysed using morphology index (Mi). The antimicrobial effect of amphotericin B, nystatin and chlorhexidine on each biofilm was seen by determining minimum inhibitory concentration (MIC). The expression of target genes in *C. albicans* was analysed by quantitative real-time PCR (qPCR) while their respective proteins by Western blot technique. CV and XTT analysis of static biofilms showed a significant increase in biomass and cellular metabolic activity of two dual species and mixed species in comparison to single species biofilm. The results were validated by CFU count of microorganisms on biofilms formed under continuous nutrient flow. SEM analysis revealed candida hyphae formed under the influence of streptococci in dual and mixed

species biofilm, whereas, only blastoconidia were visible in single species. The MIC values of amphotericin B (AmB), nystatin (Nt) and chlorhexidine (CHX) was 4µg/mL against single species whereas the values were higher against dual and mixed species (64 and 125µg/mL respectively for AmB) (32 and 125µg/mL for Nt) (4 and 32µg/mL for CHX). The genomic expression revealed that *ALS1* and *ALS3* were significantly over expressed in mixed species biofilm, in comparison to dual species and single species biofilm. However, *ALS2* was not significantly expressed in dual or mixed species. Western blot of single species showed clear bands of als1, als2 and als3p whereas, in dual and mixed species clear bands of only als1 and als3p were visible. In conclusion, *C. albicans* demonstrated enhanced biofilm characteristics when grown with *S. mitis* and *S. sanguinis* in both static and dynamic flow conditions. The role of *ALS1* and *ALS3* genes is enhanced in dual and mixed species. This study proposes substantial contribution of bacteria in propagation of *C. albicans* biofilm. Hence, in oral fungal infestations, promotion of the infection may be contingent upon the bacterial constituent, a prospect which is frequently neglected and requires more research.

**Keywords:** bacteria, biofilm, candida, oral cavity, saliva,

## ABSTRAK

Terdapat lebih daripada 700 jenis mikroorganisma berbeza yang berada di permukaan mukosa mulut manusia. Interaksi di antara mikroorganisma ini menyumbang kepada penghasilan komuniti polimikrobial di atas permukaan tisu keras dan lembut termasuk gigi palsu dan implan. Candida albicans merupakan fungi pleomorfik yang mendiami kaviti mulut dan saluran gastrousus (GIT) yang boleh menjadi patogen dan menyebabkan penyakit lokal atau sistemik. Oleh kerana streptokoki adalah koloni pemula, *Candida albicans* berupaya untuk melekat kepada streptokoki untuk memulakan pengkolonian dan penyebaran candida. Kajian ini bertujuan untuk menyiasat secara *in vitro* untuk interaksi polimikrobial di antara *C. albicans*, *S. sanguinis* and *S. mitis* ke atas biofilem mulut dan mengenalpasti perbezaan ekspresi turutan 'Agglutinin-like' gen *ALS1, ALS2 & ALS3* dan protein yang berkaitan. Spesis tunggal, dwi dan campuran biofilem mulut telah dibentuk secara in dibentuk secara *in vitro* di dalam keadaan aliran statik dan dinamik selama 24 jam. Biomas setiap biofilm ditentukan menggunakan pewarna kristal violet (CV) dan aktiviti metabolik selular dalam biofilem ini ditentukan menggunakan assay XTT tetrazolium. Pertumbuhan mikrob biofilem dikira dengan mengira unit pembentukan koloni (CFU) pada model aliran berterusan. Morfologi organisma dalam biofilem ini dilihat di bawah pemeriksaan mikroskop electron (SEM) dan dianalisis menggunakan indeks morfologi (MI). Kesan antimikrob amphotericin B, nistatin dan chlorhexidine pada setiap biofilm dilihat dengan menentukan kepekatan perencatan minimum (MIC). Ekspresi gen sasaran di *C. albicans* dianalisisa dengan menggunakan PCR kuantitatif (qPCR) manakala protein menggunakan teknik Western Blot. Analisis CV dan XTT biofilem statik menunjukkan peningkatan yang ketara dalam biomas dan selular daripada spesies dwi dan campuran berbanding spesies biofilem tunggal. Keputusannya telah disahkan oleh kiraan mikroorganisma CFU pada biofilem yang terbentuk di bawah aliran nutrien yang berterusan. Analisa SEM menunjukkan

hiphae candida  yang terbentuk di bawah pengaruh streptokoki dalam biofilm spesies dwi dan campuran, sedangkan hanya blastoconidia yang dapat dilihat dalam spesies tunggal. Nilai MIC amphotericin B (AmB), nistatin (Nt) dan chlorhexidine (CHX) adalah 4µg / mL berbanding spesis tunggal manakala nilai MIC lebih tinggi berbanding spesis dwi dan campuran (64 dan 125µg / mL masing-masing untuk AmB) (32 dan 125µg / mL untuk Nt) (4 dan 32µg / mL untuk CHX). Hasil ekspresi genomik menunjukkan *ALS1* dan *ALS3* lebih ketara  dalam biofilem spesis campuran, berbanding biofilm spesis dwi dan tunggal. Walaubagaimanapun, *ALS2* tidak dinyatakan dengan jelas dalam spesis dwi atau campuran. Hasil Western blot spesis tunggal memperlihatkan kumpulan yang jelas als1, als2 dan als3p sedangkan dalam spesis dwi dan campuran jelas hanya als1 dan als3p kelihatan. Kesimpulannya, *C. albicans* menunjukkan ciri biofilem yang dipertingkatkan apabila hidup bersama *S. mitis* dan *S. sanguinis* dalam keadaan aliran statik dan dinamik. Peranan gen *ALS1* dan *ALS3* dipertingkatkan dalam spesis dwi dan campuran. Kajian ini mencadangkan sumbangan besar bakteria dalam penyebaran biofilem *C. albicans*. Oleh itu, dalam infestasi fungi mulut, promosi jangkitan mungkin bergantung pada konstituen bakteria, prospek yang sering diabaikan dan memerlukan lebih banyak penyelidikan.

**Keywords:** bakteria, biofilem, candida, kaviti mulut, air liur

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| | | |
|---|---|---|
| ALS | : | Agglutinin-like sequence |
| Amp-B | : | Amphotericin B |
| bp | : | Base pairs |
| $CO_2$ | : | Carbon dioxide |
| CWP | : | Cell wall proteins |
| CFU | : | Colony forming unit |
| cDNA | : | Complementary DNA |
| °C | : | Degree Celsius |
| $dH_2O$ | : | Distilled water |
| *et al.* | : | et alia (and others) |
| *e.g.*, | : | Exampli gratia |
| g | : | Gram |
| GPI | : | Glucosylphosphatidylinositol |
| h | : | Hour |
| HWP | : | Hyphal wall protein |
| *i.e.*, | : | In other words |
| µg | : | Microgram |
| µg/mL | : | Microgram per millilitre |
| µL | : | Microlitre |
| µM | : | Micromolar |
| mL | : | Millilitre |
| mg | : | Milligram |
| mg/mL | : | Milligram per millilitre |
| mm | : | Millimeter |

| | | |
|---|---|---|
| nm | : | Nanometer |
| NAM | : | Nordini's artificial mouth |
| OD | : | Optical Density |
| % | : | Percentage |
| PBS | : | Phosphate Buffer Saline |
| PIR | : | Plasmodium interspersed repeats |
| qPCR | : | Quantitative Real-Time Polymerase Chain Reaction |
| RT-PCR | : | Reverse transcription –polymerase chain reaction |
| RNA | : | Ribonucleic Acid |
| RNase | : | Ribonuclease |
| TS | : | Tryptic soy |

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# CHAPTER 1: INTRODUCTION AND OBJECTIVES

## 1.1 Introduction

Human oral mucosa is inhabited by diverse groups of microbial species. It has been estimated that more than 700 different types of microorganisms may dwell in human oral mucosal surfaces (Deo *et al.*, 2019). *Candida albicans* is a pleomorphic fungus that inhabits the genitourinary and gastrointestinal (GI) mucosal epithelium of humans, colonizing these habitats as a commensal organism in approximately 60% of healthy population (Barnett, 2004). Any unusual changes in bacterial flora, host immunity or surrounding environmental factors, *e.g.*, salivary flow, are considered as determinants of candidal transformation from a benign commensal organism to an infectious opportunistic pathogen, adept at causing a broad range of local mucosal or lethal systemic diseases (Morales & Hogan, 2010). In current times, *Candida albicans* infections represent an increasing challenge for clinicians. The diseases caused by *C. albicans* may be systemic or local (superficial). Invasive candidiasis is a severe infection that has potential to affect the heart, brain, blood, eyes, bones and various other parts of body (Puel, 2020). Candidemia, a bloodstream infection with *Candida*, is a common infection in hospitalised patients (Wang *et al.*, 2014). Superficial (local) infections of the mucocutaneous structures comprise of oropharyngeal candidiasis (affecting the palate, buccal mucosa and tongue), conjunctivitis, esophagitis, vaginitis or gastrointestinal candidiasis (Patil *et al.*, 2015). According to a previous study, candidal adherence to mucosal surfaces is considered as critical initial step in the pathogenesis of oral candidiasis (Tsui *et al.*, 2016). A very important feature of *C. albicans* pathogenicity is the expression of virulent genes. Attention has been focused on Agglutinin-like sequence *ALS*1, 2 & 3 genes that encode large surface glycoproteins. Their encoded proteins are localized at the *C. albicans* cell surface (Hoyer *et al.*, 2008). Acrylic dentures and

implants placed in oral cavity also act as a cistern and play a pivotal role in enhancing danger of candidal colonisation (Gleiznys *et al*., 2015).

The increase in worldwide occurrence and incidence of oral mucosal candidiasis may be accredited to the high usage of prescribed drugs like cytotoxics, broad-spectrum antibiotics, corticosteroids and also due to an increasing statistics of immuno-compromised individuals and people suffering from serious nutritional deficiencies or endocrine disorders (*e.g*., diabetes mellitus) (Bongomin *et al.*, 2017).

Antimicrobial resistance is an increasingly crucial phenomenon which is defined by the World Health Organization (WHO) as the ability of a pathogen to resist antimicrobials to which it was initially sensitive (Prestinaci *et al.*, 2015). It is considered a consequence of imprudent use of antimicrobial agents and develops when a pathogen mutates or acquires a resistance gene. Recently, it has been inferred that an increasingly popular cause of resistance is the formation of polymicrobial species communities (Ferrer-Espada *et al.*, 2019). Formation of these communities does not only increase chances of infection, but also renders the polymicrobial biofilm, resistant to specific drug types. Resistant organisms have the ability to resist antimicrobial attack thus causing severe problems, usually leading to treatment failure, which would have serious consequences, particularly in critically ill patients where infections are difficult to control (Tanwar *et al.*, 2014). This problem is also posing as a constant challenge to contain fungal diseases adequately as the resistant fungus can diffuse and cause wider problems, not only within health care institutions, but in larger communities as well.

Recent evidence suggests that, the majority of *Candida* infections are associated with biofilm growth (Lohse *et al.*, 2018). The biofilm resistance has been observed to increase constantly compared to planktonic cells. Indeed, biofilm cells may become 10–1000 times more resistant to antimicrobial agents (Stewart, 2015). Biofilm is defined as a complex

interaction of unicellular organisms, encased in an extracellular matrix of polysaccharides, proteins and nucleic acids (Kostakioti *et al.*, 2013). *Candida* species can form drug-resistant biofilms. This reflects as an imperative factor in the contribution of *Candida* to human diseases (Silva *et al.*, 2017). The formation of the candidal biofilm is an initial step in the series of events leading to candidal infections. The stages of biofilm formation consist of the adherence of yeast cells to the surface and the establishment of a monolayer of cells, followed by proliferation, filamentation and maturation (Ramage *et al.*, 2001) .

Inside oral cavity, *Candida albicans* forms mixed-species infections that include angular cheilitis, denture-induced stomatitis, gingivitis, periodontal diseases and prosthetic implant infections (Holmes *et al.*, 1995). *Candida albicans* often coexists with numerous bacterial species. Previous studies have shown adherence of candida with a variety of *Streptococcus* species (Kolenbrander *et al.*, 2002). Hence, it can be stated that when candidal infections emerge, they often arise in affiliation with bacteria. The number of bacteria colonising mucosal and skin surfaces exceeds the number of cells forming human body. Commensal bacteria co-evolve with their hosts, however, under specific conditions they are able to overcome protective host responses and exert pathologic effects (Tlaskalova-Hogenova *et al.*, 2004). Resident bacteria form complex ecosystems; whose diversity is enormous. Mutualistic, antagonistic and synergistic interactions that occur between different microorganism species contribute towards formation of polymicrobial communities, developing a biofilm on oral structures and prosthetics (Peters *et al.*, 2012). In particular, the capability of abettor organisms to adhere physically or metabolically with each other is known to be fundamental to the survival, growth and virulence of each individual species.

Streptococcus species are amongst the initial colonisers of soft and hard oral tissues, which makes up more than two-thirds of the initial biofilm composition (Kreth *et al.*, 2009). They express an intricate collection of cell wall polypeptides that determine adherence interactions. Several species of streptococci have been found in the oral cavity. *Streptococcus sanguinis* is a gram positive, non-motile, non-spore forming member of the viridans group. *Streptococcus mitis* is a commensal bacterium, a member of the oral flora that colonises teeth as well as mucous membrane. *S. mitis* and *S. sanguinis* collectively comprise of two-third primary colonisers in oral cavity (Xu *et al.*, 2014). Intergeneric microbial co aggregation mediates biofilm formation and facilitates closer propinquity for cell-cell interactions through the manufacturing and expression of cell surface signalling molecules. The adherence mechanism plays a significant role in governing the species constitution in polymicrobial communities (Podbielski *et al.*, 2004).

Since streptococcal species are amongst the first to colonise the oral cavity, the competency of *Candida albicans* to co-aggregate with streptococci can produce an added platform for fungal growth and colonisation. Microbial community agglomerates on the soft (mucosa, tongue) and hard (dental) surfaces, on prostheses and implants in the form of sessile biofilms. In general, the adhesion mechanism includes physicochemical or thermodynamic forces that provide specific surface-surface and high affinity interactions amongst receptor and adhesion (Alem *et al.*, 2006). The oral streptococci, which are primary colonisers of salivary pellicle, elaborate both adhesins and receptors for co-aggregation. *C. albicans* adheres to a variety of bacterial genera and in particular exhibits high-affinity and saturable binding to a range of oral streptococci (Diaz *et al.*, 2012). *Candida albicans* is capable of co-aggregating with different types of oral bacteria and based on the known *in vivo* and *in vitro* co-aggregation studies between oral Streptococci and *C. albicans*, it can be hypothesized that these two microorganisms form an interspecies alliance that could assist host mucosal colonisation and infection.

## 1.2 Objectives of research

The goal of this work is to evaluate the impact of *Streptococcus mitis* and *Streptococcus sanguinis* on colonisation of *Candida albicans* in an *in vitro* study.

The specific objectives of the study were:

1. To assess the growth of *C. albicans* in single (*C. albicans*), dual (*C. albicans* with *S. sanguinis*) (*C. albicans* with *S. mitits*) and mixed species (*C. albicans* with both *S. sanguinis* and *S. mitis*) biofilms by quantifying and comparing biofilm biomass, cellular metabolic activity and colony forming units of microorganisms under both static and dynamic conditions.
2. To visualise the morphology of aggregated microorganisms in single species (*C. albicans*) (*S. sanguinis*) (*S. mitis*), dual species (*C. albicans* with *S. sanguinis*) (*C. albicans* with *S. mitits*) and mixed species (*C. albicans* with both *S. sanguinis* and *S. mitis*) biofilms using scanning electron microscope SEM.
3. To determine the effect of commonly used antimicrobials like amphotericin B, nystatin and chlorhexidine on single species (*C. albicans*) (*S. sanguinis*) (*S. mitis*), dual species (*C. albicans* with *S. sanguinis*) (*C. albicans* with *S. mitits*) and mixed species (*C. albicans* with both *S. sanguinis* and *S. mitis*) biofilms.
4. To evaluate the dominant *ALS* 1, 2 and 3 gene expression and respective als1, als2 and als3 proteins of *C. albicans*, in single species (*C. albicans*), dual species (*C. albicans* with *S. sanguinis*) (*C. albicans* with *S. mitits*) and mixed species (*C. albicans* with both *S. sanguinis* and *S. mitis*) involved in biofilm formation and growth.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 The oral cavity and commensal microbiota

Human oral cavity is an appropriate site for microbial species to grow and dwell (Jenkinson *et al.*, 2005). As early as 1674, Antony Van_Leeuwenhoek observed his own dental plaque and reported "little living animalcules prettily moving". This was the hallmark from which other studies evolved in relation to oral microflora in disease and health (Aas *et al.*, 2005; Dewhirst *et al.*, 2010). The types include gram positive and negative bacteria, yeasts, mycoplasms and protozoa (Patil *et al.*, 2013). A lot of surfaces in oral cavity *e.g.* teeth, tongue, palate, mucosa *etc*. provide unique habitats owing to their biological and physical properties. Each surface possesses unique features that favour the most suitable microorganisms to inhabit the region. The function of various microorganisms in an environment is called "ecological niche". A variety of ecological niches are present on the oral mucosa, including subgingival plaque, supragingival plaque and on the tongue and teeth (Samaranayake *et al.*, 2017).

The inhabitant oral microflora forms a dynamic relationship with salivary constituents and oral environment. Majority of these organisms prevail in the buccal cavity as symbiotic species, developing mutual beneficial affiliation with the hosts (Jenkinson *et al.*, 2005). Not only are these organisms harmless, the commensal inhabitants may also keep a check on the pathogenic species by resisting their attachment to oral mucosa. These microorganisms do not cause infection by becoming pathogenic, until they break the barrier of commensals. A substantial alteration in an environmental parameter governing microbial growth could negatively affect and disturb the harmonious balance of oral microflora, thus resulting in colonisation and invasion of host tissues. Recently, *C. albicans* infections are receiving booming attention, seemingly because of the intensifying prevalence worldwide.

## 2.2 *Candida albicans*

### 2.2.1 Taxonomy and etymology

Research on *Candida albicans* has been sufficiently reported in literature, describing all prospects of factors and virulence properties that interpret its contribution to infections (Calderone *et al.*, 2001; F. L. Mayer *et al.*, 2013; Tsui *et al.*, 2016). *Candida albicans* is one of the 163 species under the genus *Candida*. Kurtzman et al., (2011) classified yeasts in a comprehensive manner, placing *Candida* species subclass Saccharromycetaceae, in the order Hemiascomycetes (Kurtzman *et al.*, 2011). The chronicles of the discovery and nomenclature of *Candida* begins from the primeval Greek scientists to modern time researchers. There is almost 200 years' worth of medical history records obtained before the causative factor of oral thrush (candidiasis) was first identified as a "fungal pathogen" (Barnett, 2004). In 1853, Charles Robin isolated microorganism from infection of oral and vaginal mucous membrane and named it "Oidium albicans" using *albicans* ("to whiten") to name the fungus causing thrush. The genus *Candida* was first created in 1923 by Christine Berkhout (Berkhout, 1923). "Candida" is a derivative of Latin tradition where toga candida, a white robe was attired by Roman Senators. Berkhout's diagnosis of the genus, written in Dutch, was as follows:

"Few hyphae, prostrate, breaking up into shorter or longer pieces. Conidia, arising by budding from the hyphae or on top of each other, are small and hyaline." (McGinnis, 1980).

This was the beginning of an era dedicated to research on *Candida albicans*. Since 1980s, there has been a massive surge of keenness and subsequent research on candidal infections. These yeast-like structures are anamorphic fungi (sexual imperfect) that belong to a form-class called "Blastomycetes". They are discriminated due to their

polymorphic behaviour and ability to form blastoconidia (budding yeast cells), blastospores, mycelia and pseudomycelia (Jacobsen *et al.*, 2017).

### 2.2.2 Ecology

Its normal habitat is found in the alimentary tract of warm-blooded vertebrates, where it lives as budding yeast on the mucosal surface and in the mucosal secretions. Due to its diverse nature, *C. albicans* is perpetually inoculated on the skin surface, yet it rarely ever establishes habitat on it. *C. albicans* exists as an opportunistic pathogen in the normal microflora of human gastrointestinal tract (Noverr & Huffnagle, 2004). Van Uden distributed the gastro-intestinal fungus into 3 groups: (1) facultative saprophytes (2) obligate saprophytes and (3) passers-by (Van Uden *et al.*, 1958). As a commensal microbe, *C. albicans* produces enzymes that digest carbohydrates by process of fermentation and release carbon dioxide and alcohol, required for their sustenance and growth. If oral rinses were used for sampling the oral carriage, approximately 50% of adults would show *C. albicans* as part of their normal oral flora (Kaur *et al.*, 2017). *C. albicans* colonise oral surfaces such as teeth, mucosa, prosthetic devices and implants and form intricate polymicrobial biofilms (Thein *et al*., 2009).

### 2.2.3 Morphology

*Candida* is a polymorphic species that can grow as yeast cells (single cells), hyphae, pseudohyphae, and forms chlamydospores (Figure 2.1). Yeast cells (single cells) exhibit oval shape and two types of budding patterns, *i.e*., both bipolar and axial. Hyphae and pseudohyphae are generally known as “filamentous” morphologies. Their cells commonly grow like a polarized structure which is elongated in shape with end to end attachment. Pseudohyphal cells develop in ellipsoid shape (*i.e*., the cell is wider in the centre in comparison to the distal ends) and are constricted at their septal junctions (P.

Sudbery *et al.*, 2004). Contrary to this, hyphal cells commonly display parallel sides, with unvarying width, and develop true septa which lack constrictions (Sudbery, 2011). Hyphal cells contain pores in the septa for intercellular communication (Gow, 1994; van der Klei *et al.*, 2011). In yeast and pseudohyphae, division of nuclei and formation of septum ring occurs at the mother-bud neck. In contrast, hyphae display these characteristics only inside the germ tube (the initiatory small filament). Whilst the pseudohyphae appear morphologically akin to hyphae, its properties are more relatable to yeasts instead. Hyphae are large spores with thick walls, high content of lipid and carbohydrate, and it may be formed when the growth environment is low in oxygen, light, temperature and nutrients (Enjalbert *et al.*, 2005). Last but not the least, rarely seen chlamydospores are round, thick-walled, large cells (about triple in size than yeast), that commonly develop at the hyphal ends. These types of cells have a tendency to develop as a reaction to nutrient-poor situations (Staib *et al.*, 2007; Whiteway *et al.*, 2007).

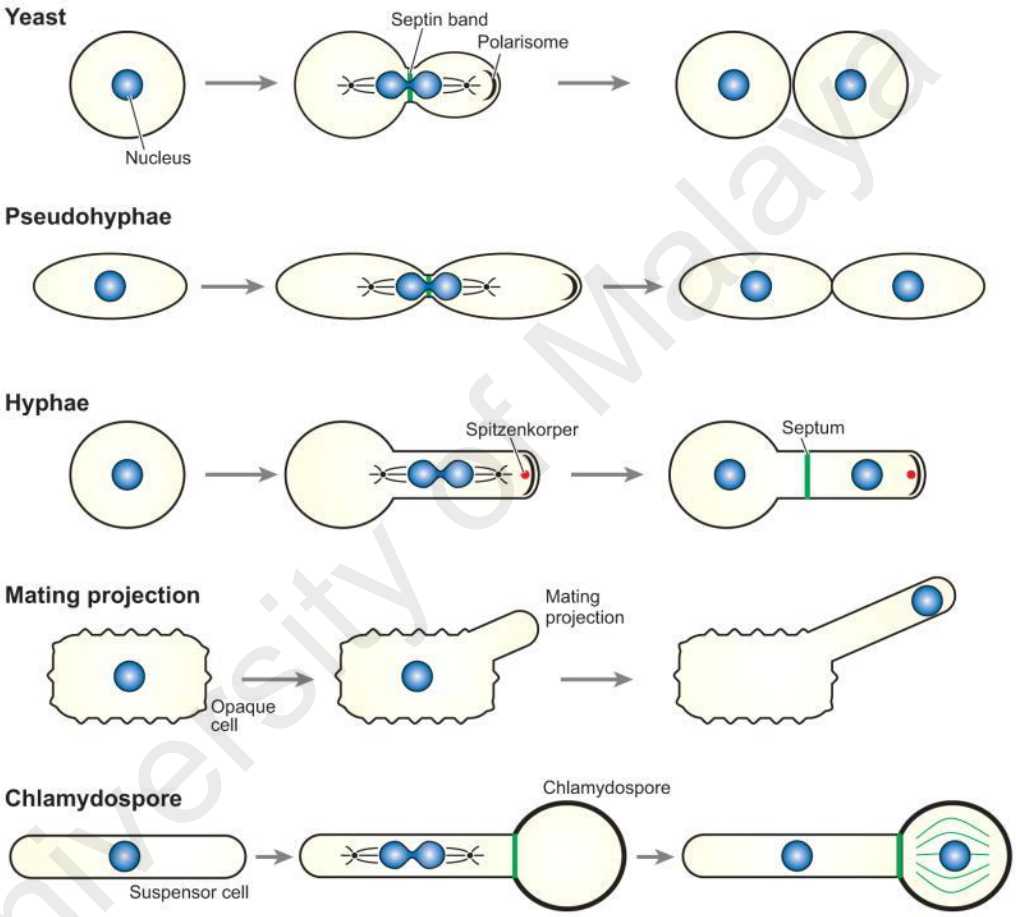


**Figure 2.1:** Common morphologies of *C. albicans* and their cell division patterns
Source: (Whiteway *et al.*, 2007)

A major virulent attribute of *Candida* is its ability to alter its morphology under different circumstances, from blastosphore (single budding cells) to hyphae form (filamentaous growth). This switching referred to as "dimorphic transition", propagated by cell-wall constituents, is a feature pivotal for adherence to host surfaces (Cutler, 2001; Gow, 1997; Liu, 2001). Although the terminologies "dimorphic fungus" or "dimorphism" are generally acknowledged when inferring about *Candida albicans*, the unique ability to display a large scale of morphologies has rendered this organism as "pleomorphic" or 'polymorphic". These morphological transformations are manifested in response to the dynamic environmental conditions of fungal cells which may empower the organism to habituate in a variety of biological niches.

In *C. albicans*, hyphal formation is known to promote virulence by several mechanisms: (i) hyphae can invade epithelial cell layers by exerting mechanical force; invasion can involve growth between epithelial cells, as well as penetration of individual cells (Kumamoto *et al.*, 2005); (ii) hyphae are adept in breaching and destroying endothelial cells (Swidergall *et al.*, 2017); and (iii) following phagocytosis, *C. albicans* hyphal growth can cause lysis of both macrophages and neutrophils (Korting *et al.*, 2003). Additionally, thigmotropism, also known as contact sensing, is presumed to favour *Candida albicans* hyphae in identifying and penetrating small crevices, weak points and grooves present in host surfaces during infection (Noble *et al.*, 2017). Thus, in studies determining the pathogenesis of *C. albicans*, formation of hyphae marks a hallmark for fungal virulence.

### 2.2.4 Cell wall

The fungal cell-wall functions as an exoskeleton that acts as a permeability barrier and determines the shape of the cell and helps to withstand turgor pressure. The cell wall is essential to nearly every aspect of the biology and pathogenicity of *Candida albicans*.

Although it was initially considered an almost inert cellular structure that protected the protoplast against osmotic offense, more recent studies have demonstrated that it is a dynamic organelle (Mancuso *et al.*, 2018). Also, as the most external part of the cell, the wall propitiates the early physical interaction between the host's environment and the microorganism. It has been documented that the formation of a biofilm is largely concerned with the extracellular components which are mainly formed by cell-wall polysaccharides i.e., glucose and mannose residues (Limoli *et al.*, 2015; Pierce *et al.*, 2017). The cell wall thus plays crucial part in the pathogenesis of microspecies and presents a favourable target point for binding cell-wall ligands of other species, thus developing a polymicrobial biofilm. For these reasons, the cell wall of *C. albicans* is the focus of study by numerous research groups (Cottier *et al.*, 2020).

The cell wall of *C. albicans* has a layered structure. It consists of a homogeneous inner layer of about 100 nm and an outer protein layer of about 180 nm, consisting of densely packed fibrillae organized perpendicular to the cell surface. The inner layer functions as a scaffold for an external protein layer that limits the permeability of the cell wall for large molecules, and determines the antigenic properties and the hydrophobicity of the cell surface (Klis *et al.*, 2001). Almost 80 to 85% of cell-wall of *Candida albicans* is composed of carbohydrates. There are three main constituents of polysaccharides that form the cell wall: (i) Outer part consisting of polymers of mannan (mannose) which are covalently bonded to proteins (glyco[manno]proteins); (ii) unbranched polymers of *N*-acetyl-D-glucosamine(GlcNAc) comprising of β-1,4 bonds(chitin) and (iii) branched polymers of glucose comprising of β-1,3 and β-1,6 linkages(β-glucans) (Figure 2.2). Additionally, the structure of *C. albicans* cell wall includes 6-25% proteins along with small quantity of lipid (about 1 to 7%) (Garcia-Rubio *et al.*, 2020).

The microfibrillar molecules (β-glucans and chitin) illustrate the structural constituents of the cell wall. They represent a tough skeleton that provides strength and turgor to the cell. From a structural viewpoint, β-glucans represent the main component, making up for about 47 to 60% of dry weight of cell wall. A minor constituent is chitin. It composes about 0.6 to 9% of cell wall, yet it is a particularly important part of budding scars, the septa between individual cell compartments, and the band around the septal contraction where new bud emerges from mother cell (Bowman *et al.*, 2006). On the other hand, mannose polymers (mannan), which do not exist as such but are found in covalent association with proteins (mannoproteins), represent about 40% of the total cell wall polysaccharide and are the main material of the cell wall matrix (Nett *et al.*, 2020). The percentage composition of yeast cell wall and hyphal filamentous cell wall is similar; however, the relative amounts of mannan, chitin and β-glucans differ in relation to variation in the *Candida albicans* growth form (Ruiz-Herrera *et al.*, 2006).

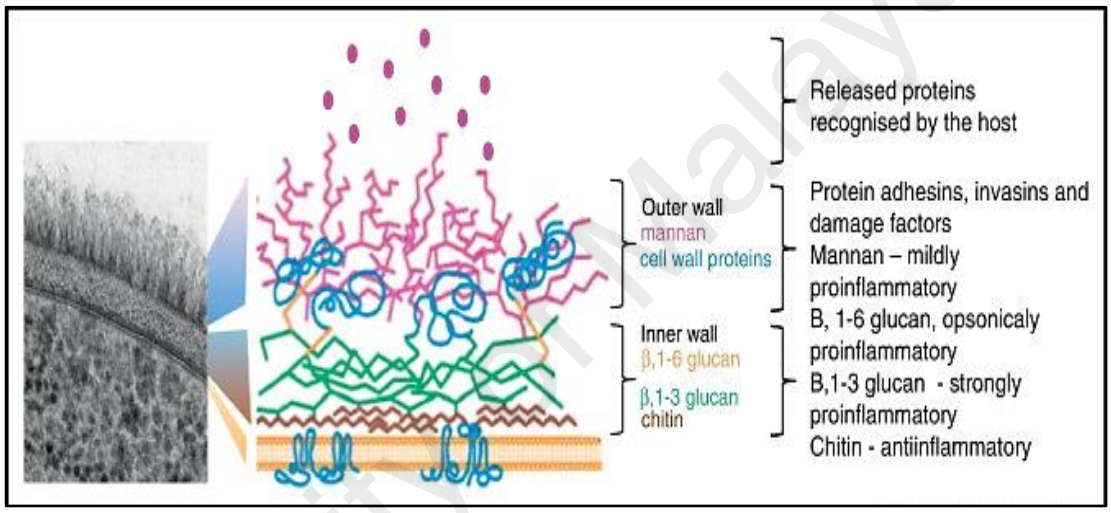


**Figure 2.2:** Transmission electron micrograph showing a section of the *C. albicans* cell wall (left) and a schematic arrangement of the major components (centre) and the role of those components in immune activation and suppression (right)
Source: (Gow et al., 2012)

### 2.2.5 Cell wall proteins (CWP)

Cell surface proteins are of considerable interest in *C. albicans* because of their immediate exposure to and potential interaction with the host. Proteins at the surface function as adhesins, elicit an immune response and alter with morphological state (Chaffin, 2008). Wall proteins show a variety of functions ranging from adhesion and iron acquisition (Almeida *et al.*, 2008) to tissue invasion (Schaller *et al.*, 2005) and defence against the immune response (Frohner *et al.*, 2009). Mannoproteins are considered as the most important antigenic component of *Candida* strains composing 10-30% of the cell wall. This group of proteins is mainly composed of carbohydrate polymannose containing more than 150 strongly bonded mannosyl units. Most wall proteins are covalently linked to β-1,6-glucans by modified glycosylphosphatidylinositol (GPI) anchors. Thus the cell wall proteins are classified as GPI-anchor proteins and PIR proteins (Klis *et al.*, 2001). A recent review presents a list of 115 putative GPI proteins (Plaine *et al.*, 2008). GPI proteins possess two signal peptides located at either end of the polypeptide chain. The N-terminal signal peptide directs them to the endoplasmic reticulum (ER). In the ER, the N-terminal signal is removed and the C-terminal signal is replaced by a GPI anchor, a preassembled lipid anchor that links them to the lumenal leaflet of the membrane. The CWP coat determines two major collective cell surface properties: cell wall permeability and cell surface charge. Restriction of cell wall permeability is due to the close packing of CWPs, the presence of bulky N-linked protein side-chains, and the formation of intermolecular disulfide bridges (Cassone, 1989; Klis *et al.*, 2009). This protects the structural polysaccharides against degradation by foreign glycanases. The other main cell-surface characteristic is because of phosphodiester linkages present on N-linked carbohydrate side-chains of cell wall proteins, which impose negative charge to the cell wall (Cutler, 2001; Fradin *et al.*, 2008; Thieme *et al.*, 1971).

### 2.2.6 Properties of CWP families

1. **Coat-forming CWPs**: As mentioned above, Pga59 is a likely candidate for a coat-forming CWP that restricts the permeability of the cell wall (Moreno-Ruiz *et al.*, 2009).

2. **Hydrophobicity-conferring CWPs**: Eap1 promotes adhesion to styrene, a hydrophobic polymer derived from ethenylbenzene (Li *et al*., 2003).

3. **The Als adhesin family** consists of nine GPI proteins, seven of which have been experimentally validated as covalently linked CWPs. Als proteins bind to diverse mammalian proteins. One of the reasons is that the recognition of peptide ligands by the als proteins is degenerate and that their specificities overlap each other only partially. This allows *C. albicans* to bind to a large variety of host proteins (Klotz *et al.*, 2004). This quality probably augments intercellular attachment and biofilm cohesion (Bastidas *et al.*, 2009; Nobile *et al.*, 2006). Als proteins have also been investigated in the formation of mixed species aggregates containing fungal and bacterial cells. The details of which are still unknown.

4. **Hwp1** is an adhesion protein that represents an interesting event of molecular mimicry. The N-terminal domain of Hwp1 is perceived as a familiar substrate by transglutaminases present on the host's epithelium. In reaction to which, *Candida albicans* bonds covalently to host epithelial / mucosal surface and resists detachment (Staab *et al.*, 1999). Hwp1 is also known to play a supplementary role in biofilm development, along with als1 and als3, the exact mechanism of which is still unknown.

5. **Pir1** is a cross-linking cell wall protein. It is present in the inner layer of cell wall. Pir1 is presumed to form cross-links between β-1,3-glucan chains. Its exact function is yet unrecognised (Ecker *et al.*, 2006; Kapteyn *et al.*, 2000).

6. **Carbohydrate-active enzymes**: A considerable number of CWPs have (predicted) glycosylase/transglycosylase activity (Cantarel *et al.*, 2009; de Groot *et al.*, 2004).

7. **Heme-iron acquisition**: Some CWPs (Als3 and Rbt5) are involved in the acquisition of iron (Frohner *et al.*, 2009).

8. **Coping with oxidative stress**: *C. albicans* incorporates the GPI-modified superoxide dismutases Sod4 and 5 into its wall, which help the cell to cope with oxidative stress originating from innate immune cells (Frohner *et al.*, 2009).

9. **Invasion-related CWPs**: Other CWPs, such as Als3, have been shown to act as an invasin, thereby facilitating endocytosis (Phan *et al.*, 2007).

10. **The yapsin-like proteins:** Sap 9 and 10 possess proteolytic activity, and in their absence, normal cell wall construction is affected, but their specific substrates are still largely elusive (Albrecht *et al.*, 2006).

Most of these proteins are only expressed under specific conditions and previous work suggests that there are no more than approximately 20–30 wall proteins expressed at any given time (Klis *et al.*, 2009). It is however, not yet defined which of these proteins are over / under expressed in polymicrobial species interaction of *C. albicans.*

## 2.3 *C. albicans* in human body

### 2.3.1 Life as a successful saprophyte

*Candida albicans* is basically an asexual yeast but nonetheless, it is physiologically and morphologically an alterable and adaptable organism. *Candida albicans* can survive in a variety of host niches (vagina, gut, skin, oral mucosa,) without causing damage to the structure (Hall *et al.*, 2017). In light of this observation, it can be suggested that *C. albicans* is suited for commensalism (Tang *et al.*, 2016). Oral mucosal and gastrointestinal tract colonisation appears to occur due to the accumulation of yeast cell

type (Douglas, 2003; Vautier *et al.*, 2015). The posterior surface of tongue, cheek, gums and other remaining mucosa of buccal cavity are deliberated as the prime locations for candidal colonisation. As a commensal organism, *C. albicans* releases enzymes to digest carbohydrates by fermentation and release carbon dioxide and alcohol for its growth and survival. Other naturally present microbial flora prevents the propagation and colonisation of *C. albicans* in the buccal cavity by releasing signalling molecules that inhibits fungal adhesion and by competing for oxygen and nutrients in the oral environment to limit fungal colonisation (Frey-Klett *et al.*, 2011). However, observations done on murine with steady gastrointestinal candidiasis revealed that carriage of *Candida* in the oral cavity and gut leads to the transformation of fungal cell into a 'GUT phenotype' yeast cell (gastro intestinally induced transformation), that expresses a distinct transcriptome in the alimentary tract to promote digestion of common nutrients in the gut. These modified cells are physiologically adapted to repress the tendency for mucosal incursion and expression of some virulence traits (Pande *et al.*, 2013). Hence it can be inferred that the commensal stage does not purely depend on the host immune system, it is also assisted by organism-specific adaptations conducted by changes in the host (Neville *et al.*, 2015).

In a nutshell, investigations suggest that mucosal commensalism of *C. albicans* is dependent upon both extrinsic factors (host immune condition, diet, and competitive microbiome) and intrinsic factors (cell morphology, fungal burden, fungal gene regulation, adaptation). Candida species act simultaneously opportunistic pathogens (Jabra-Rizk *et al.*, 2016); they are able to invade the host mucosa and cause considerable damage to tissues. This usually occurs when several predisposing factors are activated and cause pathogenesis of infection *i.e.*, oral thrush *etc.*

### 2.3.2 Life as a successful pathogen

A good hygiene is eminent in the oral cavity not only to ensure that gums and teeth are in perfect health, but also to defend oral tissues from augmented growth of microorganisms thus preventing oral infections like caries, periodontitis and plaque accumulation. *Candida* species are capable of transitioning from a benign environment friendly commensal to a serious pathogen whenever the chemistry of buccal cavity changes drastically. It is the combined effect of both host and candidal factors that ultimately contribute to the development of oral candidiasis. An imbalance of the normal microbial flora, breakage of epithelial barriers or dysfunction of the immune system favour the transition of the human pathogenic yeast *Candida albicans* from a commensal to a pathogen. The excessive growth of *C. albicans* then initiates a series of events leading to progressive crippling of body's defence system, which in response permits *C. albicans* to disseminate even further (Stappers *et al.*, 2017).

Throughout the course of disease, *Candida albicans* inhabits several host niches according to the differences in their physical properties such as, pH, nutrient availability, $CO_2$ levels, oxygen carrying capacity etc. (Hall, 2015). *C. albicans* is capable of adapting and successfully thriving in such different situations; which is a key feature that establishes *C. albicans* as a successful pathogen (Brown *et al.*, 2014). Host tissue environment has multiple carbon sources so, when *C. albicans* traverses through various host niches, it adapts by using alternative carbon source immediately for its survival and progression (Ene *et al.*, 2013). This adaptation occurred because of rearrangement of ubiquitination sites in metabolic enzymes causing catabolite inactivation (Sandai *et al.*, 2012). The metabolic adaptability of *C. albicans* assists in various cellular process *e.g.*, the modification of cellular secretome and proteome, and its capability to transform from yeast to hyphal form, known as the "white-opaque switching" (Miller *et al.*, 2002), it also

affects biofilm formation along with its adhesion attributes, and the ability for cell-wall remodelling. A change in the cell-wall polysaccharide arrangement, modifies the microbe's sensitivity to antifungal and environmental stress, it also influences pathogen's immunogenicity by modifying the expression of pivotal pathogen-associated molecular patterns (PAMPs), thus forming candida a moving target for host's immune cells (Hall *et al*., 2013).

The host tissue conceals micronutrients *e.g*., Cu, Zn, Mn and Fe, from the microorganism in a mechanism known as 'nutritional immunity' (Crawford *et al.*, 2015). These micronutrients are fundamental for several vital cellular operations in the organism. Countering this, candida has evolved a system to surmount host's nutritional immunity by expressing either micronutrient transporters (*e.g*., Zrt1/Zrt2/soluble Pra1 for Zinc and Rbt5/Als3 for Iron), or other superfluous enzymes that utilize auxiliary micronutrients as cofactors (Gerwien *et al.*, 2018).

*Candida albicans* expresses different types of esterases, proteases, lipases and phospholipases which take part in the breakdown of human immune defence polypeptides, degradation of human connective tissues, and thus support in nutrition acquisition, evasion and invasion of the organism from human immune system (Schaller *et al.*, 2005). These hydrolases are members of multigene families; each member having unique substrate specificity. Recently, 'candidalysin', an entirely unique secretory host-cell lysing factor from *C. albicans*, has been discovered. It can cause damage to mucosal epithelium directly and activate danger response in host immune system (Moyes *et al.*, 2016). These are the evolutionary adaptive attributes that enable *Candida albicans* to thrive in different host niches, resist immune defences of host and aid microorganism to be established as a virulent pathogen (Figure 2.3).

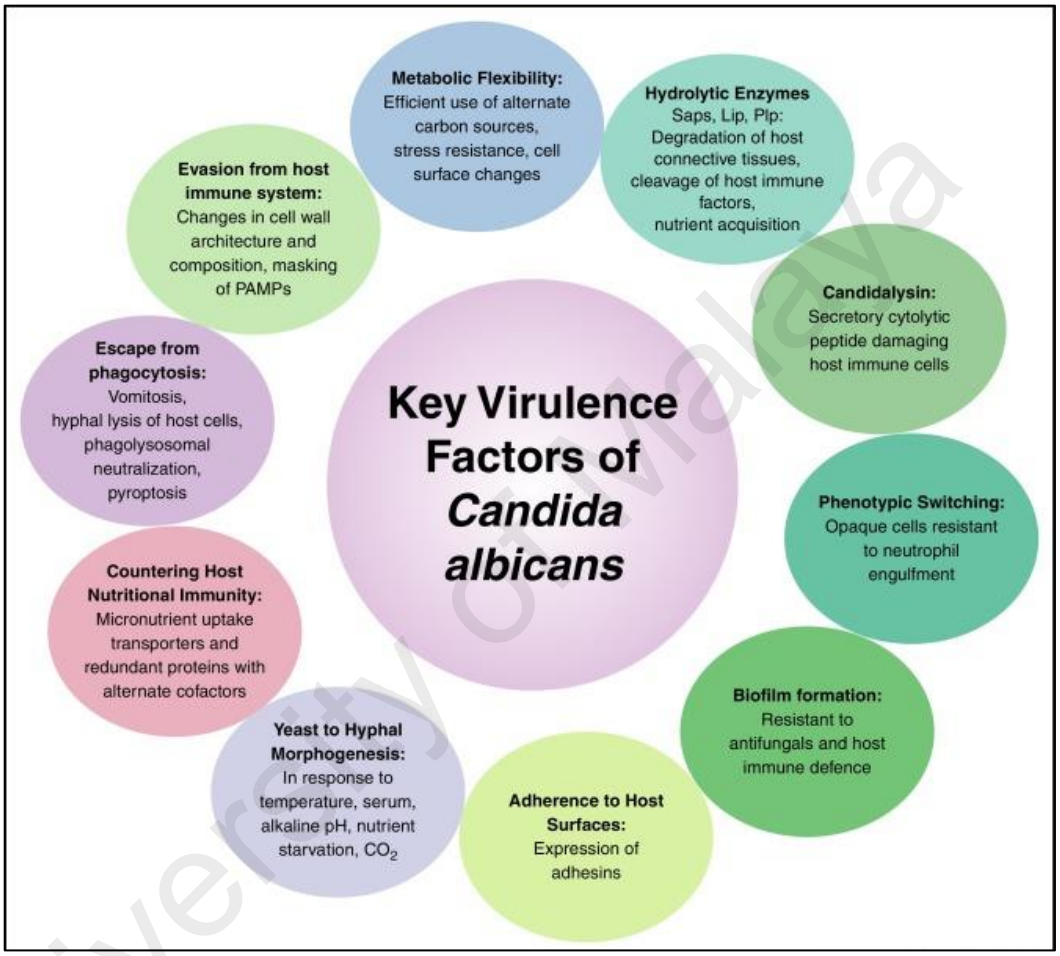


**Figure 2.3:** Factors governing virulence of *C. albicans*
Source: (da Silva Dantas *et al.*, 2016)

## 2.4 Oral Candidiasis

Oral candidiasis, commonly referred to as “thrush” is a commonly occurring opportunistic infection, the causative agent of which is *C. albicans* of genus Candida (Krishnan, 2012). Oral presentations of candidiasis vary from the large white curd-like plaques of pseudo membranous candidiasis on the tongue and buccal mucosa to the palatal erythematous lesions of chronic atrophic candidiasis, and to angular cheilitis on the labial commissures (Vainionpää *et al.*, 2019). The disease is more frequently visible in patients who are concurrently afflicted from other infections. Oral candidiasis can also infect neonates and infants because their immune systems are under developed (Pammi *et al.*, 2019).

### 2.4.1 Classification of Oral Candidiasis

Oral candidiasis was initially classified systematically by Lehner in 1966. He classified the disease based on histological, clinical and therapeutical criteria. Recently, oral candidiasis has been proposed to be classified as Group 1 (Primary) and Group 2 (Group 2). Group 1 is further subdivided into acute (erythematous and pseudomembranous) and chronic (nodular, erythematous, hyperplastic, pseudomembranous and plaque-like candida associated lesion), whereas, Group 2 includes oral manifestations of systemic diseases like endocrinopathy, HIV and thyroid aplasia (Mallya *et al.*, 2019).

## 2.5 Biofilm formation

The entry of Candida cells into the oral cavity is not sufficient for colonisation; they must be stably maintained. Since the oral cavity is a continuous-flow environment, yeast cells will be washed out by saliva and swallowed unless they adhere and replicate. Growth conditions in the oral cavity are so poor there is practically no growth in saliva unless it is supplemented with glucose that cells have to adhere to be maintained (Valentijn-Benz

*et al.*, 2015). Adhesion is therefore of critical importance in colonisation. Recent evidence suggests that the adherence and propagation of *Candida albicans* in oral cavity is associated with biofilm formation (Cavalheiro *et al.*, 2018; Nikawa *et al.*, 2005). *Candida albicans* could form biofilms on oral mucosa, teeth, dentures, inert polymers of various filling materials and implants. It has been known to adhere to basal epithelial cells, other microorganisms and even saliva molecules (Ganguly *et al.*, 2011; Serefko *et al.*, 2012).

To colonise surface, fungal cells must first adhere to biomaterial surfaces and the initial attachment of candida cells is mediated by both nonspecific factors (cell surface hydrophobicity and electrostatic forces) and promoted by specific adhesins present on the fungal surfaces that recognise ligands such as serum proteins and salivary factors (McCall *et al.*, 2019). A salivary coating containing glycoproteins, albumin, sialic acids, mucins, acidic proline–rich proteins and several other components forms a film over the oral mucosa (Hannig *et al.*, 2017). This nutrient enriched film, known as acquired pellicle, displays receptor surface for early colonisers. Biofilm formation correlates with cell surface hydrophobicity. The initial attachments of individual cells to substrate are followed by cell division, proliferation, and biofilm development (Mirani *et al.*, 2018) .

Various studies conducted on *C. albicans* have revealed that a typical biofilm consists of a basal substrate-bound layer of yeast cells that ranges from 20 to 100 microns in depth under many conditions. Filamentous cells project from the basal layer and can extend for several hundred microns (Figure 2.4). Yeast cells are often found to be produced by the filamentous cells, especially in the apical regions of the biofilm. Amorphous extracellular matrix material is found throughout the biofilm, which can appear aggregated or dispersed. A three-dimensional reconstruction has revealed a very dense basal region beneath the loosely packed filamentous cells. The loose packing of the upper region may facilitate solvent access to the basal region (Desai *et al.*, 2015).

Biofilm cells inherently have limited exposure to immunological defence and antimicrobial drugs, which could account for the emergence of resistant microorganisms. Slow growth, altered cell metabolism regulation due to nutrient limitation and stress conditions, and cell density are other suggested mechanisms of biofilm resistance (Borghi *et al.*, 2016). Biofilms are distinctive for being about 1,000 fold more resistant to common antifungal than their planktonic counterparts (Taff *et al.*, 2013).

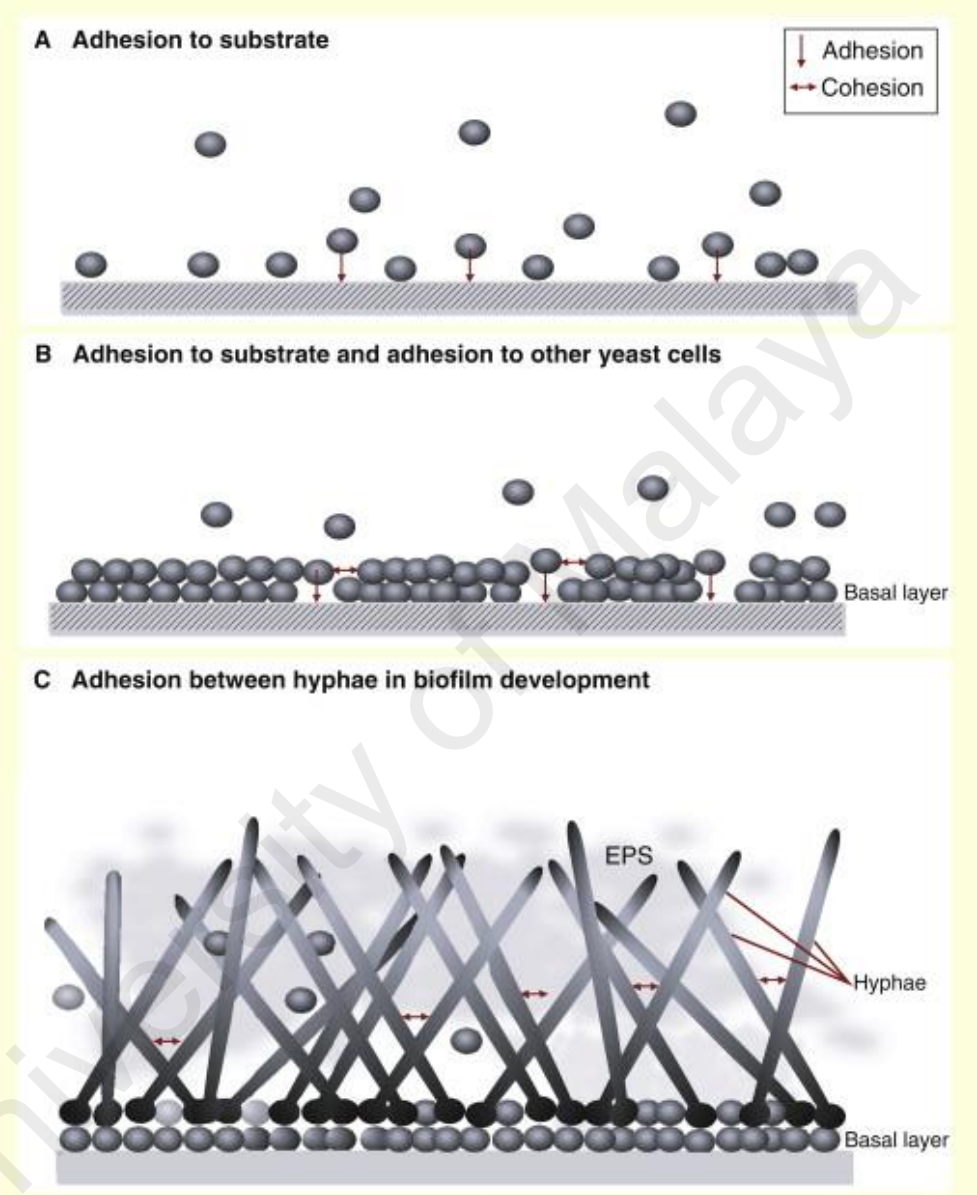


**Figure 2.4:** Development of typical *C. albicans* biofilm on host surface
Source: (Soll, 2008)

### 2.5.1 Polymicrobial biofilms

Over the past two decades there has been a revolutionary paradigm shift in the field of microbiology with the appreciation that microorganisms present in most biological systems exist in biofilms, rather than in a free-living state (McLean *et al.*, 2013). Studies utilizing these systems have demonstrated time and time again that *C. albicans* behave very differently when in a biofilm than during planktonic growth. In many ways, it is imperative to relearn everything about candidal behaviour through the view of the biofilm lens. Oral cavity is home for more than 500 different species of microbes (Aas *et al*., 2005). So, it is logical to believe that in natural environment, *C. albicans* would form biofilm consisting of coexistent microbial species. It is now believed that microbes act in concert to establish biofilms, which in turn can increase tolerance to antimicrobials, exacerbate of the host's immune response and increase persistence at the infection site (Murray *et al.*, 2014). These biofilms are difficult to diagnose and treat and have the potential to serve as an infectious reservoir for a variety of other micro-organisms.

Interactions between microbes are complex and highly dependent on context. They can range from fierce competition for nutrients and niches e.g., in lung infections, the association between *C. albicans* and *Pseudomonas aeruginosa* is an example of an antagonistic interaction between fungi and bacteria, where *P. aeruginosa* kills yeast hyphae and biofilms of *C. albicans* (Morales & Hogan, 2010). Similarly, *Lactobacillus rhamnosus* is being used as a treatment therapy for vaginal candidiasis because it interferes with the growth, morphogenesis and adhesion of *C. albicans* (Jeavons, 2003). Some interactions, however, have evolved cooperative mechanisms between different species that support their mutual growth in specific environments (Peters *et al.*, 2012). For instance, *Staphylococcus aureus* forms otherwise poor monoculture biofilms in serum, but in the presence of *C. albicans*, it forms a substantial polymicrobial biofilm. In

terms of architecture, *S. aureus* formed micro colonies on the surface of the biofilm, with *C. albicans* serving as the underlying scaffolding. Thus in this scenario, *C. albicans* aids propagation of *S. aureus* (Harriott *et al.*, 2009). Similarly studies have shown synergistic interactions of *C. albicans* with *Streptococcus gordonii, Streptococcus sanguinis* and *Streptococcus oralis* where *C. albicans* enhanced the ability of bacterial species to develop biofilms on buccal mucosal analogues or on abiotic sites (Diaz *et al.*, 2012). It is hypothesized that the presence of streptococci increased the ability of *C. albicans* to adhere and invade oral tissues by enhancing its virulence factors, but it has not yet been studied in detail.

Genetic diversity of microbes within biofilm communities is thought to increase the fitness of the residing community, making them more equipped to survive environmental stresses. In large part, this is due to an expanded gene pool, which can be more easily shared within the confines of a biofilm community (Gabrilska *et al.*, 2015). Community composition and interactions within the community can have huge influences on behaviour of *C. albicans*. Thus, just as the behaviour of planktonic versus biofilm-associated candida is dramatically different, so is that of candida in single species versus multispecies systems. Details of how one species affect the other is yet to be determined and is topic of interest amongst researchers.

## 2.6 Oral *Streptococci*

### 2.6.1 Taxonomy

Streptococcus is a genus of facultative anaerobic gram-positive cocci and member of phylum Firmicutes. *Streptococcus* species are amongst the earliest colonisers of hard and soft oral tissues, which forms more than 80% of germinal biofilm composition (Kreth *et al.*, 2005). In 1937, Sherman proposed a scheme for placing the streptococci into four

categories. These categories were organized by hemolytic reaction, group carbohydrate antigens, and phenotypic tests (primarily fermentation and tolerance tests). Sherman's four divisions were the pyogenic division, the viridans division, the lactic division, and the enterococci (Sherman, 1937). Shortly before the publication of Bergey's Manual in 1986, the genus Streptococcus was split into three genera (Enterococcus, Lactococcus, and Streptococcus) (Schleifer *et al.*, 1987).

Currently, the genus Streptococcus comprises 99 recognised species. Oral streptococcal bacteria are commonly known to be “viridans streptococci” because they are either α-hemolytic, producing a green colouration on blood agar plates (hence the name "viridans", from Latin "vĭrĭdis", green), β-hemolytic, the group that causes complete hemolyisis of red blood cells RBCs (Subgroup A; *S. pyogenes* and subgroup B; *S. agalactiae*) or nonhemolytic,γ-hemolytic. Oral streptococci however, can be subdivided into five groups: (1) Salivarius (*S. salivarius*) group, (2) Mutans (renowned bacteria are *S. mutans* and *S. sobrinus*) group, (3) Sanguinis (*S. sanguinis* and *S. gordonii*) group, (4) Mitis group (*S. mitis* and *S. oralis*) and (5) Anginosus (*S. anginosus* and *S. intermedius*) group (Facklam, 2002).

This study is concerned with two streptococcal species i.e., *Streptococcus mitis* and *Streptococcus sanguinis.*

### 2.6.2 Ecology

The oral cavity of the new-born infant is microbiologically sterile at birth. The process of acquisition of resident oral microflora begins soon after the birth within few hours. The predominant ‘pioneer species’ in the oral cavity are streptococci which appear within 48 hours after birth. *S. sanguinis* and *S. mitis* together, comprise 60–80% of the primary colonisers of clean tooth surfaces (Xu *et al.*, 2014). In the healthy adult oral cavity, *S.*

*sanguinis* and *S. mitis* are commonly associated with the buccal mucosa. These bacteria are the initial adherers of oral mucosa. In the adult oral cavity, on an average, streptococci represent 28% of the total cultivable microflora from supragingival dental plaque, 29% from the gingival crevice, 45% from the tongue and 46% from saliva (Aas *et al.*, 2005). Less commonly, they also colonise pharynx, teeth, dorsum of tongue and prosthetics.

### 2.6.3 Morphology and growth

The genus streptococcus, which defines the common morphology of its members, is derived from the Greek words streptos "twisted" and kokkos "sphere" (Nobbs *et al.*, 2009). Both concerned streptococci are smooth surfaced cocci ranging from 0.6-1.2µm in size (Mehanny *et al.*, 2020). *S. sanguinis* and *S. mitis* cells grow in pairs, chains and clusters similar to other members of its genus. Being primary colonisers of the oral cavity, *S. sanguinis* and *S. mitis* serve as a tether for the attachment of several oral bacteria and other microorganisms that contribute to the development of oral biofilm or dental plaque and may contribute to periodontal diseases (Heller *et al.*, 2016).

### 2.6.4 Pathogenesis

In the healthy oral cavity viridans streptococci prevent colonisation / invasion by more pathogenic exogenous microorganisms; a phenomenon called 'colonisation resistance. By competing with other microorganisms, these streptococci maintain physiological ecosystem of the oral cavity. However, any disruption in the ecological balance can shift the paradigm, making commensal streptococci into opportunistic pathogens (Bryskier, 2002). Both *S. mitis* and *S. sanguinis* can directly bind to oral surfaces and along with other oral microorganisms, colonise the tooth surface to form dental plaque, and contribute to the aetiology of both caries and periodontal disease (Zhu *et al.*, 2018). *S. sanguinis* can attach to the bloodstream and damage heart valves by secreting $H_2O_2$,

causing infective endocarditis. Endocarditis can proceed through entrance of oral streptococci to the bloodstream during dental procedures or even during normal daily activities such as eating (Paik *et al.*, 2005). Factors that have been implicated to contribute to the virulence and competitiveness of these streptococci include functions such as cell wall synthesis, amino acid synthesis, nucleic acid synthesis, the ability to survive under anaerobic conditions and the secretion of $H_2O_2$ (Kreth *et al.*, 2009). Streptococci have been studied extensively in polymicrobial species communities. However, the effect of these streptococci on growth profile and virulence of *C. albicans* have yet to be defined.

### 2.7 Virulence associated genes in *Candida albicans*

Like all pathogens, *C. albicans* has also possessed a plethora of imputed virulence attributes that aid in overcoming host defence system and invasion of the oral tissues. In spite the fact, that an array of virulence traits are concerned with the destructive process, no singular factor could be accounted for *C. albicans* pathogenicity. Similarly, not all defined virulence traits are essential for a specific stage of disease. Attention has been focused on Agglutinin-like sequence *ALS*1, 2 & 3 genes that encode large surface glycoproteins. Their encoded proteins are localized at the *C. albicans* cell surface (Hoyer *et al.*, 2008).

#### 2.7.1 *ALS* genes

*ALS* genes are a family of adhesions recognised to play a role in adherence and early biofilm formation. Since biofilm formation contributes to drug resistance, *ALS* genes appear to be responsible for fluconazole resistance. *Candida albicans ALS1* was first described in 1995 (Hoyer *et al.*, 1995). Since that time, seven more genes have been associated with the *ALS* family (Figure 2.5). Research efforts have focused on understanding the relatedness of *ALS* genes and exploring the function of the als proteins.

In the simplest terms, *ALS* genes have three general domains (Hoyer, 2001). The 5′ domain includes approximately 1300 bp and encodes a protein region that has a relative lack of glycosylation in the initial 320 to 330 amino acids. Following the *ALS* gene 5′ domain is a central domain consisting entirely of tandem repeated copies of a 108-bp sequence. The tandemly repeated sequence is somewhat variable, but each encodes a Ser/Thr-rich amino acid sequence and many copies contain a consensus N-glycosylation sequence. Subfamilies of *ALS* genes were proposed based on cross-hybridization between the tandem repeat sequences. Majority of the als proteins function as adhesins. The binding region for substrates is present on the N terminus. The last als domain is the C-terminal domain, which is the least conserved in length and sequence, but in all cases, encodes a Ser/Thr-rich protein with many consensus N-glycosylation sites (Hoyer *et al.*, 2016; Sheppard *et al.*, 2004). Because of their hydrophobic nature, the tandem repeats are directly able to mediate attachment of few substrates, like polystyrene.

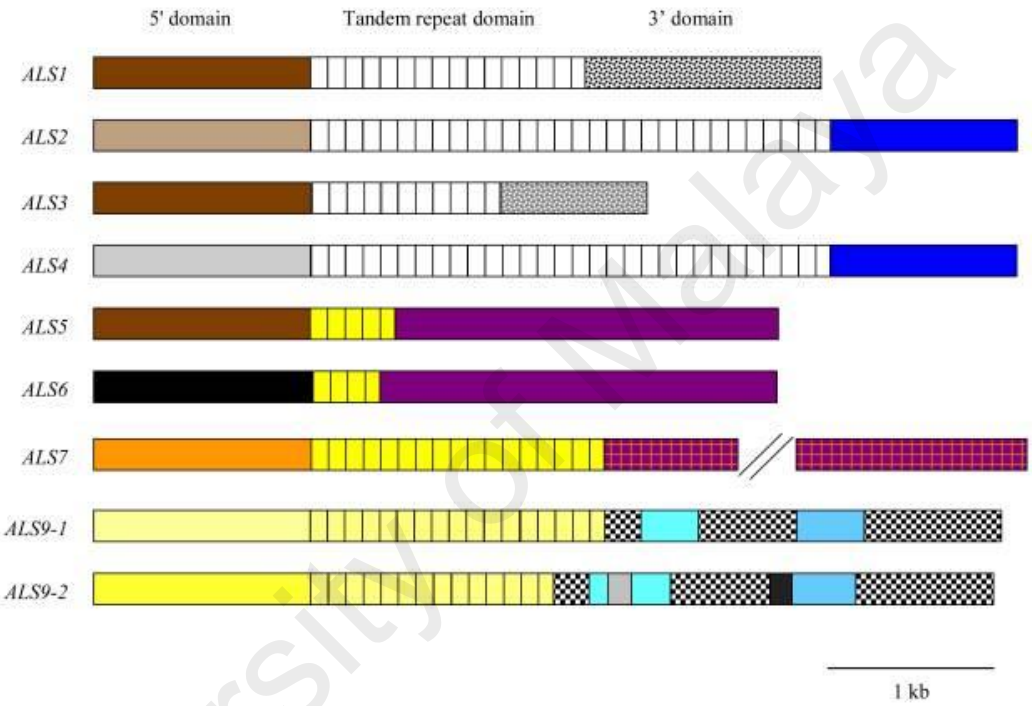


**Figure 2.5:** Figure showing selected domains of the *C. albicans ALS* genes. Colour coding is used to show regions of similarity between genes
Source: (Hoyer *et al.*, 2008)

### 2.7.2 *ALS*1

Agglutinin-like sequence 1 (*ALS*1) is amongst the most researched and frequently detected *C. albicans* adhesins. It expresses cell wall protein called als1p. Since host tissue attachment and hyphal development are two extremely important virulent factors and are a prerequisite for *C. albicans* infection, the expression of adhesins is crucial for the establishment and progression of candidiasis (Sundstrom, 2002). Als1p functions in adhesion to vascular endothelial cell monolayers facilitating hematogenous dissemination of *C. albicans* causing blood borne infections (Fu *et al.*, 1998). Several studies have proved adhesion of als1p to buccal epithelial cells, monolayer cells, fibronectin and laminin of oral epithelium (Hoyer *et al.*, 2008).

Recent studies prove that *ALS*1 not only mediates attachment of *C. albicans* to host tissue, it is actively involved along with *ALS*3 in formation of candida biofilm in oral cavity (Araújo *et al.*, 2017). It has been studied that *ALS*1 undergoes a heterotypic interaction with *ALS*3 between the surfaces of adjacent cells to maintain the integrity of a developing biofilm (Kamai *et al.*, 2002; Soll, 2008). This observation endorses previous studies which suggested that adherence to the host and pathogenicity of *C. albicans* are two positively correlated attributes. Although *ALS*1 gene expression and regulation of als1p synthesis has been vastly investigated for *C. albicans* (Finkel *et al.*, 2012; Fu *et al.*, 2002; Loza *et al.*, 2004) however, there is no sufficient evidence to prove whether interaction of *C. albicans* with other microorganisms have the potential to express discrete patterns of *ALS*1 expression, and ultimate secretion of als1 during polymicrobial biofilm formation.

### 2.7.3 *ALS*2

Previous work conducted to investigate functions of *ALS*2 suggests that als2p, expressed by *ALS*2 contributes to *C. albicans* adhesion to vascular endothelial cells. Like all other *ALS* family genes, *ALS*2 functions as an adhesive of *C. albicans*. It has substantial contribution in candida biofilm formation, germ tube induction and cell wall regeneration of the candida during fungal adhesion to host surface and propagation (Zhao *et al.*, 2005). Unlike *ALS*1 and *ALS*3, this gene is considered relatively benign in regard to pathogenesis of *C. albicans*. To date, not enough studies have been conducted to define the role of *ALS*2 in candida related polymicrobial species interactions.

### 2.7.4 *ALS*3

*ALS*3 gene is expressed by *Candida albicans* hyphae and pseudohyphae but not yeast-phase organisms. Researchers have shown special interest in the biology and physiology of biofilm formation by *C. albicans*, since it is the foundation of microbial acquisition and subsequent growth on oral surfaces. It has been suggested that *ALS*3 is one of the most important genes associated with *C. albicans* biofilm production (Hoyer *et al.*, 2008). Although the exact mechanism of how *ALS*3 induces biofilm formation is still unknown, many *in vivo* and *in vitro* studies using *ALS*3 mutant gene have shown significant decrease in biofilm formation or have produced scant, disorganized biofilms authenticating the importance of *ALS*3 in biofilm formation and maintenance (Zhao *et al.*, 2006).

Several studies have shown that als3p plays an important role in several processes which are essential for the microorganism to inhabit the host tissue and cause infection (Liu *et al*., 2011). These processes are; biofilm formation, adhesion to host tissue, iron acquisition and invasion of host cells. Furthermore, since als3 is greatly expressed *in vivo*, researchers are targeting it for vaccine development and therapeutic antibody production

(Liu *et al*., 2011). Als3p has a wide range of substrate specificity, thus it can mediate attachment to different host structures like oral epithelial cells, endothelial cells, fibronectin, type IV collagen, gelatin, laminin, fibrinogen, and salivary pellicle (Sheppard *et al.*, 2004). This feature renders *C. albicans* capable of adhering to multiple natural or prosthetic surfaces present in oral cavity.

Although not studied in detail, but in lieu of the previous studies performed on *ALS*3, it will be interesting to investigate the expression of *ALS*3 in polymicrobial species interaction of *C. albicans*.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 MATERIALS

### 3.1.1 Fungal species

The following candidal strain was used for experiment:

*Candida albicans* (ATCC® 14053™)

### 3.1.2 Bacterial species

The following bacterial strains were used for experiment:

i. *Streptococcus mitis* (ATCC® 49456™)
ii. *Streptococcus sanguinis* (ATCC® BAA 1455)

## 3.2 RESEARCH METHODOLOGY

### 3.2.1 Research outline

The study was based on formation and evaluation of *Candida albicans* polymicrobial species biofilms with two bacterial species i.e., *Streptococcus mitis* and *Streptococcus sanguinis*. Polymicrobial biofilms were formed in-vitro in static and dynamic conditions. The influence of streptococci on *C. albicans* biofilm and morphology was estimated using CV assay, XTT assay and SEM analysis. The antimicrobial effect of amphotericin B, nystatin and chlorhexidine was evaluated. The expression of *ALS*1, 2 and 3 genes along with their respective proteins was also determined for *C. albicans*. The methodology of research is summarised in Figure 3.1.

### 3.2.2 Preparation of agar plates and broth media for *Candida albicans* and Streptococci

Yeast Peptone Dextrose (YPD) agar and broth were used as growth media for the yeast. Brain Heart Infusion (BHI) agar and broth were used as growth media for *S. mitis* and *S. sanguinis*. The media was appropriately weighed, dissolved in distilled water, boiled and later sterilized at 121°C for 15 min in an autoclave. The agar plates and broth media were kept refrigerated at 4°C for later use.

### 3.2.3 Microbial strains and growth condition

The lyophilised candidal species in Section 3.1.1 were rehydrated in sterile distilled water and inoculated onto Yeast Peptone Dextrose (YPD) agar media. The lyophilised streptococcal species in Section 3.1.1 were rehydrated in sterile distilled water and inoculated onto Brain Heart Infusion (BHI) agar media.

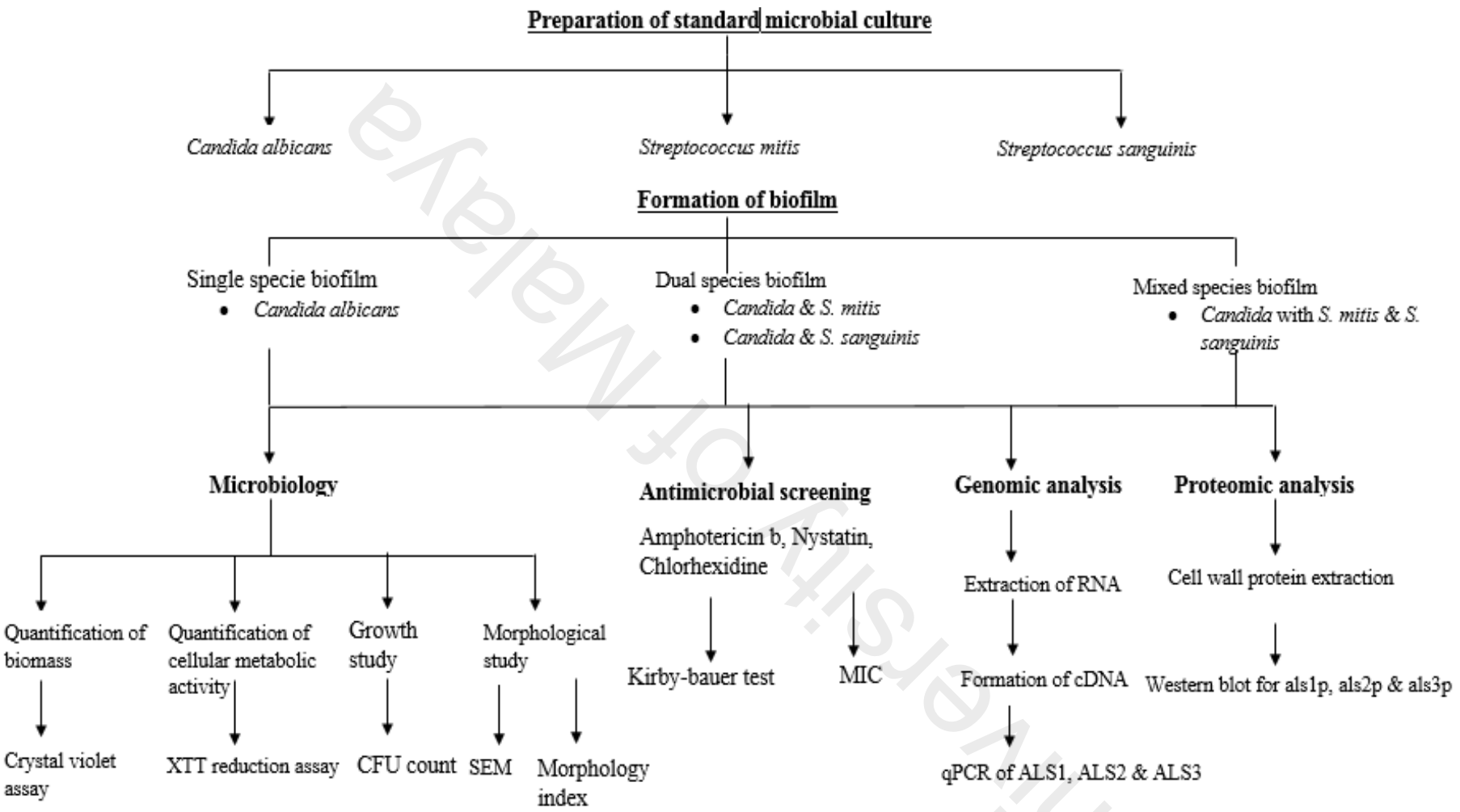


**Figure 3.1: An outline of research project**

Following incubation at 37°C, the colonies were sub-cultured on fresh YPD and BHI agar slants respectively and stored at 4°C for further use in the experiment. Stock for long storage was prepared in 20% glycerol and stored at -70°C. The purity of the candidal and bacterial strains was validated with the use of the Biolog Identification kit. Regular sub-culturing was carried out every two weeks to maintain viability of the cells. These growth colonies were later used in the preparation of candidal and bacterial suspension respectively.

### 3.2.4 Preparation of the standard microbial cell suspension

For use in an experiment, the fungal colonies were harvested and dispensed in 10 mL YPD medium and allowed to grow overnight at 37°C in an orbital shaker (150-180rpm). Bacterial colonies were harvested and dispensed in 10mL BHI broth and allowed to grow overnight at 37ºC in a standard incubator. Cell cultures were harvested by centrifugation at 2000 ×g, washed twice with phosphate-buffered saline (Sigma-Aldrich) and re-suspended in Tryptic soy (TS) broth. TS broth was used as study media for both yeast and bacteria to standardize experimental conditions. From here onwards, both microbial species were cultivated in tryptic soy agar. The turbidity of the suspension was adjusted to an optical density ($OD_{550nm}$) of 0.144 which is equivalent to $1x10^6$ cells/mL for candida and $1x10^8$ cells/mL for streptococci or to #0.5 McFarland standard.

### 3.2.5 Formation of *in vitro* salivary biofilms

Saliva is continually produced from salivary glands in the oral cavity. Saliva thus coats all hard and soft tissues inside the oral cavity. It forms the foundation of salivary pellicle and dental plaque. Oral microorganisms adhere to these foundations thus forming microbial biofilms and subsequently progress to cause infection. Thus, saliva plays an important role in meditating attachment of microbes to oral surfaces by providing specific

adhesion mechanisms that involve various ligand-receptor reactions of protein-protein and/or protein-carbohydrate between the acquired salivary pellicle and the surface of microbial cell wall.

In this study, *in vitro* salivary biofilms were formed using static and dynamic conditions to mimic oral environment. Using the Nordini artificial mouth system (NAM) (Rahim *et al*., 2008), experimental pellicles were developed on glass surfaces to mimic the acquired pellicles on the tooth surface. Single, dual and mixed species biofilms were formed on glass surfaces for 24 h and were subsequently assessed as described later.

#### 3.2.5.1 Collection and preparation of saliva for biofilm

Ethical approval for the collection of saliva was obtained from Ethical Committee, Faculty of Dentistry under the code of (Ref: 40550/AN/SU). Unstimulated saliva was obtained from a 30 year old, medically healthy donor with no persistent oral condition, and collected into ice-cold sterile falcon tubes. One donor was asked to contribute throughout the whole experiment to minimize any variations that may arise because of different subjects as previously done in other studies (Li *et al*., 2017; Velsko *et al.*, 2018). The donor was instructed to rinse the oral cavity with distilled water prior to the collection of saliva at 9:00 am to 11:00 am, to reduce bacterial transfer from the oral cavity. The collected saliva was then clarified using centrifugation for 30 min at 17,000 g. The pellet was discarded, and the clarified saliva was filter-sterilized using sterile 0.2 µm cellulose acetate syringe filter and stored at -20$^{o}$C prior to use.

#### 3.2.5.2 Formation and growth of biofilm in dynamic condition

Dynamic biofilms are those that provide continuous flow of saliva and nutrients thus mimicking the conditions in oral cavity. Maintaining the temperature of the flow system

as that of oral cavity and continuously supplying nutrients could help develop a mature biofilm quite similar to the one formed in oral cavity.

Using the prepared inoculums as described in section 3.2.4, single, dual and mixed species (*C. albicans* plus *S. mitis* and *S. sanguinis*) biofilms were formed. The development of experimental pellicle and dynamic biofilm was carried out in the Nordini's artificial mouth (NAM) model using the procedure defined by Rahim *et al*., 2008. The model contained 6 glass beads (3mm in diameter) that mimic the surface of the tooth (Hasnor *et al*., 2008; Rahim *et al*., 2008). Temperature of 37ºC was maintained to replicate temperature within oral cavity. Sterilized saliva was allowed to flow into the NAM model for 2 min at a flow rate of 0.3 mL/ min to coat the glass beads for the development of the experimental pellicle. This was followed by a flow of sterile distilled water to rinse off the excess saliva on the glass beads. Single, dual and mixed species suspensions were pumped into the system and left to circulate for 24 h at 37ºC and speed of 0.3 mL/ min respectively. The microorganisms would attach to saliva coated glass beads to form a dynamic biofilm.

#### 3.2.5.3 Formation and growth of biofilm in static condition

Static biofilm systems are formed quite frequently in experiments to evaluate microbial growth for several reasons. Static assays are particularly useful for examining early events in biofilm formation. The evaluation techniques can quantify even smaller biofilms with high reproducibility. Another major advantage of these systems is the simplicity of the protocols: these assays can be executed primarily using common laboratory equipment. Furthermore, these assays have a relatively high throughput and can easily be adapted to study a variety of biofilm formation conditions, making them excellent tools for performing genetic screens.

Using the prepared cell inoculums as described in section 3.2.4, single, dual and mixed species biofilms were developed on commercially available flat-bottom, polystyrene 96-well microtiter plates. The surface of wells was coated with 50 µL of clarified saliva and incubated for 90 min at 37°C. Excess saliva was aspirated. For single species biofilm, 200 µL of *C. albicans* cell suspension (1 x $10^6$ cells/mL cells) diluted in Tryptic soy medium, was poured in saliva coated wells as shown in Figure 3.2. Two more single species biofilms were formed using 200 µL of *S. mitis* and 200 µL of *S. sanguinis* cell suspension (1 x $10^8$ cells/mL) respectively diluted in TS broth. Single bacterial species biofilms were made as control to compare biomass and cellular metabolic activity with dual and mixed species biofilms. Two dual species biofilms were made; first; of *C. albicans* with *S. mitis* (1:1) and the second; *C. albicans* with *S. sanguinis* (1:1). Equal volume of each species (OD550nm) was used to give a total volume of 200 µL in each well. For mixed species biofilms, ratio of *C. albicans* : *S. mitis* : *S. sanguinis* species was 1: 1: 1, to give a total volume of 200 µL. The plates were incubated for 24 h at 37°C. After the formation of biofilm, medium was aspirated, and the biofilms on microtiter plates were lightly washed twice with PBS to remove non adherent cells. Biomass of biofilms and the cellular metabolic activity were measured quantitatively using crystal violet (CV) staining assay and tetrazolium (XTT) reduction assay, respectively.

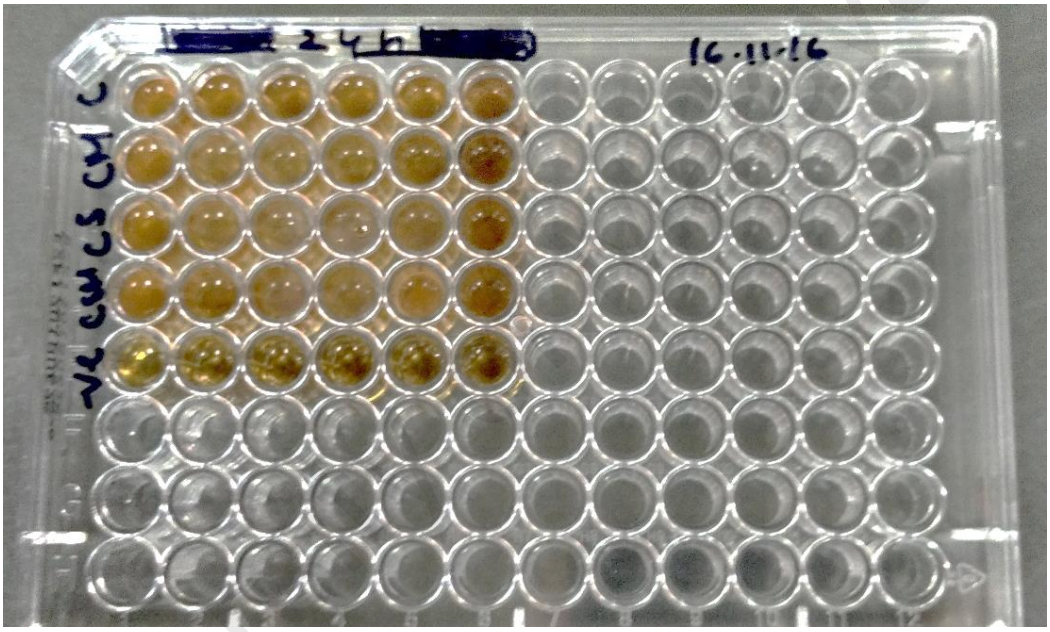


**Figure 3.2:** Microbial cell suspensions were diluted in tryptic soy broth and poured in 96-well plate to form static biofilm

*Candida albicans* alone was used in single species biofilm as control for the experiment. Results of dual and mixed species biofilms, when compared to it, will determine the effect exhibited by both bacteria and fungal biofilm growth and propagation. *S. mitis* and *S. sanguinis* alone was also used to make single species biofilms. Results of which will further help in comparing results of dual species biofilms and justifying the role of each bacteria in biofilm growth and propagation.

### 3.2.6 Determination of microbial growth in biofilm

#### 3.2.6.1 Experimental design

Adherence of microbial species on the mucosa, teeth and denture surfaces is mediated by specific adhesion mechanism that involves various ligand-receptor reactions of protein-protein and/or protein-carbohydrate between the acquired pellicle and the surface of the candidal cells. In the oral cavity, saliva plays an important role in promoting adherence of these cells to hard surfaces that is providing specific receptors for adhesion to the acquired pellicles. Using the Nordini's Artificial Mouth (NAM) model (Rahim *et al.*, 2008), experimental pellicles were developed on glass surfaces (Figure. 3.3) to mimic the acquired pellicles on the tooth surface. Attachment of each species on salivary pellicle was evaluated by counting the CFU. Dual and mixed species could have different CFU than single species. Each species could compete for its place on the pellicle, be washed away by the continuous flow or they could have a mutualistic behaviour. Increase or decrease in the CFU count of single, dual and mixed species on the constant flow system could establish how well that organism can withstand oral environment.

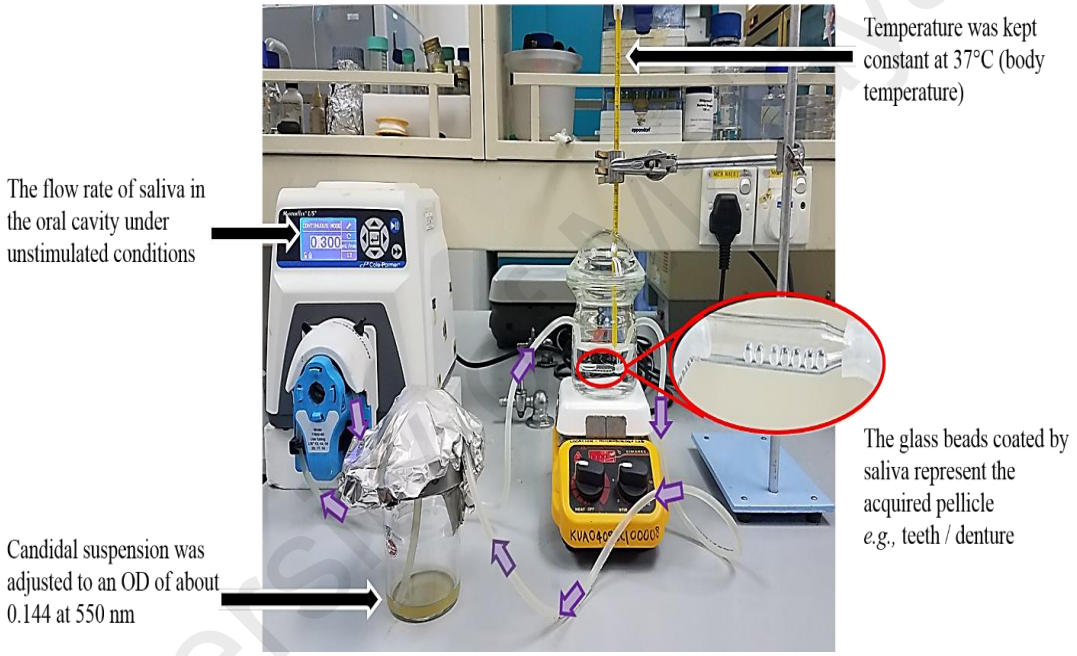


**Figure 3.3:** An illustration of *Nordini's artificial mouth* (NAM) model for formation of dynamic biofilm

### 3.2.6.2 Protocol for CFU count

After the formation of dynamic biofilm on glass bead as described in section 3.2.5.2, the CFU was counted as shown in Figure 3.4. The glass beads were rinsed with sterile distilled water to rinse off the excess saliva on the glass beads. The population of adherent cells was determined by transferring the beads to a sterile microcentrifuge tube containing one mL of phosphate-buffered saline (PBS). The tubes were sonicated for a few seconds and vortexed for 1 min to dislodge attached cells. The tube containing the dislodged species was marked as Tube 1 (T1). The content of T1 was then serially diluted using PBS to a final dilution of 1:10 (6th dilutions) contained in Tube 7 (T7). A 100 µL of suspensions from each tube was pipetted out and inoculated on three separate TS agar plates. Following incubation at 37ºC for 24 h, the plates with dilution that gave a CFU count of between 30 to 300 cells were selected for enumeration. The growth population (CFU/mL) were calculated using the formula:

$$\text{Total CFU/mL} = \frac{\text{number of formed colonies}}{\text{dilution factor X volume used (mL)}}$$

The results were expressed as the mean of CFU/mL of triplicate determinations.

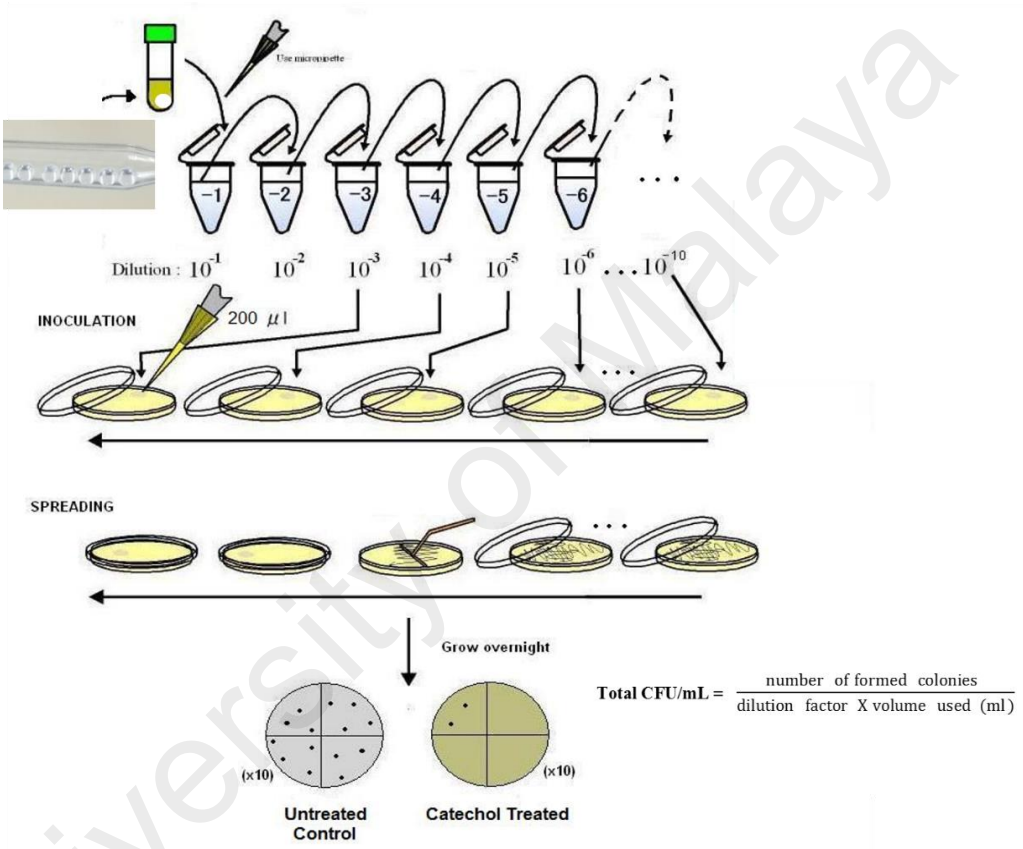


**Figure 3.4:** An illustration showing method of CFU count

### 3.2.7 Determination of biofilm biomass

#### 3.2.7.1 Experimental design

In this experiment, static salivary biofilms were formed on 96-well microtiter plates as described in section 3.2.5.3. The collective biomass of formed biofilms was quantified using crystal violet (CV) staining procedure using the method described by O'Toole (O'Toole, 2011). Crystal violet is a basic dye that binds non-specifically to negatively charged surface molecules such as polysaccharides and eDNA in the extracellular matrix. Because it binds to cells as well as matrix components, it can generally be used to evaluate biofilm biomass in toto. When grown in combination with bacteria, the overall biomass of *Candida* biofilm might be different. The change in growth conditions, presence of a different species in the vicinity may influence the overall growth of biofilm. An increase or decrease in the size of biofilm could determine organism's potential to sustain in host environment. Crystal violet (CV) assay was performed to assess and compare the difference in biofilm biomass for single species *Candida* biofilm and biofilms formed in combination with bacteria.

#### 3.2.7.2 Sample preparation and protocol for CV assay

Biomass of biofilms was determined by crystal violet assay. Single species (*C. albicans* alone) (*S. mitis* alone) (*S. sanguinis* alone), dual species (*C. albicans* with *S. mitis*) (*C. albicans* with *S. sanguinis*) and mixed species (*C. albicans* with *S. mitis* and *S. sanguinis*) biofilms were developed on polystyrene, commercially available, flat-bottom 96-well microtiter plates using cells as described in the Section 3.2.5.3. After 24 h incubation, excess cells were dumped out by turning plate and shaking out the liquid. This method offers least disturbance to the formed biofilm. The wells were gently washed twice with PBS. This step removed unattached cells and media components to avoid

unnecessary staining. After washing, the biofilms were fixed with 10% formaldehyde for 10 min and stained with 100 µL of 0.1% w/v crystal violet solution for 15 min without any agitation. The biofilms were gently washed twice with PBS and destained with 95% ethanol. Destaining removed excess dye from biofilm components. Plates were allowed to air dry for 60 min as shown in Figure 3.5. Next, 75 µL of the solution was transferred into new wells of new 96-well microtiter plates, and their absorbance was measured at 595 nm using a microtiter plate reader.

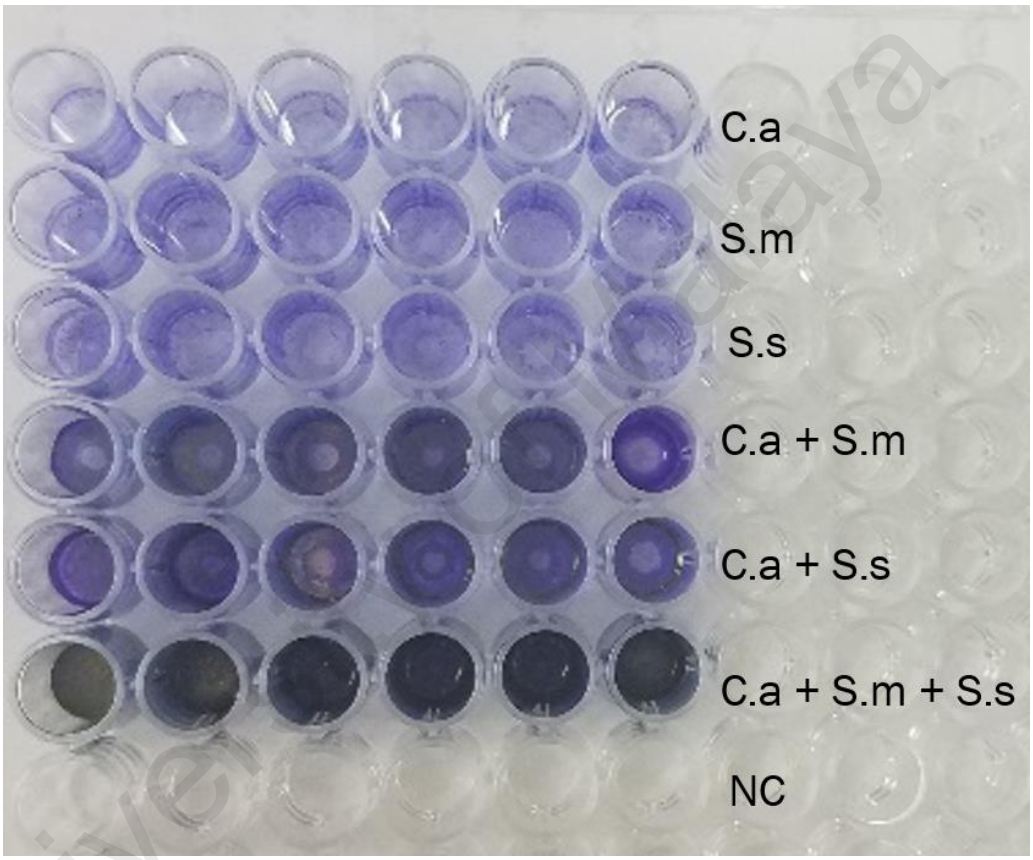


**Figure 3.5:** Crystal violet staining of single, dual and mixed species biofilms on 96-well plate after 24 h incubation period

Note: C.a; *Candida albicans*
S.m; *Streptococcus mitis*
S.s; *Streptococcus sanguinis*
NC; negative control

### 3.2.8 Determination of cellular metabolic activity in biofilms

#### 3.2.8.1 Experimental design

The cellular metabolic activity of *in vitro* static salivary biofilms was determined by XTT (tetrazolium salt 2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide) reduction assay using the method as described by Koban with slight modifications (Koban *et al.*, 2012). The XTT assay is based on the reduction of the XTT dye (tetrazolium salt) to a water-soluble formazan. Colourless XTT compound, when reduced to a formazan derivative by mixture of cellular effectors, turns bright orange. XTT penetrates rapidly into living cells and directly into subcellular membranes with dehydrogenase activity, where it is converted to coloured formazan derivatives. The reduction occurs by breaking apart the positively charged quaternary tetrazole ring. XTT requires the presence of an electron-coupling agent to obtain a good correlation between the formazan production and the number of viable cells. In this study, menadione was used as a nonenzymatic electron transporter to enhance the reduction process. The resultant formazan product of XTT reduction is soluble and can be detected by ELISA reader. The absorbance of the cell supernatant is proportional to the number of metabolically active microbial cells. Quantification of the overall biomass of biofilm is not enough, it is imperative to determine cell viability within biofilms. Living cells will have the potential to invade host tissue and spread disease. XTT assay was undertaken to examine the effect of *S. mitis* and *S. sanguinis* species on the viability of *C. albicans* cells within the biofilms.

#### 3.2.8.2 Sample preparation and protocol for XTT Assay

The XTT (Sigma) solution was prepared in PBS (1 mg/mL), filter-sterilized through a 0.22 μm pore size filter and stored at -80°C until required. Menadione (Sigma) solution

(dissolved in acetone 0.4 mM) was filtered and mixed with XTT solution at a ratio of 1 to 10 by volume before the assay. Single species (*C. albicans* alone) (*S. mitis* alone) (*S. sanguinis* alone), dual species (*C. albicans* with *S. mitis*) (*C. albicans* with *S. sanguinis*) and mixed species (*C. albicans* with *S. mitis* and *S. sanguinis*) biofilms were developed on polystyrene, commercially available, flat-bottom 96-well microtiter plates using cells as described in the procedure above (Section 3.2.5.3). After 24 h incubation, excess cells were pipetted out gently from the wells. This method offers least disturbance to the formed biofilm. The wells were gently washed twice with PBS. This step removed unattached cells and media components from formed biofilm. After washing twice with PBS, a total of 100 µL XTT-menadione (10: 1) solution, composed of XTT sodium salt (Sigma-Aldrich) mixed with menadione (Sigma-Aldrich) solution was dispensed into each well. The plates were covered in aluminium foil and incubated in the dark for 2 h at 37°C. Following this, 75 µL of the solution from each well was transferred into new wells of new 96-well microtiter plates as shown in Figure 3.6, and the amount of colorimetric change (a depiction of the metabolic activity of biofilm cells) was measured at 490 nm using a microtiter plate reader (FC-Bios µQuant). Since there are four different groups of cells, it is expected to visualize different opacities caused by single, dual and mixed species (Kuhn *et al.*, 2003).

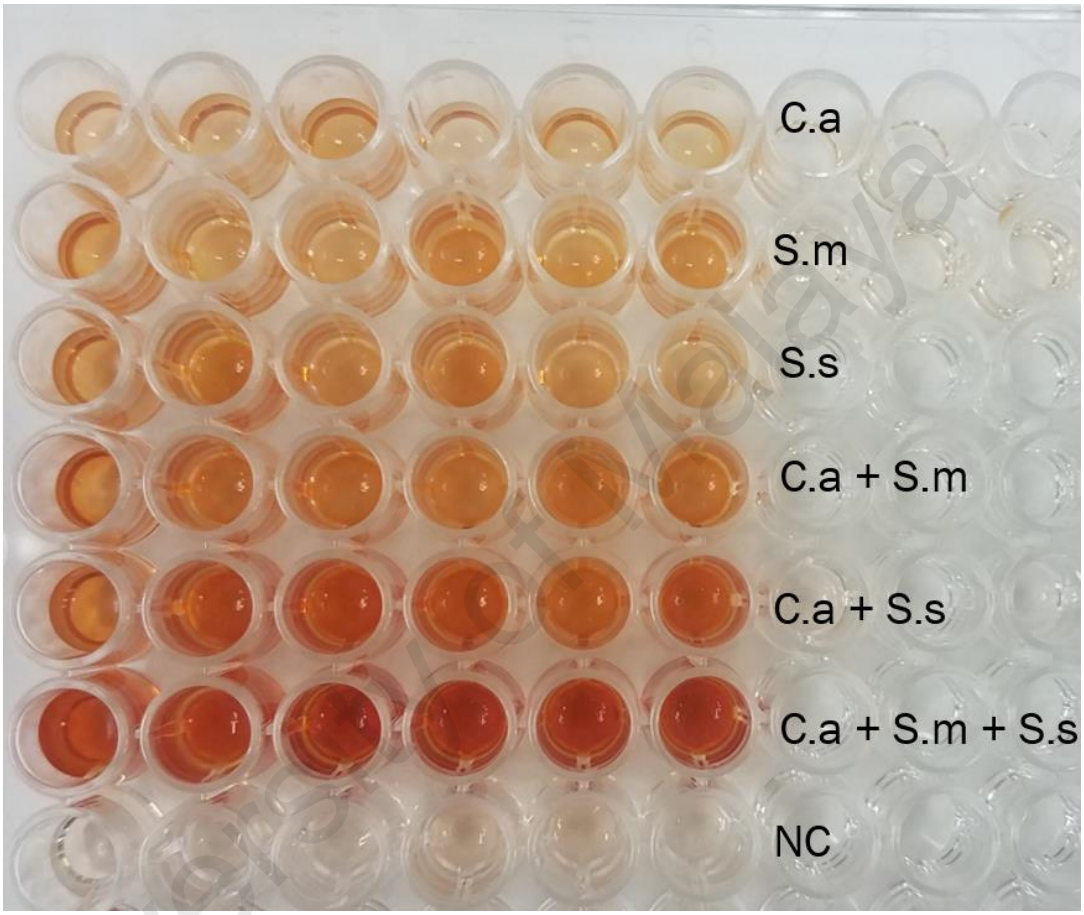


**Figure 3.6:** Colorimetric change observed on 24 h single, dual and mixed species biofilm following XTT reduction assay

Note: C.a; *Candida albicans*
S.m; *Streptococcus mitis*
S.s; *Streptococcus sanguinis*
NC; negative control

### 3.2.9 Assessment of morphology of *C. albicans*

#### 3.2.9.1 Experiment design

In this experiment, static *in vitro* salivary biofilms were formed consisting of *candida* alone and in combination with streptococci. In the presence of streptococci in its environment, *Candida albicans* might have to alter its cellular structure in order to survive. The streptococci, to co-aggregate with *C. albicans* may orchestrate phenotypic changes. The new environment could facilitate or prohibit *C. albicans* growth. Changes in morphology of the fungus could have influence its nutrient acquisition, redox modulation, and virulence attributes. Ultrastructural study using the scanning electron microscopy (SEM) was performed to investigate changes in the physical appearance of the candidal cells. Any alteration in the morphology of candida cells was later determined by calculating the morphology index (Mi) of cells.

#### 3.2.9.2 Sample preparation and protocol for scanning electron microscopy

To examine the physical appearance of cells, sample preparation was done as previously described by Oliviera with slight modifications (Oliveira *et al.*, 2010). To visualise the morphology of coaggregated yeast cells and streptococci, the colonies were grown for 24 h. Since streptococci appear the same under scanning electron microscope, only relevant SEM pictures were included. Using the inoculum prepared as described in section 3.2.4, single species *Candida albicans,* single species streptococci and one mixed biofilm with all three species biofilms were formed and grown in glass cover slips placed in sterile wells of 6-well plates. After 24 h, fixation of the samples was done with 4% glutaraldehyde solution at 4 °C overnight. After which, samples were washed with 0.1 $mol \cdot L^{-1}$ concentration of sodium cacodylate buffer (pH 7.4). The samples were then secondarily fixed overnight after with 2% osmium tetroxide diluted in buffer solution.

The following day, samples were washed gently with distilled water twice, for 15 min. Dehydration of samples was done with an ascending series of ethanol concentrations (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 95%) for 15 min each. Finally, the samples were dehydrated twice for 15 min in 100% ethanol, succeeded with dehydration in ethanol–acetone mixture at ratios of 3:1, 1:1, 1:3 and pure acetone for 15 min each. Critical point drying of the samples was done in liquid $CO_2$ for 2 h under 95 bar pressure (Balzers CPD 030; Bal-Tec AG, Balzers, Liechtenstein). Finally, an ion sputter coater (JOEL JFC1100; JEOL, Tokyo, Japan) was used to gold-coat samples under low pressure. Any physical differences in the morphology of the candidal cells in mono species (candida alone) and mixed species (with streptococci) biofilms was observed with a scanning electron microscope (TESCAN VEGA3).

**3.2.9.3 Determination of morphology index**

A reproducible and objective method for investigation of cell morphology is by calculating "Morphology Index". The morphology index (Mi) (as shown in Figure 3.7) originally described by Merson-Davies and Odds was used in the study to characterize the various pleomorphic forms of *Candida albicans, Streptococcus mitis* and *Streptococcus sanguinis* in four groups, i.e., single species, two dual species and mixed species biofilm (Merson-Davies *et al.*, 1989). Since streptococci appear similar on SEM, only images with single species (candida alone) and mixed species biofilm (*C. albicans* with both *S. mitis* and *S. sanguinis)* was represented here. The morphology index of randomly selected cells (n=25 for each group) was calculated for the observed cell type based on the given formula:

$Mi = ls/d^2$.

Where “l” is the length of the cell from apex to septum, “d” is the maximum diameter of the cell and “s” is the diameter at the septal junction in “µm”. This gave the value of morphology index which approaches zero for spherical yeast cells with small septal diameters and becomes large for elongated cells with no septal constrictions. Cell dimensions in all samples were measured with the Olympus DP80 microscope digital image analysis system. Microscopic fields were examined with a phase contrast objective of x 100 and viewed under a video camera with their image displayed on the connected monitor. Classification of pleomorphic forms of candida was based on Mi value: blastoconidia, 1.0-1.5; germ tube, 1.6-2.4; pseudohyphae, 2.5-3.4; true hyphae > 3.4 (Merson-Davies *et al.*, 1989).

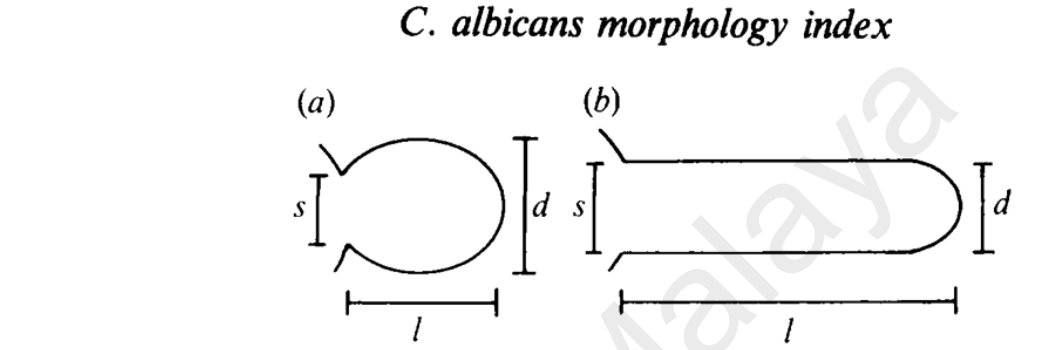


**Figure 3.7:** An illustration showing calculation of morphology index of candida cell (a) yeast, (b) hyphae (Source: Merson-Davies & Odds, 1989)

### 3.2.10 Effect of antimicrobials on single, dual and mixed species

Antimicrobial response of single, dual and mixed species was screened *in vitro* against three drugs which are commonly prescribed in clinics for candidal infections, i.e., Amphotericin B, nystatin and chlorhexidine. Paper discs impregnated with the respective drugs were laid on top of an inoculated agar plate of each group for Kirby-Bauer susceptibility testing (Hudzicki, 2009). The drugs diffuse through the agar, setting up a concentration gradient. Inhibition of colony growth which is the measure of antifungal activity was indicated by a clear growth inhibition zone surrounding the discs. The size of the clear zone is dependent upon the rate of extract diffusion and colony growth. The antimicrobial response of each drug was further validated using a broth dilution.

#### 3.2.10.1 Preparation of antimicrobial agents

Standard powder of nystatin was obtained from Sigma-Aldrich (Germany) and was dissolved in 5% DMSO; the stock solution was kept at −4 °C until use. Amphotericin B (Sigma Chemicals, U.S.A), and Chlorhexidine (Sigma Chemicals, Germany) were obtained from their respective manufacturers as liquid solution.

#### 3.2.10.2 The Kirby-Bauer susceptibility test

The antifungal activity of amphotericin B (AmB), nystatin (Nt) and chlorhexidine (CHX) were carried out based on the disc-diffusion concept of Kirby-Bauer susceptibility test (Bauer *et al.*, 1966) as shown in Figure 3.8. Single, dual and mixed species suspension was swabbed evenly over a Mueller Hinton agar (MHA) plate. Then, sterile paper discs of 6 mm in diameter (Sterile Blanks Concentration Discs, Difco Bacto, Detroit, MI, USA) were impregnated with AmB (250 µg/mL), Nt (250 µg/mL) and CHX (0.12% w/v) respectively. The discs were air-dried prior to placement on the agar surface. Throughout this experiment, a blank disc impregnated with sterile distilled water represented as

negative control. The volumes of the agents impregnated onto the discs were standardized at 100 µL. Discs were soaked with antimicrobials prior to placement on agar plates to minimize excess agent on agar plate. All plates were incubated at 37° C for 24 h according. The susceptibility of each group was determined by the diameter and pattern of the inhibited zone surrounding the disc. The experiment was done in triplicate and carried out three times to ensure reproducibility of results.

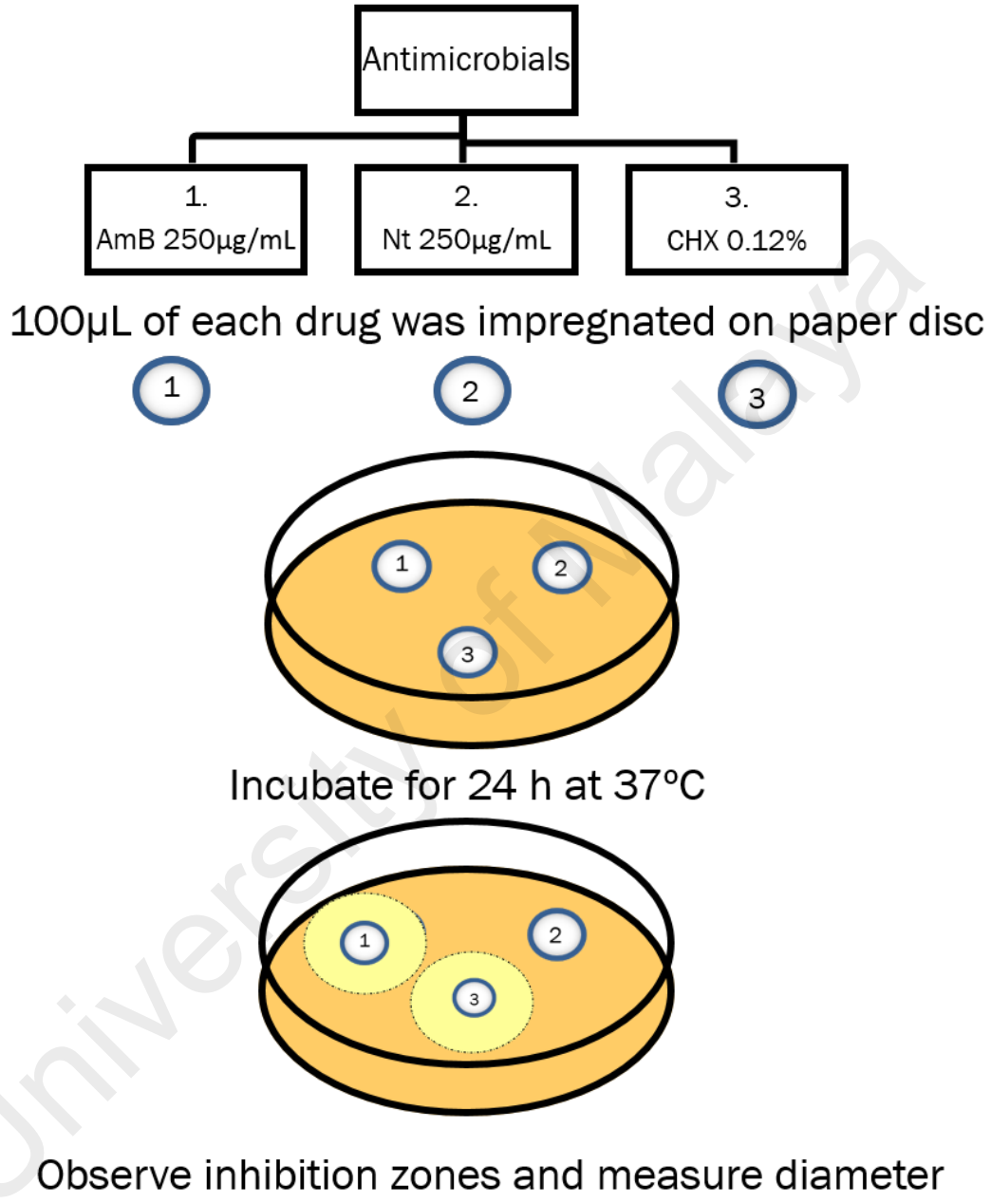


**Figure 3.8:** An illustration of screening method using Kirby-Bauer susceptibility test

### 3.2.10.3 Determination of MIC

Based on the results obtained from Kirby-Bauer test the minimum inhibitory concentration MICs of AmB, Nt and CHX for single, dual and mixed species were determined using the Clinical and Laboratory Standards Institute broth microdilution method (CLSI-BMD) described in NCCLS guidelines. To validate the antimicrobial activity of drugs, 100 µL of single (candida alone), two dual and one mixed (candida with both streptococci) (ratio of 1:1:1) species inoculum prepared in TSB (as previously described in 3.2.5.3) was poured in 96-well plates, followed by 100 µL of different concentrations of AmB (250 - 0.24 µg/mL), Nt (250 - 0.24 µg/mL) and CHX (1200 - 0.2 µg/mL) respectively. Drug free broth was used as positive control for comparison with the inoculated broth. After 24 h incubation at 37$^{o}$C, microtiter plates were agitated and organism growth was assessed by optical density $OD_{550nm}$ using a spectrophotometer (PowerWave 200, Bio-Tek Instruments, and Winooski, VT, USA) (Multiscan EX). Spectrophotometric readings were based on the reduction of growth compared to that in a growth control well for each isolate. The background optical density of the sterility check control well was subtracted from the optical densities of all of the other wells.

The MIC (in micrograms per millilitre) was determined as the lowest concentration of antifungal agent that inhibited ≥50% of growth inhibition compared to that of the agent-free growth control using a modified Gompertz model (Lambert *et al.*, 2000). The determination was carried out in triplicate and repeated three times to ensure that the result obtained is reliable.

### 3.2.11 Expression of agglutinin-like sequence *ALS* 1, 2 and 3 genes in single, dual and mixed species

#### 3.2.11.1 Experimental design

The expression of interest genes such as the agglutinin-like sequence (*ALS*) is considered a key attribute of *Candida albicans* that aids in host adhesion and invasive pathogenesis. In this study, the expression of these genes in candidal cells was determined in single, dual and mixed species biofilm. For an accurate measurement and reproducible expression profiling of target genes in qRT-PCR analysis, use of stable housekeeping genes, also called as 'internal control' is essential to normalize the expression level. Housekeeping genes work for the basic cellular and metabolic functions and maintains the stable and constitutive expression throughout, irrespective of any external physiological conditions (Zhang *et al.,* 2015). The *C. albicans ACT1* gene was used as an internal mRNA control for evaluation of the efficacy of the qPCR analysis and served as the house keeping gene to confirm the presence or absence of *Candida* species.

#### 3.2.11.2 Total RNA extraction

Expression of *ALS1, ALS2* and *ALS3* genes was analysed in salivary biofilms of *Candida* with *S. mitis* and *S. sanguinis*. Primers that were used for analysis are shown in table 3.1 (Green *et al.,* 2004). For RNA extraction, easy-RED™ total RNA extraction kit was used. A 24 h biofilms of single, dual and mixed species were developed on glass coverslips placed in flat bottom 6-well plate as described in a similar procedure in section 3.2.5.3. Microbial cells were scraped off from glass coverslip biofilms and transferred to 1.5 mL tubes respectively. A prelysis buffer of 250 µL volume was added. The tube was incubated for 3 min at 95$^{o}$C. Following that, 750 µL of easy-RED™ solution was added, vortexed and then incubated for 5 min at (RT) room temperature. Then, 200 µL chloroform was added, vortexed and incubated for 5 min. After centrifugation, aqueous

phase was pipetted and transferred to a clean tube and then 1 mL of cold isopropanol was added and left at −20°C for 15 min. Samples were washed by 70% ethanol. RNA was dissolved using 50 µL RNAse free water and stored in -70$^{o}$C.

### 3.2.11.3 RNA quantification

It is vital to get high quality and intact RNA to ensure good results obtained in the experiments. Total RNA integrities of the samples were analysed using RNA quantification machine (Thermoscientific Nanodrop 2000 Spectrophotometer) and all samples were considered to have high quality RNA by referring to the RIN number (Schroeder *et al.*, 2006).

### 3.2.11.4 cDNA synthesis

Complementary DNA (cDNA) was synthesized using 1-step RT-PCR kit (SuprimeScript RT-PCR kit & Premix 2x) according to instructions provided by manufacturer in Thermocycler (Eppendorf Mastercycler gradient). Reverse transcription reactions for synthesizing cDNA were done using 5µL total RNA template (200 ng µL-1), 1 µL 50 µg ml−1 random primers, 1 µL 10 mM dNTPs, 5 µL M-MLV reaction buffer (× 5), 1 µL 25 U µL−1RNase inhibitor and 1 µL 200 U µL−1Reaction mix (SuprimeScript RT-PCR kit). A final reaction volume of 25 µL was made by adding RNAse free water. The final reaction mix was then incubated for 5min at 70°C, followed by incubation for 60 min at 37°C. The resultant cDNA was stored in freezer at –20°C preceding qPCR.

### 3.2.11.5 Specific primers

Primers that were used in qPCR analysis are displayed in table 3.1 and were designed from full-length gene sequences obtained from the nucleotide platform in PubMed using Primer3 software (Koressaar *et al.*, 2007; Untergasser *et al.*, 2012).

**Table 3.1:** Forward (F) and Reverse (R) primers used for qPCR analysis

| Gene name | Sequence (5´ ⟶3´) | Product size (bp) | Accession no. |
|---|---|---|---|
| ALS1 Agglutinin-like sequence | **F-** GAC TAG TGA ACC AAC AAA TAC CAG A<br>**R-** CCA GAA GAA ACA GCA GGT GA | 318 | L25902 |
| ALS2 | **F-** CCA AGT ATT AAC AAA GTT TCA ATC ACT TAT<br>**R-** TCT CAA TCT TAA ATT GAA CGG CTT AC | 366 | AF024580 |
| ALS3 | **F-** CCA CTT CAC AAT CCC CAT C<br>**R-** CAG CAG TAG TAG TAA CAG TAG TAG TTT CAT C | 342 | U87956 |
| ACT1 *C. albicans* actin housekeeping gene | **F-** CCA GCT TTC TAC GTT TCC<br>**R-** CTG TAA CCA CGT TCA GAC | 200 | HM997110 |

#### 3.2.11.6 Quantification of genes using qPCR.

Quantitative PCR was performed using 96-well qPCR plates as triplicates in an Applied Biosystems 7500 Fast Realtime PCR instrument. Each 20 µL reaction comprised of 2 µL cDNA, 1 µL of each primer (10 mM), 10 µL (×2) Titan HotTaqEvaGreen® qPCR mix SYBR-Green PCR Master Mix and 6 µL RNAse free water. Thermal cycler protocol consisted of initial denaturation step for 2 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, then primer annealing for 30 s at 58 °C and primer extension for 30 s at 72°C. A final extension for 2 min at 72°C was conducted, succeeded by chilling at 4°C. To verify the amplified product, a dissociation step at 60°C was done and a melting curve was generated. After finishing qPCR, a threshold cycle was set in accordance with amplification curves of evaluated ALS genes. The control and experimental groups were compared based on cycle number at which the reference and the target genes attained the threshold cycle (Ct). Relative gene expression analysis was achieved according to the double delta Ct analysis (ΔΔCt) method (Bustin *et al.,* 2009).

### 3.2.12 Expression of agglutinin-like sequence als 1, 2 and 3 proteins in single, dual and mixed species

#### 3.2.12.1 Experimental design

Agglutinin-like sequence genes *ALS* act by encoding proteins and those proteins, in turn dictate cell function. Although study of *ALS* gene expression *in vitro* could reveal several characteristics of the gene family, documentation of als protein production is essential to support the idea that als proteins are cell surface adhesion glycoproteins with the potential to promote *C. albicans* pathogenesis. In this study, the expression of the als 1, 2 and 3 proteins in candidal cells was determined in single, dual and mixed species

biofilm. The three proteins were quantified according to their molecular weight in each group with the help of western blot technique.

### 3.2.12.2 Total protein extraction

Expression of als1p, als2p and als3p was analysed in salivary biofilms of *Candida* with *S. mitis* and *S. sanguinis.* For protein extraction, ReadyPrep™ Protein extraction kit (BIO-RAD) was used (Zarnowski *et al.*, 2016). Biofilms of single, dual and mixed species were developed on glass coverslips placed in flat bottom 6-well plate for 24 h as described in a similar procedure in section 3.2.5.3. Microbial cells were scrapped off from glass coverslip biofilms and transferred to 50 mL falcon tubes containing TSB respectively. Suspension was centrifuged at approx. 3000 x g for 5 min at 4ºC. Supernatant was discarded and pellet was re-suspended in appropriate volume of lysis buffer reagent according to the weight in mg of the pellet. With the sample on ice, the suspension was sonicated with an ultrasonic probe to disrupt the cells and fragment the genomic DNA. The sample was sonicated using 30 sec bursts, 4 – 5 times. The tube was then centrifuged for 10 min at ~3,000 x g in a microcentrifuge at 4°C to pellet insoluble material and unbroken cells. The supernatant was removed and diluted in a beaker or bottle containing 60 mL of the ice-cold Membrane Protein Concentrating Reagent. The suspension was slowly stirred on ice for 60 min. The sample was then centrifuged at 100,000 x g for 60 min at 4ºC to pellet the membranes and membrane proteins. The supernatant was discarded. Each pellet was washed briefly with 3 mL of cold lysis buffer and incubated on ice for 3 min. The membrane-protein pellet(s) from each extraction were re-suspended in 1.0–2.0 mL of complete 2-D rehydration/sample buffer and vortexed to solubilize protein in the buffer. Sample was centrifuged at 16,000 x g for 15 min at 20ºC. Supernatant was removed and ready for determination of protein concentration.

### 3.2.12.3 Total protein quantification

Total extracted protein quantity from each sample was assessed using Bio-Rad protein assay kit. Bovine serum albumin (BSA) protein was used as standard. Five dilutions of the extracted sample protein (s) were prepared. Then, 10 µL of each protein standard and sample was pipetted in 96-well microtiter plate wells. Following which, 200 µL of diluted dye reagent was dispensed in each well and mixed. Plate was incubated at room temperature for 15 min. Absorbance was measured at 595 nm using a microtiter plate reader (FC-Bios µQuant). Protein lysate was quantified in µg/mL of solution. Protein solutions were assayed in triplicate. Protein samples aliquot were stored at -20$^{o}$C for long term use. To reduce and denature samples, separation by their molecular weight was carried out. Each lysate was diluted in equal volume of 2X laemmli loading buffer. Each sample was then boiled for 5 min at 95$^{o}$C and centrifuged at 16,000 x g for I min to completely denature proteins.

### 3.2.12.4 Protein separation by gel electrophoresis

SDS-PAGE (discontinuous) was performed in a commercially available polyacrylamide mini-gel (8.6 x 6.7 cm) format on 12.5% w/v separating and 4% w/v stacking slab gel at a constant voltage of 65 V for I h. Three or more gels were prepared for simultaneous electrophoresis. Each lane contained 20 µg of protein of an extract preparation along with pre stained molecular weight ladder. The proteins will be separated according to their molecular weight and ready for transfer to membrane.

### 3.2.12.5 Protein transfer from gel to membrane

The gel was removed from cassette and placed in 1x western transfer buffer for 15 min. Filter paper and nitrocellulose membrane were activated by soaking in transfer buffer for 10 min. Transfer sandwich was prepared by stacking sponge, filter paper,

nitrocellulose membrane, gel, filter paper and sponge in a cassette. The cassette was placed in tank along with ice pack and begin transfer at voltage of 100V for 30 min.

### 3.2.12.6 Immunoblotting

The proteins were transferred to nitrocellulose membrane. The membrane was rinsed thrice in PBS to remove excess gel pieces. All areas of membrane that do not contain protein were blocked by 5% BSA in tris-buffered saline tween solution at room temperature for 1 h to prevent nonspecific binding of antibody and to reduce overall background signal.

### 3.2.12.7 Addition of specific antibodies

Primary anitbodies *i.e.*, anti-als1, anti-als2 and anti-als3 (source*: E.coli*) (Creative bio-labs, NY, USA) was diluted in blocking buffer at 1:1000. Nitrocellulose membrane was placed in diluted primary antibody and incubated overnight at 4$^{o}$C. The blot was then rinsed five times with TBST for 5 min each to remove stringent background signals. Secondary antibodies; goat anti-rabbit horse raddish peroxidase (HRP) conjugate (ThermoFisher scientific, Germany) were diluted in blocking buffer at 1:300. Incubate the membrane in this solution for 1 h at room temperature. The blot was then rinsed five times with TBST for 5 min each.

### 3.2.12.8 Imaging and data analysis

Chemiluminescent substrate was applied to the blot and incubated for 5 min without agitation. Enhanced chemiluminescent (ECL) was decanted and excess solution was removed. The membrane was placed on clear plastic wrap to prevent complete drying. Image was captured and analysed using software (Gel Doc$^{TM}$ Gel imaging system, Bio-Rad, U.S.A).

### 3.3 Statistical analysis

All results were computed and expressed as mean ± standard deviation (SD) from three determinations performed in triplicate (n = 9). Statistical analyses were performed using SPSS software (version 22.0). A one-way ANOVA test was used to compare the significant differences between control (single species) two dual species (*C. albicans* with *S. mitis*) (*C. albicans* with *S. sanguinis*) and mixed species (*C. albicans* with both *S. sanguinis* and *S. mitis*) sample. An independent t-test was used to compare significant difference between two dual species groups. The morphology index (Mi) of various pleomorphic forms of *Candida albicans* and streptococci were analysed by descriptive statistics (number of cells measured, average, SD, ANOVA test). A *P* value of $< 0.05$ was considered statistically significant.

# CHAPTER 4: RESULTS

## 4.1 Biofilm biomass and cellular metabolic activity

Crystal violet analysis shows a significant increase in biofilm biomass of two dual species and mixed species in comparison to single species biofilm ($p<0.05$). *C. albicans* forms denser biofilms in combination with *S. sanguinis* and *S. mitis* respectively when compared to *C. albicans, S. mitis* and *S. sanguinis* alone. Figure 4.1 shows the absorbance values of different biofilms. Data was represented as mean ± SD of three independent experiments performed in triplicate.

Although, there is no significant difference between biomass of two dual species (*C. albicans* with *S. mitis* and *C. albicans* with *S. sanguinis*) ($p>0.05$), however, CV analysis shows that *C. albicans* form a slightly denser biomass with *S. sanguinis* when compared to *S. mitis*. Analysis shows that *C. albicans* forms an even denser biofilm when combined with streptococci as mixed species ($p<0.05$). Suggesting that streptococci benefit from this synergy by exhibiting enhanced biofilm mass under the influence of *C. albicans.* It also suggests that greater the number of symbiotic organisms in a biofilm, greater the biomass.

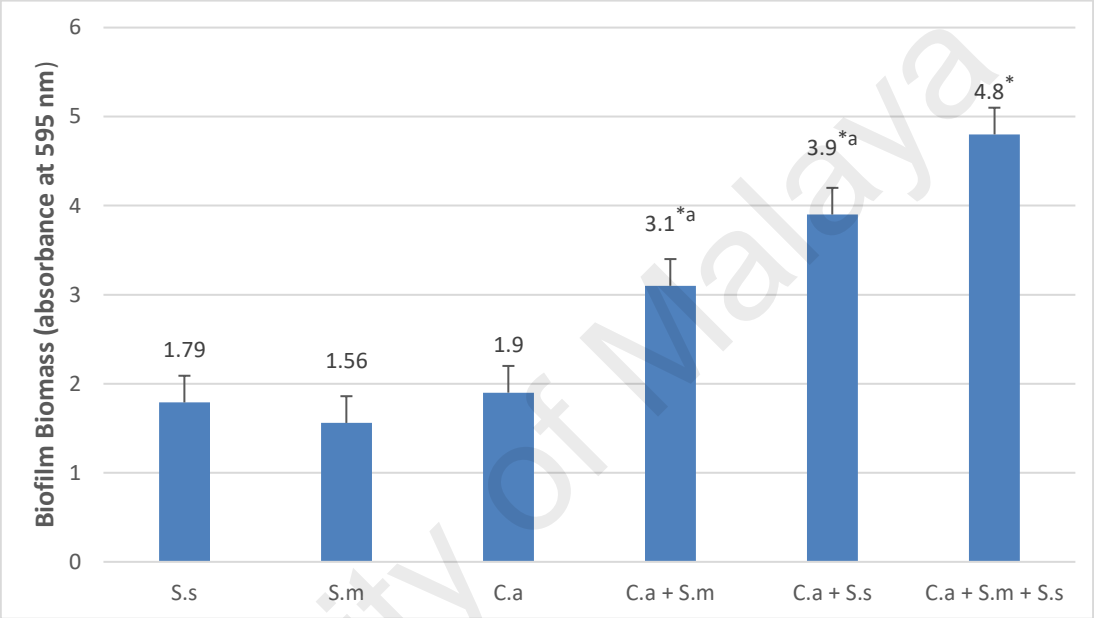


**Figure 4.1:** Absorbance values of crystal violet solutions obtained from *C. albicans* biofilm formation in 24 h single, dual and mixed species biofilms. Data was represented as means ± SD of three independent experiments performed in triplicate. Microbe free broth was used as control.
*C.a, Candida albicans; S.m, Streptococcus mitis*; *S.s, Streptococcus sanguinis*;
(*), significant when compared to single species (One-way ANOVA; $p < 0.05$)
(a), no significant difference between dual species groups ($p < 0.05$)

The XTT (tetrazolium salt) assay accurately detected the observed differences in the extent of biofilm formation between the control biofilm, the two dual species biofilm and the mixed species biofilm. The results were consistent on XTT analysis as well, (Figure 4.2). Based on XTT assay, mixed species biofilm showed highest metabolic activity as compared to single and dual species ($p<0.05$). The two dual species showed significantly higher cellular metabolic activity as compared to single species ($p<0.05$), but not higher than each other ($p>0.05$). The results suggest that each of the streptococci have a mutualistic behaviour towards *C. albicans*, especially when all three organisms are combined in mixed species biofilm. All three organisms take advantage of this environment and substantially prosper in salivary biofilm.

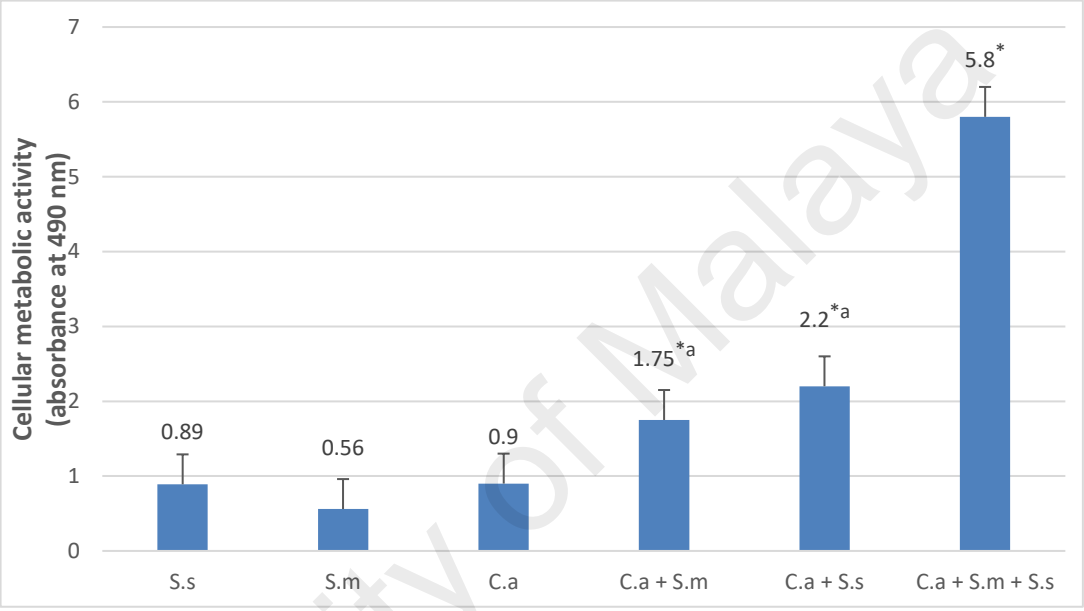


**Figure 4.2:** Absorbance values of XTT reduction assay obtained from *C. albicans* biofilm formation in 24 h single, dual and mixed species biofilms. Data was represented as means ± SD of three independent experiments performed in triplicate. Microbe free broth was used as control.
*C.a, Candida albicans; S.m, Streptococcus mitis*; *S.s, Streptococcus sanguinis*;
(*), significant when compared to single species (One-way ANOVA; $p < 0.05$).
(a), no significant difference between dual species groups ($p > 0.05$)

### 4.2 Microbial growth on a dynamic biofilm

The results showed similar pattern of adhesion as seen in static biofilm (Figure 4.3). The adhering capacity of dual species was greater when compared to single species. CFU/mL of mixed species was significantly greater than that of dual or single species ($p < 0.05$). This suggests that candida and streptococcal interactions are not only strong amongst the two different species, this candida-bacteria combination has high affinity to adhere to surfaces coated with salivary pellicle. Greater adherence to oral surfaces is a hallmark for high virulence capacity of the organism.

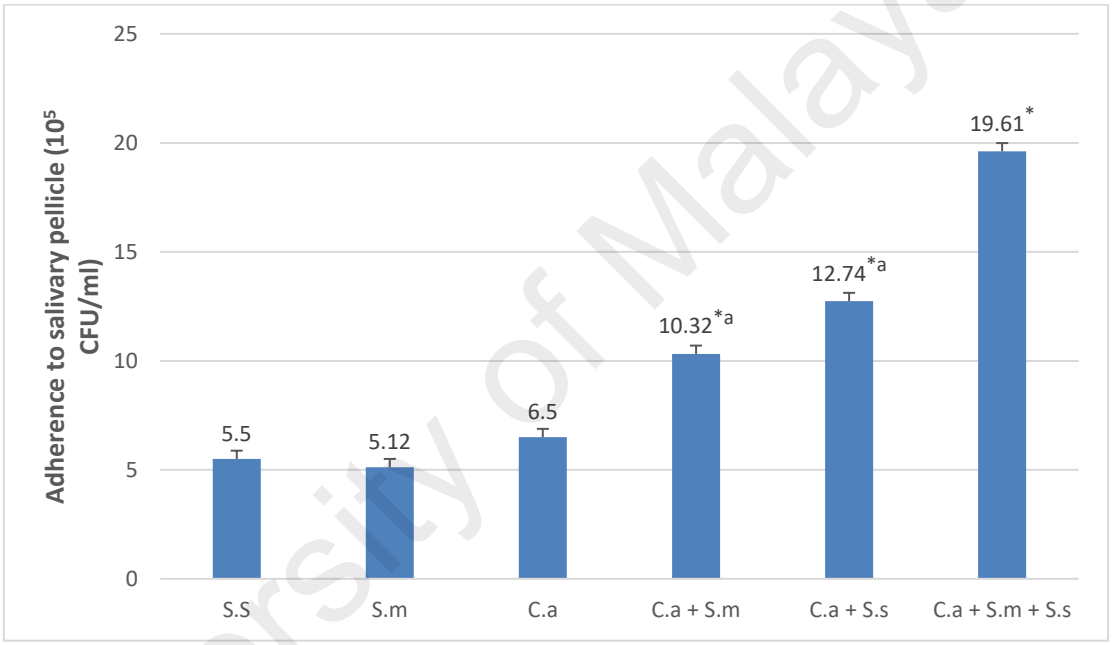


**Figure 4.3:** *C. albicans* adhered to saliva coated glass surface in the form of single, dual and mixed species biofilms. Data was represented as means ± SD of three independent experiments performed in triplicate
*C.a, Candida albicans; S.m, Streptococcus mitis*; *S.s, Streptococcus sanguinis*;
(*), significant when compared to single species (One-way ANOVA; $p < 0.05$).
(a), no significant difference between dual species groups ($p > 0.05$)

### 4.3 Ultrastructure under scanning electron microscope

Photomicrograph of *Candida albicans* species alone was represented in Figure 4.4. The figure shows candidal blastoconidia as smooth-surfaced, ovoid and spherical cells. A ring of bud scars was visibly located at one pole (tip) of the blastoconidial cell and buds were seen emerging from the opposite pole of the cell. Fragile blastoconidial septum was also seen. The average length-to-width ratios of candida in monospecies sample (n=25) was 1.14.

Ultrastructure of *Candida albicans* in mixed species biofilm was represented in Figure 4.5. In high contrast to the previous image, cells of candida were now visible as true hyphae. These true hyphal forms displayed homogenously elongated structure with lack of constrictions at the solid septum. Streptococcal diplococci cells were found as clusters, close to candidal hyphae (Figure 4.5). The average length-to-width ratio of candida in two dual species sample i.e., with *S. sanguinis* and *S. mitis* was 4.62 and 4.15 respectively (n=25). The average length to width ratio of candida in mixed species sample (n=25) was about 6.45. There was significant difference in morphology index of *Candida albicans* ($p<0.05$) (Table 4.1) in single versus mixed species sample.

Electron micrograph of streptococci in single species biofilm is illustrated in Figure 4.6 as reference. Both streptococci revealed similar morphology and hence, only one image is shown. Round to ovoid, smooth surfaced streptococci were visible, grown in chains of variable lengths. Growing cells were shown with distinctive plane of division. Fragile cell septum was visible between two cells. The average length to width ratio of candida in mixed species sample (n=25) was about 6.45. The average length to width ratio of streptococci in single and mixed species sample (n=25) was the same i.e., 1.0. The size and shape of streptococci did not change.

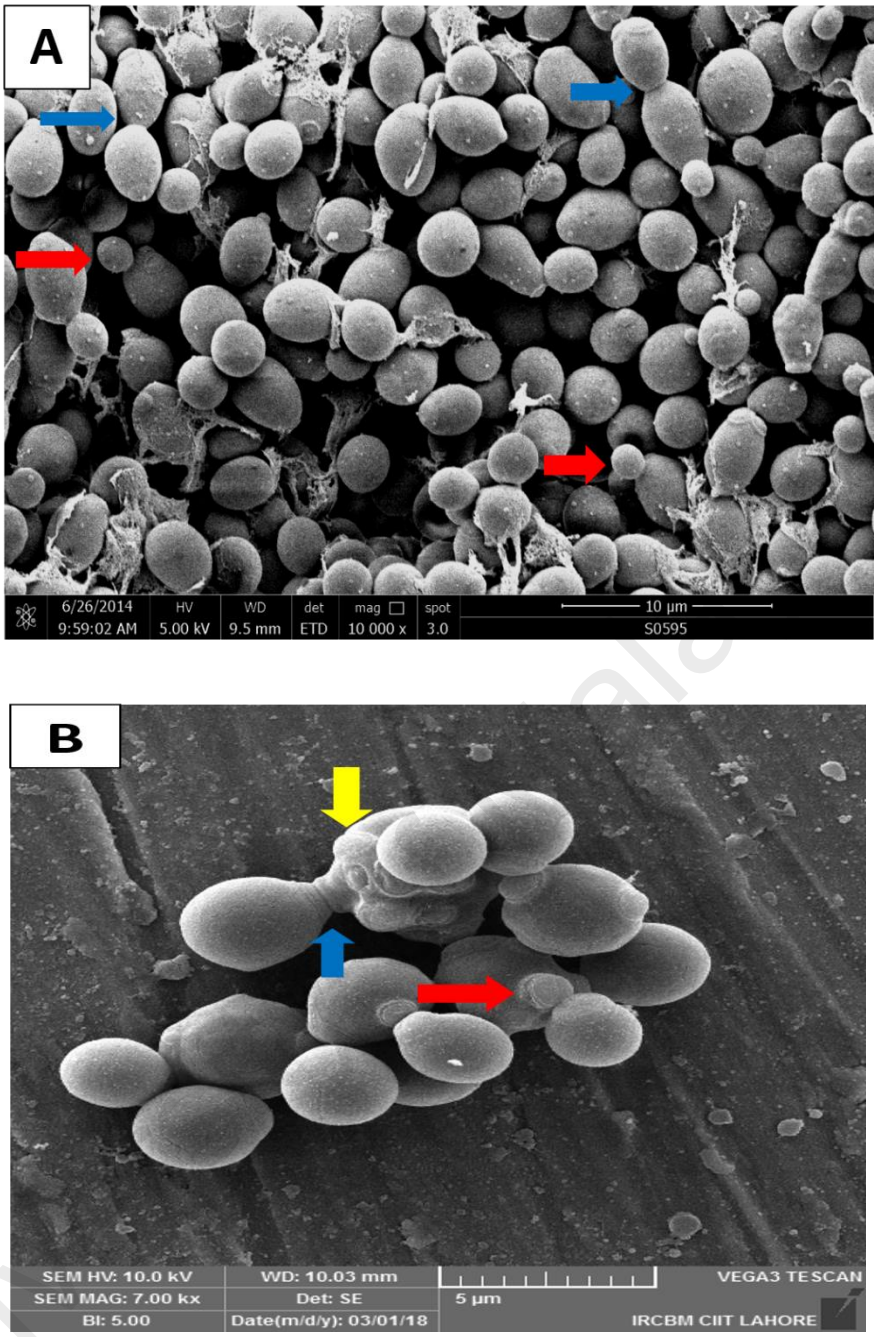


**Figure 4.4:** Scanning electron micrograph of *Candida albicans* in a 24 h single species biofilm Ovoid blastoconidia are visible

**A:** low magnification. **B:** high magnification.

Note: the ring of scars (shown in yellow arrow ) located on one pole. A bud (shown in red arrow) emerging from opposite side of pole. Fragile blastoconidial septum (shown in blue arrow ) is also seen.

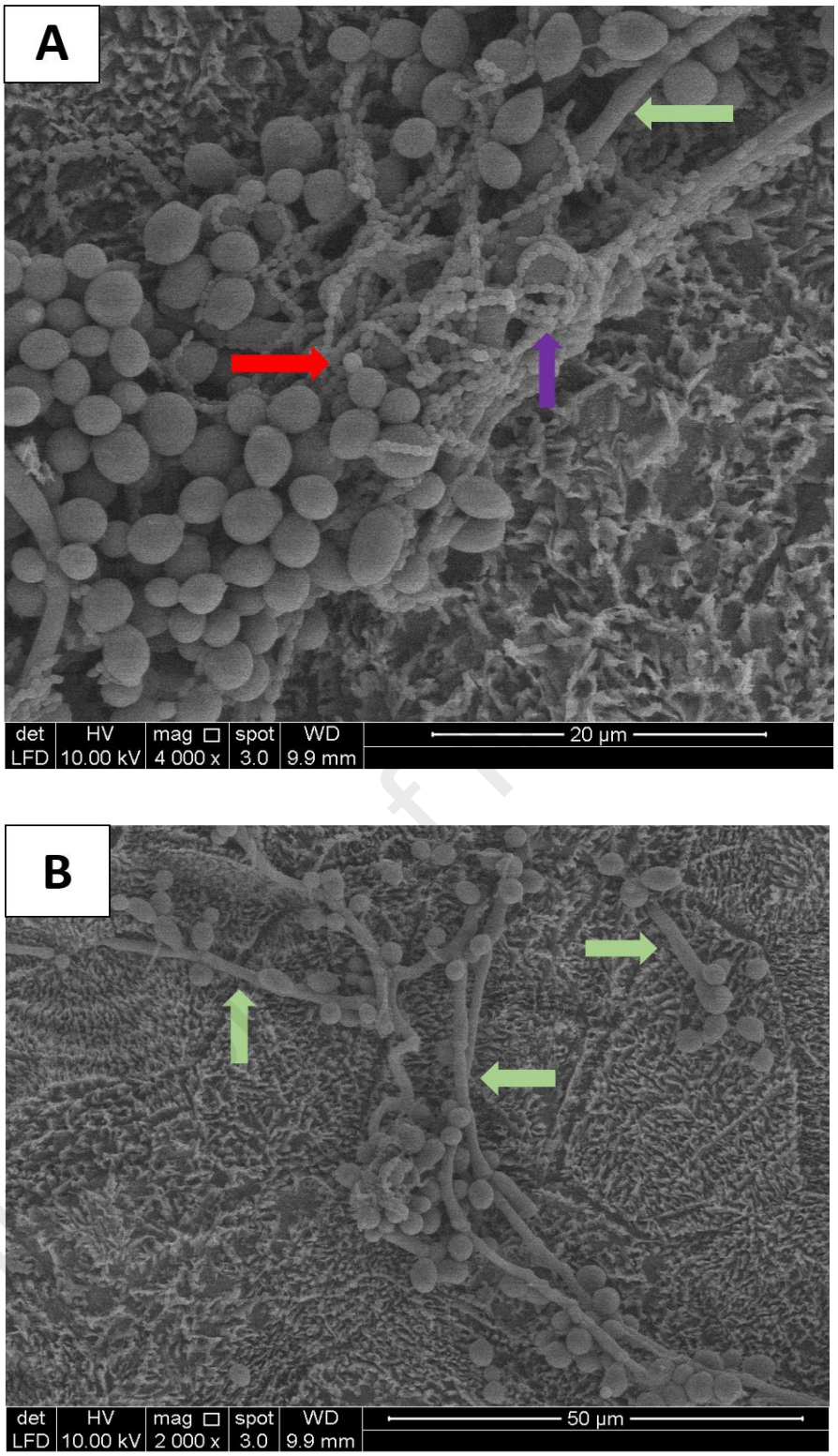


**Figure 4.5:** Scanning electron micrograph of *Candida albicans* in a 24 h mixed species biofilm at different magnification A, B & C

Note: True hyphal form of candida is visible (shown in green arrow ). New blastoconidia can be seen budding off of hyphal cell (shown in red arrow ). Chains of streptococci can be seen near candida hyphae (shown in purple arrow --).

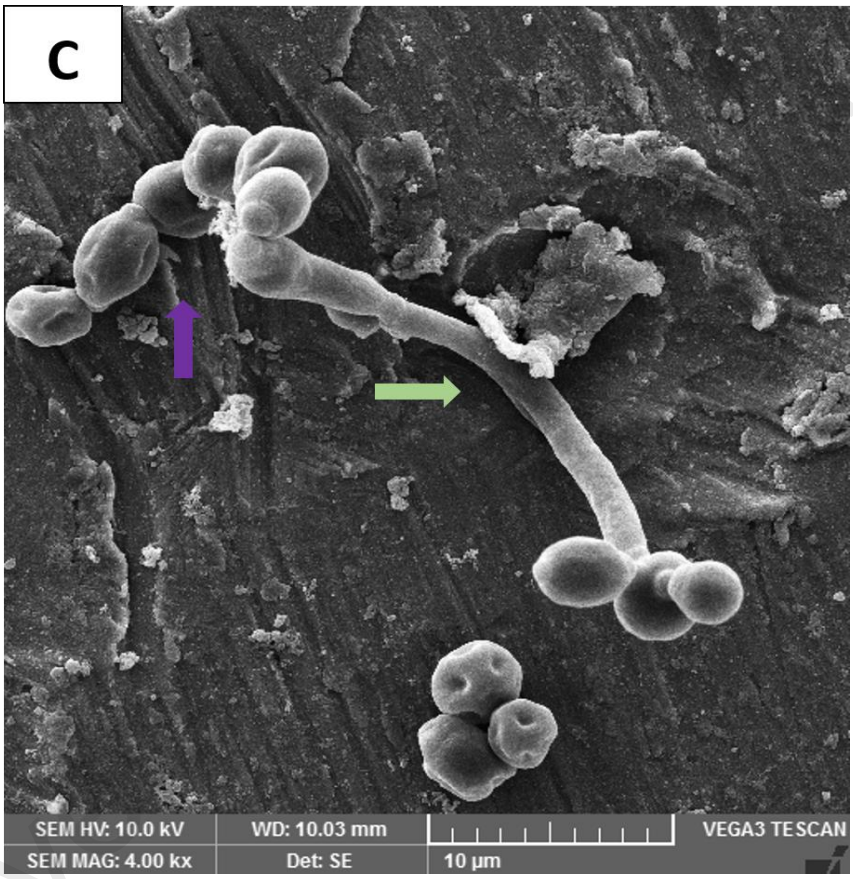


**Figure 4.5, continued**

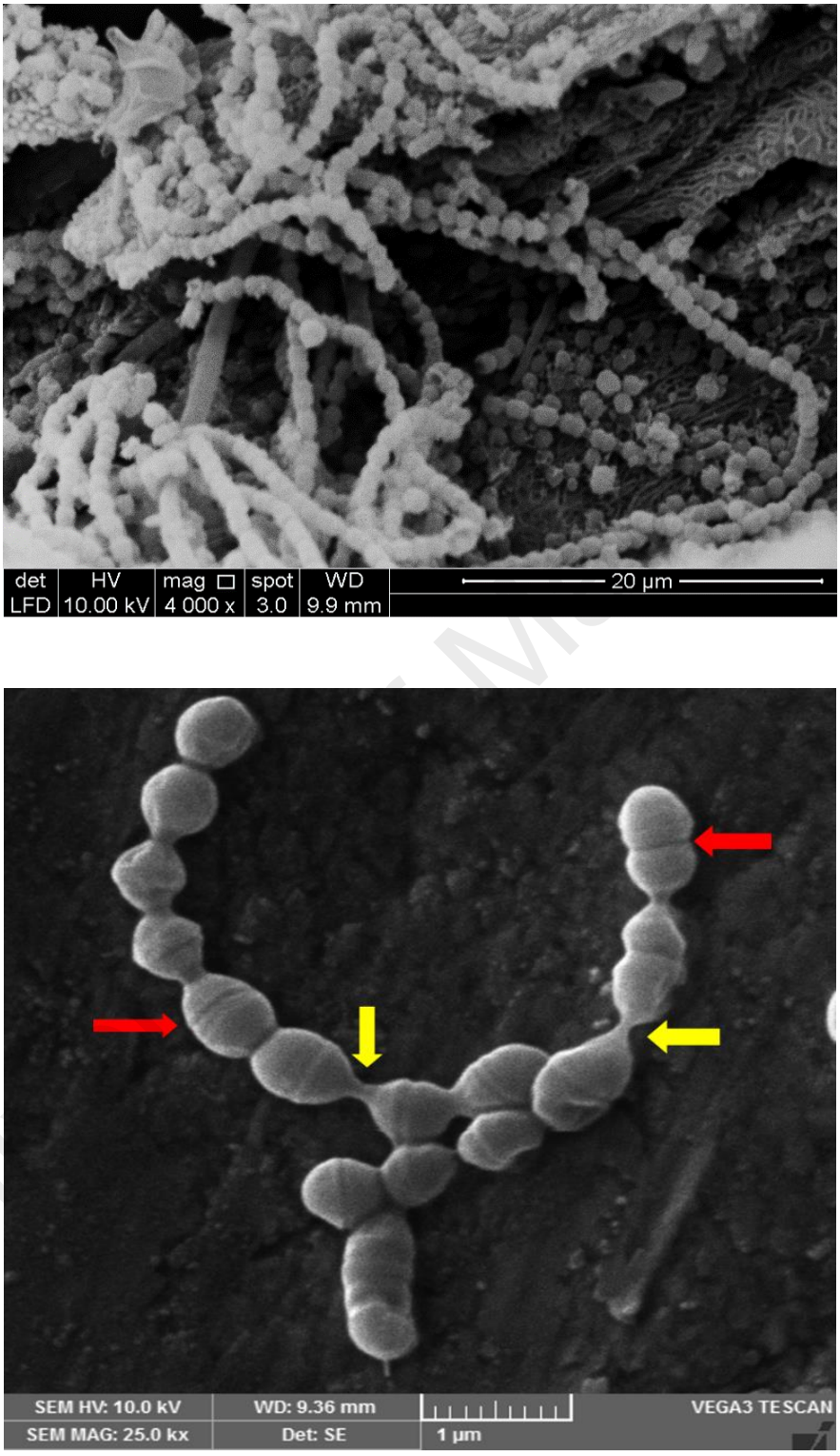


**Figure 4.6:** Scanning electron micrograph of streptococci in a 24 h single species biofilm. Image shows chains of streptococcal bacteria with plane of division (shown in red arrow) and intercellular septa (shown in yellow arrow).

**Table 4.1:** Morphology index (Mi) values for pleomorphic cells of *Candida albicans* and streptococci in 24 h single species (*C.a*), two dual species (*C.a + S.m*) (*C.a + S.s*) and a mixed species (*C.a + S.s + S.m*) biofilm. Data was estimated by measuring the cells documented by scanning electron micrographs

| **Group** | **Pleomorphic form of *Candida albicans*** | **Morphology index (Mi) of *Candida albicans*** | **Morphology index (Mi) of streptococci** |
|---|---|---|---|
| | | Mean ± S.D | Mean ± S.D |
| *C.a* single species | Blastoconidia | 1.14*± 0.25 | |
| Streptococci single species | | | 1.0 ± 0.30 |
| *C.a + S.m* | True hyphae | 4.15**± 2.12 | 1.1 ± 0.2 |
| *C.a + S.s* | True hyphae | 4.62**± 3.01 | 1.0 ± 0.4 |
| *C.a + S.m + S.s* | True hyphae | 6.45***± 3.22 | 1.1 ± 0.4 |

Mi: Morphology index
Classification of pleomorphic forms based on Mi value: blastoconidia, 1.0-1.5; germ tube, 1.6-2.4; pseudohyphae, 2.5-3.4; true hyphae > 3.4 (Merson-Davies and Odds, 1989).
Note: Each value represents the mean and S.D of three independent experiments performed in triplicate.
(*) Not significant ($p>0.05$)
(**) Significant when compared to (*) ($p<0.05$) but not significant when compared to (**)
(***) Significant when compared to (*) and (**)

### 4.4 Effect of drugs on microorganisms

In light of the inhibition zones created by AmB, Nt and CHX on four groups, it was evident that AmB, Nt and CHX created larger zones on single species as compared to dual and mixed species (Table 4.2 and Figure 4.7).

**Table 4.2:** The diameter of inhibition zone produced by amphotericin B (AmB), nystatin (Nt) and chlorhexidine (CHX) on single, dual and mixed species

| **Groups** | **Antimicrobial activity (mm) of:** | | | |
|---|---|---|---|---|
| | AmB **(250 µg/mL)** | Nt **(250 µg/mL)** | CHX **(0.2%w/v)** | **Sterile $dH_2O$** |
| (*C. albicans* alone) | 16 | 17 | 22 | NS |
| *C. albicans* + *S. mitis* | 12 | 12 | 18 | NS |
| *C. albicans* + *S. sanguinis* | 12 | 12 | 18 | NS |
| Mixed species | 9 | 9 | 18 | NS |

NS; Normal Saline

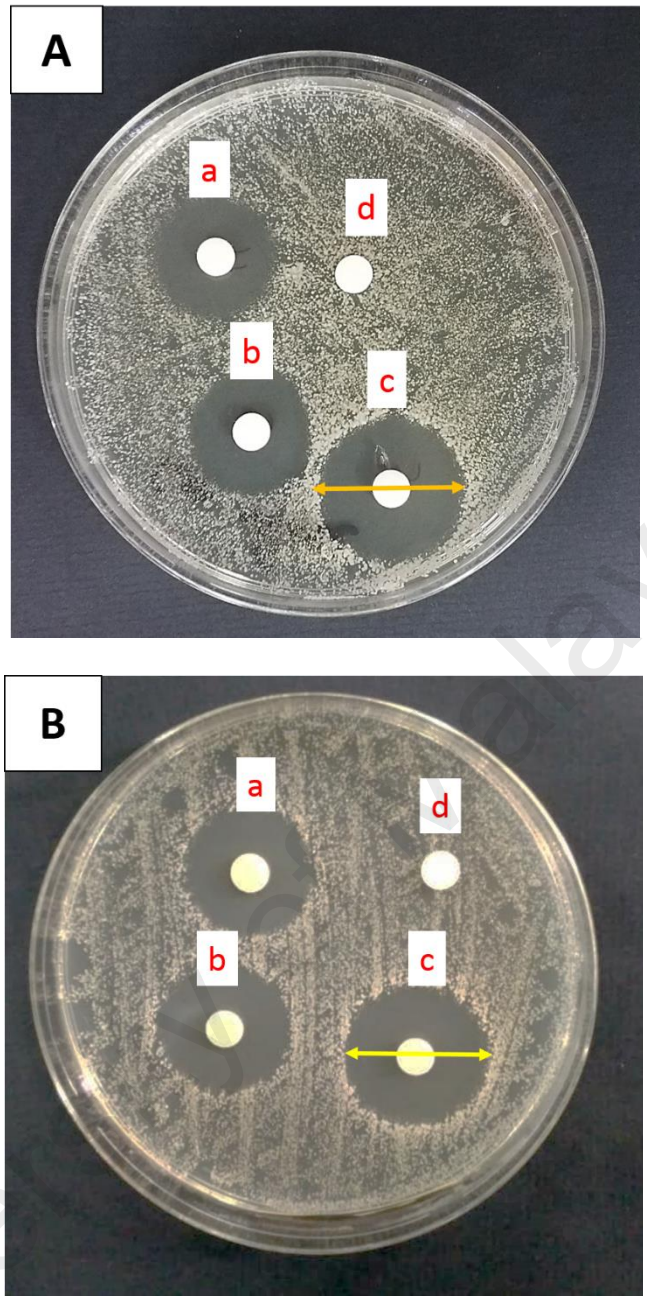


**Figure 4.7:** The antifungal responses of single, dual and mixed species using Kirby-Bauer susceptibility test

**Note:** The Mueller Hinton Agar (MHA) plates which have been seeded with (A) *C. albicans*, (B) *C. albicans* + *S. mitis*, (C) *C. albicans* + *S. sanguinis*, (D) *C. albicans* + *S. mitis* + *S. sanguinis*
Disc "a" was impregnated with 100µL of 250µg/mL amphotericin B.
Disc "b" was impregnated with 100µL of 250µg/mL nystatin.
Disc "c" was impregnated with 100µL of 0.2%w/v chlorhexidine.
Disc "d" was impregnated with 100µL of sterile distilled water.
Antifungal response was evaluated by measuring the diameter of inhibition zone of the tested group (arrow; ↔).

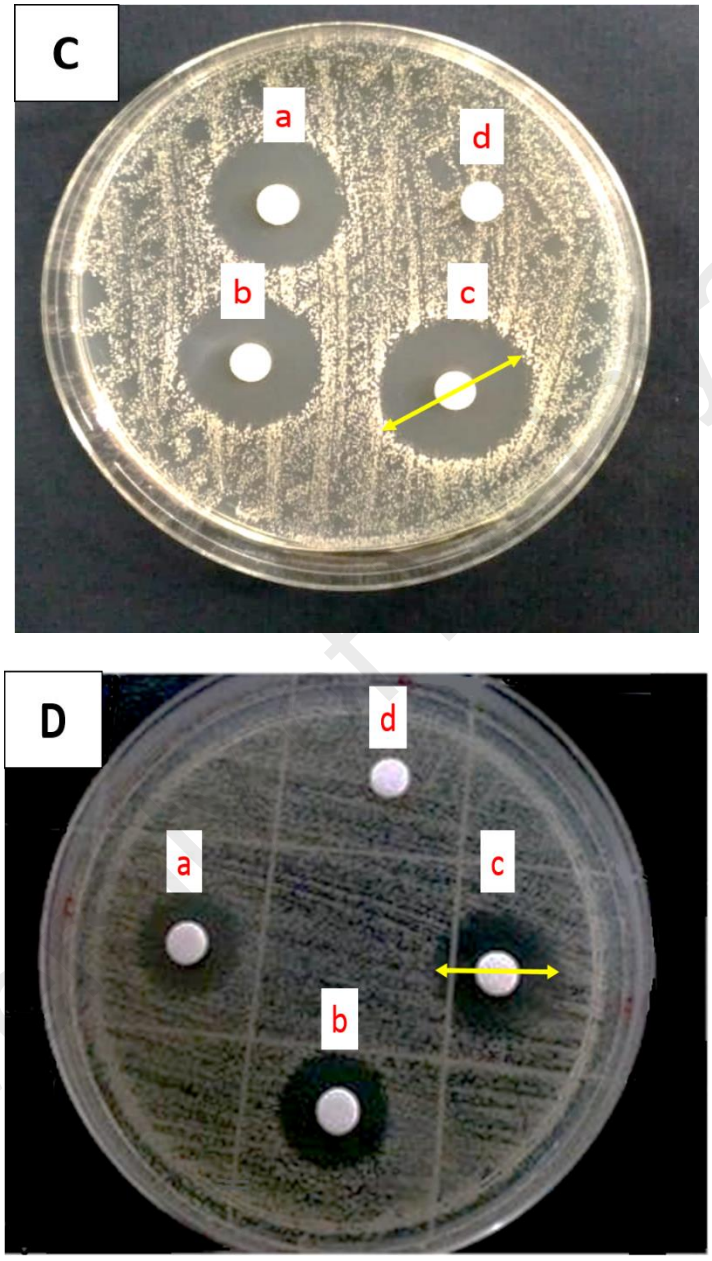


**Figure 4.7, continued**

In this study, we determined the susceptibilities of single, dual and mixed species to AmB, Nt and CHX using species-specific breakpoints in the M27-S4 document. We used the following MIC breakpoints for AmB for definitions: susceptible, ≤4 μg/mL; low-level resistance, 8 to 64 μg/mL; high-level resistance, ≥125 μg/mL. Whilst there is no clinically defined breakpoint for chlorhexidine resistance, an MIC of >4 is generally considered to be reduced susceptibility.

It is evident from the results (table 4.3) that single species candida is susceptible to AmB and Nt but dual and mixed species are resistant to even high doses of AmB and Nt. Whereas, single and dual species are susceptible to CHX, but mixed species group shows reduced level of susceptibility.

**Table 4.3:** MIC values of AmB, Nt and CHX towards single, dual and mixed species

| **Group** | **MIC values (μg/mL)** | | |
|---|---|---|---|
| | AmB **(MIC)** | Nt **(MIC)** | CHX **(MIC)** |
| Single species (*C. albicans* alone) | 4 | 4 | 4 |
| Dual spp (*C. albicans* + *S. mitis*) | 64 | 32 | 4 |
| Dual spp (*C. albicans* + *S. sanguinis*) | 64 | 32 | 4 |
| Mixed species | 125 | 125 | 32 |

### 4.5 Expression of *ALS1, ALS2* and *ALS3* genes

The ability of *C. albicans* to form dual and mixed species biofilms with *S. mitis* and *S. sanguinis* was further confirmed by genetic analysis of *ALS*1, 2 & 3 genes which encode large cell-surface glycoproteins that are implicated in the process of adhesion to host and other organisms. The resultant DNA, which was synthesized from extracted RNA of salivary biofilms, was quantified in qPCR to get fold expression of *ALS1, ALS2* and *ALS3* genes in single (*C.a* alone), two dual (*C.a + S.m*) (*C.a + S.s*) and mixed species (*C.a + S.m + S.s*) biofilm. The expression was normalised to the standard *ACT1* gene and results displayed in Figure 4.8.

Gene expression of *ALS1* was significantly high in dual species and mixed species as compared to single species ($p<0.05$). However, there was no significant difference in expression of *ALS1* between dual and mixed species biofilm ($p>0.05$). *ALS2* however, did not show significant expression in either dual or mixed species biofilm ($p>0.05$). Gene expression of *ALS3* gene however, was significantly higher in mixed species in comparison to single or dual species biofilm ($p<0.05$). *ALS3* gene expression was coherently high in dual species biofilm as compared to single species biofilm ($p<0.05$), making it the most expressed gene amongst the three tested.

The results signify that *ALS3* mainly and *ALS1* are significantly expressed when *C. albicans* forms co-aggregation with commensal *S. mitis* and *S. sanguinis* of oral cavity.

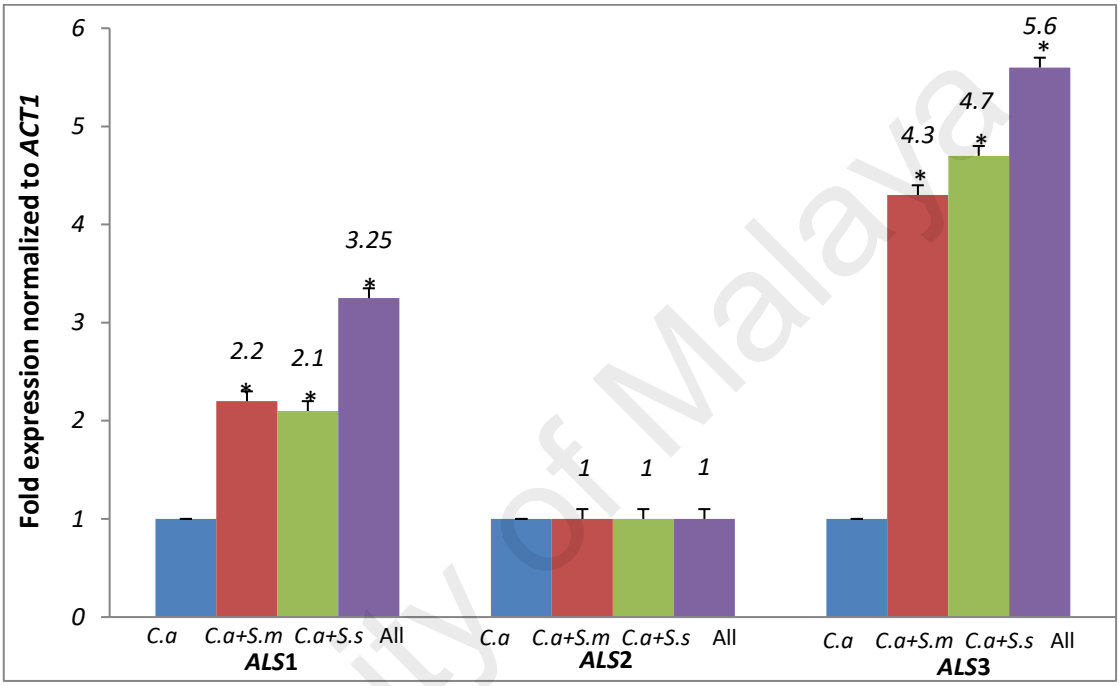


**Figure 4.8:** Relative gene expression of *ALS1*, *ALS2* & *ALS3* in single (*C.a*), dual (*C.a* + *S.m*) (*C.a* + *S.s*) and mixed-species (*C.a* + *S.m* + *S.s*) biofilms.

Note: *C. albicans* target genes were normalised using the *C. albicans* housekeeping gene (*ACT1)*. Analysis of quantitative RT-PCR was made by the ΔΔCt method. Single-species biofilms were used as reference samples for expression *C. albicans* genes. Asterisk (*) denotes the significant difference between mixed & dual species samples and the mono species sample (One-way ANOVA; $P < 0.05$).

### 4.6 Identification of als1p, als2p and als3p proteins

In order to confirm the role of *ALS* genes in single (*C.a* alone), two dual (*C.a* + *S.m*) (*C.a* + *S.s*) and mixed species (*C.a* + *S.m* + *S.s*) biofilms, western blot analysis of als proteins was done to identify each protein in subsequent biofilms.

Western blots of als proteins in single species (*C. a* alone) reveals bands of all three proteins i.e., als1p, als2p and als3p with molecular weight > 200 kDA. All three proteins form prominent bands that reveal their expression in single species. In both dual and mixed species, clear bands of als1p and als3p were visible. Bands for als2p were not shown in dual and mixed species sample (Figure 4.9). The results suggest that als2p is expressed only in single species biofilm. When candida adheres to streptococci in dual and mixed species biofilm, expression of als1p and als3p is visible. Thus, als1 and als3p are involved in candida-bacterial interactions. This explains the role of *ALS1*, *ALS2* and *ALS3* genes in single and mixed species. This concludes that *ALS1* and *ALS3* genes are significantly expressed when *C. albicans* forms co-aggregation with commensal *S. mitis* and *S. sanguinis* of oral cavity which in turn codes for als1 and als3p proteins for cell adhesion.

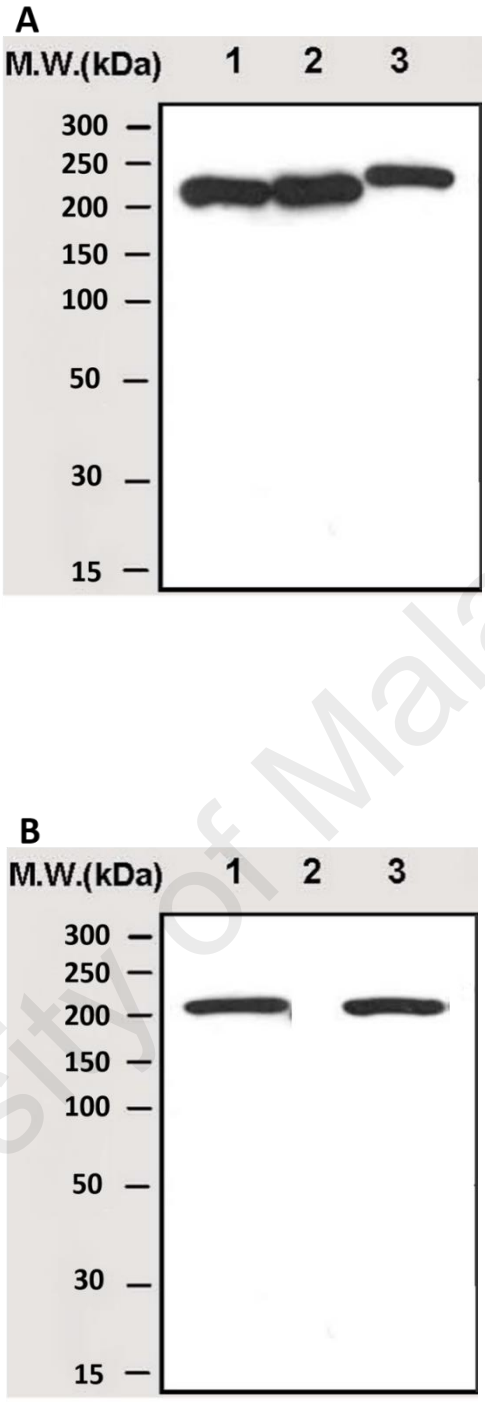


**Figure 4.9:** Western blot of candida cell wall proteins in single, dual and mixed species biofilm.

Samples were run in individual lanes where als1, als2 and als3 proteins were detected according to their molecular weight on lane 1,2 and 3 respectively
**A**; C.a alone. **B**; C.a + S.m. **C**; C.a + S.s. **D**; C.a + S.m + S.s

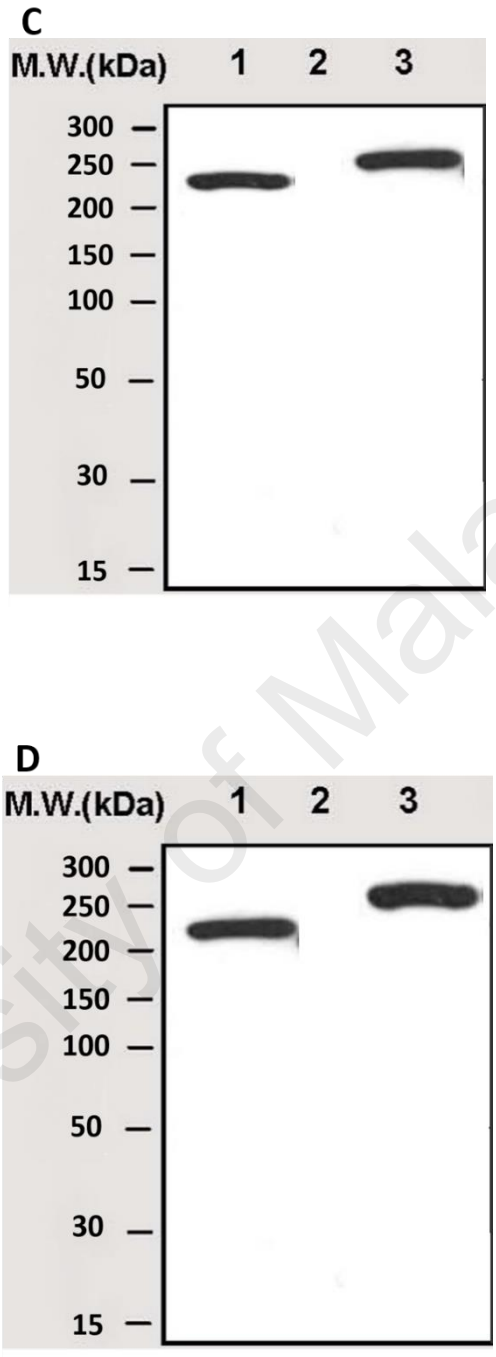


**Figure 4.9, continued**

# CHAPTER 5: DISCUSSION

Host adhesion and formation of biofilm are the key areas for the microbial virulence and antifungal property. Oral streptococci are amongst the pioneer organisms to accumulate and form salivary pellicle on oral surfaces. Although they tend to adhere first, their role has always been considered commensal (Xu *et al.*, 2014). *C. albicans*, like other organisms rarely exists individually. It forms polymicrobial interactions with a number of microorganisms. Interactions between *C. albicans* and bacteria are likely to play a role not only during *C. albicans* colonisation of mucosal surfaces but also during the course of mucosa-associated or invasive infections (Venkatesh *et al.*, 2007). However, it is not clear to what extent *C. albicans*-bacterial interactions promote or prevent disease. While the interaction of *C. albicans* with bacterial species such as *Pseudomonas aeruginosa* has been demonstrated to be antagonistic in nature, *C. albicans* seems able to coexist with *Staphylococcus* sp. and with oral streptococci (Hogan *et al.*, 2002). Few studies, however, have tried to characterize such multispecies interactions in biologically relevant models. This study aims to demonstrate the effect and influence of commensal bacteria on formation and growth of *C. albicans* biofilm.

## 5.1 Microbial growth on static and dynamic biofilm

There are a variety of systems available for examining the formation of bacterial biofilms. Static biofilm systems are formed quite frequently in experiments to evaluate microbial growth for a number of reasons. Static assays are particularly useful for examining early events in biofilm formation (Merritt *et al.*, 2005). The evaluation techniques are able to quantify even smaller biofilms with high reproducibility. Another major advantage of these systems is the simplicity of the protocols: these assays can be executed primarily using common laboratory equipment. Furthermore, these assays have a relatively high throughput and can easily be adapted to study a variety of biofilm

formation conditions, making them excellent tools for performing genetic screens (Qu *et al.*, 2017). However, static biofilms sometimes create interface with liquid-air pellicle. So to ensure continuous supply of fresh nutrient medium and to create environmental conditions as are available in oral cavity, continuous flow system was established to form dynamic biofilm as well (Azeredo *et al.*, 2017).

For the formation of static and dynamic biofilm throughout the experiment, unstimulated saliva was obtained from a single donor to minimize any variations that may arise because of different subjects as previously done in other studies (Li *et al*., 2017; Nordin *et al.*, 2013; Velsko *et al.*, 2018). Several researchers have used a pool of saliva from various donors to form *in vitro* biofilms (Chevalier *et al.*, 2017; Mira *et al.*, 2019), but it has been studied that saliva from single healthy donor gave similar findings in terms of microorganism adhesion and accumulation on hard and soft surfaces (Naginyte *et al.*, 2019). In light of the acceptable physical properties and feasibility of saliva collection from a single donor, the current study acquired saliva from one person throughout the whole experiment.

Single, dual and mixed species static biofilms were formed on sterile 96-well plates to analyse biomass and cellular metabolic activity of cells via crystal violet assay and XTT assay respectively. The crystal violet assay demonstrated that biomass of a 24 h biofilm increased with higher number of bacteria. Dual species (*C.*a+*S.m*, *C.a*+*S.s*) and mixed species (*C.a*+*S.m*+*S.s*) biofilm showed greater biomass as compared to single species biofilm. The enhancement of biofilm was further examined through XTT reduction assay. The metabolic activities of dual and mixed species biofilm increased dramatically in comparison to single species. Similar results were seen on the formation of dynamic biofilm. The adhering capacity of dual and mixed species to salivary coated glass bead was greater when compared to single species. Dynamic biofilm was created to mimic the

oral cavity environment that consists of specific temperature and a continuous salivary flow.

Studies of pellicle formation have suggested that the salivary proteins amylase, immunoglobulin A and proline-rich proteins (Delius *et al.*, 2017; Heller *et al*., 2017), as well as albumin and fibronectin, bind to oral surfaces; teeth, mucosa, denture, implant etc. (Hahnel, 2017). Pellicle proteins serve as receptors for the attachment of micro-organisms that initiate the formation of a microbial biofilm (Marsh *et al*., 2017). Previous studies have proven that streptococci bind directly to salivary proteins that are abundant in the pellicle covering teeth and mucosal surfaces, and this binding facilitates their initial colonisation under salivary flow conditions (Xu *et al.*, 2014). Previous *in vitro* studies have shown that the biofilms formed on salivary pellicle increased the metabolic activity and expression of *C. albicans* virulence factors (Cavalcanti *et al*., 2016). Thus, saliva plays a significant role in biofilm formation and propagation of microorganisms. Static and dynamic *in vitro* biofilms were formed on salivary pellicle in this study to depict the manner in which organisms would adhere to soft / hard tissues in oral cavity.

*S. sanguinis* and *S. mitis* are pioneering colonisers, aiding in the attachment of succeeding organisms, and become key players in oral biofilm development (Zhu *et al.*, 2018). Although suggested to be health-associated bacteria by 16S rRNA sequencing studies, *S. sanguinis* and *S. mitis* may potentially be binding targets for the localization of pathogens within dental plaque involved in various periodontal and gingival diseases (Maeda *et al.*, 2004). The results of the current study show that there is high co aggregation affinity between *C. albicans* and the two other types of streptococci. Undoubtedly, it has previously been observed before that oral streptococcal bacteria interact with *C. albicans* (Bamford *et al.*, 2009); Morales & Hogan, 2010). Diaz and co-workers (2012) demonstrated in their study that the ability of oral streptococci to form

biofilm on abiotic surfaces in substantially enhanced in the presence of *C. albicans*. Another study conducted by doRosario *et al*., (2018) revealed a decrease in CFU count when *C. albicans* was grown with *S. mitis* and *S. sanguinis*. This outcome was in contrast to our results that showed an increase in CFU in mixed species biofilm. The reason for this could be the conditions in which biofilms were grown. Dynamic flow systems allow provision of fresh medium to organisms facilitating their growth (Foustoukos *et al.*, 2015). Moreover, it is anticipated that these bacteria in dual and mixed species biofilms influence the local environment of *C. albicans* by altering parameters such as nutrient supply or carbon dioxide levels, which may contribute in enhancing *C. albicans* hyphal transition and subsequently virulence (Koo *et al.*, 2018). Hence, the results indicate a clear synergistic relationship between *C. albicans* and two streptococci. The organisms are not only capable of coexisting in similar environment, the interaction between them seems to enhance microbial growth. These results suggest that multi-species biofilms with these organisms may play a detrimental role in host homeostasis and may promote infection. Meaning thereby, *C. albicans* becomes more virulent in the presence of commensal organisms like streptococci in a polymicrobial biofilm.

### 5.2 Morphology of organisms

After examining the growth of biofilms in static and dynamic conditions, morphology of microorganism in those biofilms was assessed by scanning electron microscope. Scanning electron micrographs showed a significant difference in the morphology of *C. albicans* in single and mixed species biofilm. In a 24 h biofilm, *C. albicans* formed true hyphae under the influence of streptococci in mixed species biofilm, whereas, in single species, candida cells remain in the form of blastoconidia (yeast form). This result is of particular interest because hyphal form of candida is known to be critical for fungal adhesion and invasion to host surface. Morphogenesis appears to be important for

pathogenesis, because cells that are trapped in either the yeast or pseudohyphal states are less virulent in murine systemic infection models (Witchley *et al.*, 2019). Hyphal formation is well known to trigger essential degrading enzymes required to attain full virulence (Cavalcanti *et al*., 2016). The exact cause of this transition remains unknown. However, previous studies suggest that there appears to be several signal transduction pathways that could be activated to begin this developmental transition with some, such as the mitogen-activated protein kinase and cyclic AMP/protein kinase A pathways. Of course, the final outcome of any such signal, irrespective of the environmental stimulus originally received, is a change in expression of a particular subset of genes, which thereby facilitates the change in the mode of growth from one morphology to the other (Parrino *et al.*, 2017). In the adaptive immune response, Dectin-1 expressed on myeloid cells recognises β-glucan components of the fungal cell wall that are exposed during the hyphal transition. This leads to production of IL-6 and IL-23, which promote Th17 cell differentiation (Verma *et al.*, 2017). Previous studies have been conducted on infections caused by isolated *C. albicans* and do not necessarily stand true for polymicrobial species (Lohse *et al.*, 2018). Since our study includes influence of Streptococci along with *C. albicans*, the phenomenon responsible for yeast to hyphae transition and the inflammatory responses need to be studied further.

In our study, streptococcal chains seem to be in close proximity to candidal hyphae. Previous studies have shown the association of organisms to *C. albicans*. For example, the oral bacterium *Streptococcus gordonii* produces nutrient by-products that are stimulatory to *C. albicans*, enhancing the length of hyphal filaments. In turn, *S. gordonii* benefits from the reduced oxygen environment generated by *C. albicans* metabolism (Veerapandian *et al.*, 2019). Other studies reveal that biofilm growth and hyphal filament production by *C. albicans* was enhanced by *S. oralis* (Cavalcanti *et al.*, 2017). Bacterial cells of *Streptococcus mutans* have been observed

attached in chains as they adhere to and wrap around the hyphae (Metwalli *et al.*, 2013). Our study shows the interaction of *S. mitis* and *S. sanguinis* with *C. albicans* is similar to other streptococci. The interesting finding in our study is the development of candidal hyphae in 24 h under the influence of streptococci which was not found in single species biofilm, under the same growth and environmental conditions. Current study suggests contribution of commensal streptococci in candidal biofilm formation and propagation which could result in altered pathogenesis.

In symptomatic oral infections e.g., thrush, denture stomatitis, oropharyngeal candidiasis, hyphal formation is clinically imperative as it is a critical marker of pathogenesis (Millsop & Fazel, 2018). Candidal hyphae formation is regulated with secretion of adhesins, secretory proteases and cytoplasmic proteins that aid in fungal biofilm stability over host surface and its invasion and subsequent dissemination of fungal pathogen (Kumamoto & Vinces, 2005). In contrast to yeast cells**,** hyphae do not induce IL-12 production from DCs, but instead induce IL-4, resulting in a more anti-inflammatory immune response (Hasebe *et al.*, 2018). When infecting humans and animals, *C. albicans* hyphae predominate at the primary site of infiltration in oral cavity, whereas yeast cells are generally found either on the cell surface or emerging from penetrating hyphae that are infiltrating tissues (Pellon *et al.*, 2020). It has been observed that invasive disease occurs when *C. albicans* crosses tissue surfaces. Hyphae are more potent in causing cellular damage than yeast cells (Dalle *et al.*, 2010), and the damage occurs by the production of lytic enzymes, such as secreted aspartyl proteinases (SAPs) and ALS proteins (Naglik *et al.*, 2003). Studies have also shown that whereas filament formation appears to be required for the mortality resulting from a deep-seated infection, yeast cells play an important role early in the infectious process by extravasating and disseminating to the target organs (Saville *et al.*, 2003). Recent studies done on interkingdom interactions between candida and streptococci have shown the upregulation

of *efg*-1 hypha associated gene in polymicrobial biofilms (Koo *et al.*, 2018). Thus, it could be predicted that in a 24 h biofilm, *C. albicans* (alone) is in initial stages of morphogenesis and subsequent pathogenesis, whereas in the same time frame, dual and mixed species result in a more virulent and advanced morphogenetic stage of *C. albicans*. Hence, it could easily be stated that co-aggregation of *C. albicans* with streptococci in salivary biofilm poses a greater threat to local host tissues when compared to fungus alone.

### 5.3 Effect of drugs on single, dual and mixed species

After examining the microbiological properties of polymicrobial species, clinical relevance of these species interactions was determined by exposing them to drugs. Candidal infections manifest as white oral patches on oral mucosa in the clinical form of denture stomatitis or mucosal thrush. It is a common practice to treat these conditions with commercially available antifungals. CHX mouth rinses are prescribed for a variety of oral conditions like denture stomatitis, oral ulcers, oral thrush, mucositis, alveolar osteitis etc. (Ellepola *et al.*, 2001; Scheibler *et al.*, 2017). It inactivates microorganisms with a broader spectrum than other antimicrobials (e.g. antibiotics) and has a quicker kill rate than other antimicrobials. CHX kills by disrupting the cell membrane. Candida cells uptake CHX in a short amount of time and it impairs the integrity of the cell wall and the plasma membrane entering the cytoplasm resulting in leakage of cell contents and cell death (Suci *et al.*, 2002). The bacterial uptake of the CHX is also very rapid. In low concentrations it affects the integrity of the cell wall. Once the cell wall is damaged, CHX then crosses into the cell itself and attacks the cytoplasmic membrane. Damage to the cytoplasm's delicate semipermeable membrane allows for leakage of components leading to cell death. In high concentrations, CHX causes the cytoplasm to congeal or solidify (Lee *et al.*, 2016). AmB and Nt are the most commonly used drugs for the treatment for

oral candidiasis (Garcia-Cuesta *et al.*, 2014). These drugs are ionophores and act by binding to sterols (ergosterol) in the cell membrane of *C. albicans*. This creates a transmembrane channel, and the resultant change in membrane permeability allowing leakage of intracellular components causing ultimate cell death (El-Azizi *et al.*, 2015). Hence it is a common clinical practice to treat patients with oral candidiasis with antifungals adjunctive with CHX mouth rinses.

The results of our study show that single species (just candida) was susceptible to treatment with AmB. Whilst single species group was susceptible to treatment with chlorhexidine, dual and mixed species were resistant to its treatment. Nevertheless, denser biofilm, as formed under the influence of streptococci could retard access to such an extent that cells lying deep within a microcolony escape exposure. This would occur via drug adsorption or neutralization, and would depend on the thickness of the biofilm and on the chemical nature of both the antimicrobial agent and the matrix material (Al-Fattani *et al.*, 2006). Matrix polymers of biofilms are primarily exopolysaccharides, and many are negatively charged due to the presence of carboxyl, sulphate or phosphate groups. Smaller amounts of proteins, nucleic acids and lipids can also be present (Starkey, 2004). It has been studied that mixed-species biofilms of *C. albicans* and *S. epidermidis* grown statically, or under flow conditions, were highly resistant to both amphotericin B and fluconazole. At exposure times of 5 and 24 h, the drugs had no effect on the metabolic activity of the biofilms, despite the high drug concentration used (30 times MIC) (Al-Fattani *et al.*, 2006), cause of which has not yet been explained. It has been observed that there was increased resistance of *C. albicans* biofilms grown on denture acrylic to fluconazole, AmB, Nt and CHX (Chandra *et al.*, 2001). It has also been observed that oral thrush biofilms isolated from AIDS patients showed higher resistance compared to those obtained from other patients (Anibal *et al.*, 2010). The increased resistance to AmB, Nt and CHX may result from changes in the mixed species biofilm matrix, which would

reduce the permeation of the antimicrobial agents and the antibacterial protein. It is possible that interactions between the different matrix polymers might result in a more viscous biofilm. However, biofilm resistance overall is likely to be multifactorial, involving, in addition, drug-resistant physiologies such as dormant 'quiescent' cells and expression of efflux pumps.

This triggers an alarming situation when considering treatment of oral fungal infections. Since recent studies prove that oral candidal infections are in fact a result of polymicrobial community aggregation, containing both candidal and bacterial species (Costa-Orlandi *et al.*, 2017), just one treatment regimen is not sufficient to overcome microbial growth. While *C. albicans* is the fourth leading cause of mortality due to systemic infections (Mayer *et al*., 2013) the studies suggest that risk of mortality may increase upon bacterial and fungal co-infection (Harriott *et al.*, 2009; Morales & Hogan, 2010) species mixed biofilms. It has been demonstrated in several studies that candida-streptococcal biofilm causes drug resistance but previous work has been done on dual species biofilm (Chinnici *et al.*, 2019; Lobo *et al.*, 2019). In contrast, this study gives a new insight in the differences between dual and mixed species biofilm. It is also worth noting that the synergistic protective effect of the multispecies was observed in dual and mixed species which lead to antimicrobial resistance in these groups. Thus, in light of the results shown in the study, it could be concluded that these species have synergistic effect on each other, and this co-aggregation could lead to antimicrobial resistance, treatment of which needs special attention.

### 5.4 Expression of *ALS* 1, 2, 3 genes and their respective proteins

Microorganisms adhere to hard and soft tissues and to other organisms in the oral environment. In this regard, cell wall of a microbe plays a significant role in biofilm formation and propagation since interactions between pathogens and the host are typically

mediated by molecules either secreted or displayed on the cell wall. In an attempt to identify prominent genes and their respectively expressed cell wall proteins, expression of agglutinin-like sequence (*ALS1*, *2* and *3*) genes was quantified using qPCR and als1p, als2p and als3p were identified using western blot. Real-Time PCR technologies combine the sensitivity of conventional PCR with the generation of a quantifiable fluorescent signal and have been increasingly used to assess viability of microorganisms (Leach *et al.*, 2018). Western blot, an analytical technique used to pinpoint a specific protein in a given sample has advantages of being highly sensitive and specific in protein detection (Gurtler *et al.*, 2013). As emphasized in other published literatures, the use of qPCR and western blot provide authentic and discriminative tests with respect to the current microbiology, biochemical and serological diagnostic methods (Kuypers *et al.*, 2017; Schmittgen *et al.*, 2008).

In regard to the expression of virulence genes of *C. albicans*, *ALS1* and *ALS3* genes were upregulated in dual and mixed biofilms. The results were further endorsed by the identification of als1p and als3p in dual and mixed species. The expressions of *ALS* genes have been extensively studied in different experimental conditions, reflecting the growing realization of how important their role is in the various stages of oral infection (Araujo *et al.*, 2017; Hoyer *et al.*, 2016). These putative genes are concerned with substratum adhesion. *ALS1* is associated with biofilm maturation, whereas *ALS3* is a cell surface, hyphae specific protein and is known to be a receptor for the streptococcal adhesions SspB and SspA (Bamford *et al*., 2009). Previous studies suggest that als1p, expressed by *ALS1* gene might adhere to host surfaces, rather than to other *C. albicans* cells or other microbial cells (Epstein *et al.*, 2016). High expression of *ALS1* gene in dual and mixed species biofilm indicates its role in biofilm maturation rather than cell-cell adhesion (Ho *et al.*, 2019). Studies have shown that als3 protein makes an important contribution to *C. albicans* adhesion to oral epithelial cells, and subsequent epithelial damage, and that loss

of als3 results in reduced capacity of *C. albicans* to induce epithelial cytokines (Murciano *et al.*, 2012). According to studies conducted by Hoyer *et al*., 2008, *ALS3* mutant *C. albicans* showed significant downregulation in endothelial cell, buccal epithelial cell attachment and cell-cell adherence. *ALS3* has also been observed in the early phases of candida biofilm formation (Nailis *et al.*, 2009; Romo *et al.*, 2020). Recent work supported the conclusion that als3p, expressed by *ALS3* gene is a fungal invasin that mimics host cell cadherins and induces *C. albicans* endocytosis by binding to N-cadherin on endothelial cells and E-cadherin on oral epithelial cells (Phan *et al.*, 2007). This explains the role of *ALS3* in candida biofilm formation. Isolates from recurrent vaginal candidiasis indicate that there is an association between the expression of *ALS1* and *ALS3* genes and fluconazole resistance in *C. albicans* (Roudbarmohammadi *et al.*, 2016). So, the multidrug resistance shown in this study could be related to over expression of *ALS1* and *ALS3* genes and vice versa.

Increased expression of *ALS1* and *ALS3* in dual and mixed species biofilms signify that *C. albicans* adhesion to surface may also be enhanced under the influence of bacteria. Previous studies have confirmed the expression of *ALS* genes in various candida associated infections like in HIV positive patients, oral candidiasis (Green *et al.*, 2006) disseminated candidiasis, model denture and catheter biofilms (Green *et al.*, 2004) and buccal esophageal vaginal epithelial models. In all these conditions *ALS1* and 3 genes have always been expressed substantially. Nailis *et al*., (2009) stated high expression of *ALS1* and *ALS3* genes in a 48 h biofilm. The high expression of the *ALS1* and *ALS3* genes in a 24 h biofilm, as indicated in this study, underlines high adherence capacity of *C. albicans* in initial stages of biofilm formation and maturation. Hyphae formation under the influence of streptococci could be responsible for over expression of virulent genes. This trait would lead to a more stable biofilm, increasing the virulence of the concerned

microorganism. The ability of Group B streptococci to co-associate with *C. albicans* is dependent upon expression of the hypha-specific adhesin als3 (Pidwill *et al.*, 2018).

The process through which the bacterial constituent of the *in vitro* salivary biofilm propagated the above-mentioned observed changes in *C. albicans* is still under research. The co aggregation-mediated attachment plays pivotal roles in facilitating *C. albicans* biofilm development in the presence of *S. mitis* and *S. sanguinis*. Co aggregation may not only be able to promote initial attachment and retention, it may also facilitate a metabolic adhesion-interaction between the organisms that endorse *C. albicans* biofilm enhancement. Our study showed that an influential determinant in biofilm progression was the influence of the *S. mitis* and *S. sanguinis* on *C. albicans.*

# CHAPTER 6: CONCLUSION

The main conclusions of this study are:

1. *C. albicans* demonstrated enhanced biofilm characteristics when grown with *S. mitis* and *S. sanguinis.* The significant increase in biofilm biomass, cellular metabolic activity and CFU count of polymicrobial cells grown under static and dynamic flow conditions indicates a symbiotic relationship between candida and streptococci.

2. Morphology of organisms as visible under scanning electron microscope displayed *C. albicans* hyphae formation in dual and mixed species. Whereas, only yeast cells were visible in single species biofilm. The results suggest that streptococci stimulate candidal morphogenesis into a more virulent hyphal form.

3. *C. albicans* when grown in dual and mixed species with streptococci had significantly higher MIC values to nystatin, amphotericin b and chlorhexidine in comparison to single species biofilm. This implies that polymicrobial species form stronger biofilms which would be difficult to treat with conventional drugs and dosage.

4. *ALS*1 and *ALS*3 genes with their relative proteins (als1p and als3p) were significantly upregulated in dual and mixed species, while ALS2 and als2p were not significant. This indicates a significant role of these genes in biofilm thickness, hyphal transition and ultimate drug resistance.

# CHAPTER 7: SIGNIFICANCE OF THE STUDY AND FUTURE RECOMMENDATIONS

## 7.1 Significance of the study

Fungal infections are a common occurrence in the modern world. Not only elderly but young are also frequently affected by it. People with poor oral hygiene, those with dentures and/or implants in their oral cavity, pregnant women, immuno-compromised individuals are more prone to these infections than others.

Clinical appearance of candida infections is quite similar, so it is a common practice for clinicians to prescribe antifungal drugs. However, the rate of antifungal resistance has substantially increased in the last two decades. This current research points out the importance of commensal streptococcal bacteria and their potential role in culmination of *C. albicans* biofilm forming a stronger more resistant polymicrobial biofilm on oral mucosal surfaces. Medicine specialists could identify organisms working side by side *C. albicans* causing more virulent pathogenic phenomenon in the oral cavity.

Most studies on interkingdom studies have been conducted on two species. The results of current study highlight the difference between interactions of dual and polymicrobial species. It paves the way for future researchers to include more species in interkingdom studies.

## 7.2 Limitations of the study

The current study was conducted on only one strain of microorganisms. Wild type strains and mutant strains might give different results under the same experimental conditions. The study was performed on solid surfaces which can be a representative of oral hard tissues but not soft tissues.

### 7.3 Future recommendations

This study demonstrates the formation of candidal hyphae in 24 h under the influence of commensal *S. sanguinis* and *S. mitis*, which under normal conditions remains in the budding stage. This gives an important insight in the morphogenesis of *C. albicans*. Signalling molecules responsible for this transition could be studied in detail to identify the cellular mechanisms undergoing this transformation. This would help researchers device a plan to inhibit the responsible signalling molecules, and attempt to minimize candidal virulence.

The current study also demonstrated that commonly used antifungals have extremely high MIC values against mixed species. This study can be further investigated by *in vivo* clinical human trials. Research could be done to explore treatment options suitable for polymicrobial species. Natural extracts could also be studied for treatment of polymicrobial infections.

This study was conducted on only one candida species and two streptococcal species. A lot of useful information could be gathered by studying non-albicans candida species and their relationship with various types of bacteria present in the oral cavity.

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

### SYMPOSIUM

Ayesha Fahim, Wan Harun Himratul-Aznita & Puteri Shafinaz Abdul-Rahman. "Polymicrobial interactions between *S. mitis, S. sanguinis* & oral associated *C. albicans"*, Ninth annual scientific symposium Ameer ud Din Medical College, Lahore Pakistan, February 2019.

### PUBLICATIONS

Ayesha Fahim, Wan Harun Himratul-Aznita & Puteri Shafinaz Abdul-Rahman (2019). "Polymicrobial interactions between *Streptococcus mitis, Streptococcus sanguinis* and oral associated *Candida albicans* on an *in vitro* salivary biofilm and differential expression of *ALS1, ALS2 and ALS3* genes", Biotechnology & Biotechnological Equipment, 33(1):338-346. doi: 10.1080/13102818.2019.1577173

Ayesha Fahim, Wan Harun Himratul-Aznita & Puteri Shafinaz Abdul-Rahman (2019). "Allium Sativum and Bakuchiol combination: A natural alternative to Chlorhexidine for oral infections?", Pakistan Journal of Medical Sciences, 36(2). doi: https://doi.org/10.12669/pjms.36.2.1457